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This thesis is dedicated to my father, Abel Potts.

DESICCATION TOLERANCE

by

Malcolm Potts B.Sc., Ph.D. (Dunelm)

A thesis submitted in candidature for the degree of D.Sc. in the Faculty of Science, University of Durham

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Brian Whitton first drew my attention to the peculiar water-absorbing properties of some blackened, brittle, nondescript crusts, while I was an undergraduate student in the Department of Botany, University of Durham. The present thesis, and my ongoing fascination with those crusts, have their origins in work completed with Alan Donaldson and David Myers in Brian's lab. during the 1970's, and in my stay on Aldabra Atoll in 1974-75 where, as a Ph.D. student, I became to appreciate the remarkable property of desiccation tolerance.

Of the many people who have had direct or indirect bearing upon the progress made during the past two decades, I thank particularly four fine colleagues: Brian Whitton - especially for his sustained interest, support and criticism, Imre Friedmann, Alan Peat, and Siegfried Scherer.

I thank Ann, Sarah and Emma, for continuing patience, and my mother and brother Clifford for their encouragement and concern.

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ABSTRACT

Despite the fundamental significance of desiccation in determining the distributions and activities of living organisms, there is virtually no insight as to the state of the cytoplasm of an air-dried, or even a wet, cell. In bacterial cells that have been subjected to air-drying the evaporation of free cytoplasmic water ($\bar{V}_f$) can be instantaneous, and an equilibrium between cell-bound water ($\bar{V}_b$) and the environmental water (vapor) potential (Ψ_{wv}) may be achieved very rapidly. In the air-dried state some bacteria survive only for seconds, others can tolerate desiccation for thousands, perhaps for millions, of years. The means by which certain cells, the anhydrobiotes, overcome and then tolerate acute water deficit remains one of the most intractable problems in cell biology. One such anhydrobiote, the cyanobacterium *Nostoc commune,* is cosmopolitan, its colonies form visually-conspicuous and abundant growths in situ, and it constitutes an ecologically-significant component of terrestial nitrogen-fixing communities. The cyanobacteria are phylogenetically-significant organisms that provide model systems for the study of a broad range of problems in cell biology. The studies described in this thesis established the molecular ecology and cell biology of *Nostoc commune*, and they provide a chronicle of the development of this microorganism as the prokaryotic model for the anhydrobiotic cell. In the design of experiments to investigate this problem the bias was, and remains, this: to understand desiccation tolerance, understand an organism that tolerates desiccation. The thesis documents an investigation into the consequences of acute cell-water deficit and the cellular basis for desiccation tolerance. An eclectic approach has been adopted to study desiccation tolerance and it includes the application of techniques of cell biology, biochemistry, microbiology, molecular biology, structural biology and biophysics.

DESICCATION TOLERANCE - A RESEARCH PROBLEM

The Anhydrobiotic Cell

The pivotal role of water in the emergence of life is readily acknowledged. In contrast, the importance of water in cell structure and function is usually taken for granted, it is persistently understated or, more than often, it is ignored. Only recently has there been a renewed awareness that water is indespensible for the maintenance of cell integrity. In part this reawakening reflects current interest in the refinement of protein crystal structures, the appreciation that water drives conformational changes in nucleic acids and proteins, and the realisation that discrete numbers of water molecules participate in diverse enzyme reaction mechanisms.

The removal of water from cells, and the subsequent addition of water to air-dried cells, elicit responses which encompass an extreme spectrum of interactions at the structural, physiological and molecular levels. Very few organisms withstand these stresses and most succumb rapidly to the removal of only a marginal amount of their intracellular water. Certain cells do offer a degree of resistance to drying and some, when faced with the loss of the bulk of their water, not only maintain their structure and integrity during prolongued desiccation, but recover their metabolic capacities at the onset of rehydration in a rapid, seemingly highly-ordered and stringent fashion. Such anhydrobiotes constitute a diverse assemblage of organisms that includes representatives of bacteria, fungi, higher and lower plants, protozoa and invertebrates.

How do anhydrobiotic cells withstand such extreme water deficit and long-term desiccation? The question addresses one of the most intractable problems in cell biology. The difficulty in providing adequate answers, or at least the inability to devise testable hypotheses, stems from the pervasive role of water in cell function and the unique properties of the water molecule - many of the latter remain poorly understood. From both a biochemical and a biophysical perspective there is little understanding of the nature of the cytoplasm in dried (or even wet) cells, a topic that has attracted adherents with quite opposing views.

Significance of the Research Problem

Why should desiccation tolerance be of such fundamental significance? Aside from an obvious interest that reflects concerns over the depletion of water resources, the escalating desertification of arable land, and the central problem of desiccation in managed agriculture, desiccated cells provide the means to obtain a unique perspective on the nature and functional plasticity of cytoplasm. An understanding of how life survives without water can provide the yardstick with which to assess how life may have first evolved. There is a growing technology for dried cells and biomolecules - including vaccines, enzymes and liposomes, and an emerging interest in molecular (dry state) electronics. More importantly, perhaps, dried cells emphasize the dimension of time in cellular physiology. Desiccation may be equated with suspended animation and that realisation provides a further perspective on the limits of cellular evolution and function. Not surprisingly, questions as to whether anhydrobiotic (desiccated) cells are "alive," or not, has generated a needless, and predictable, amount of argument and quibbling, most of little relevance to science.

If one assumes that the properties of water are immutable, then one must assume that the constraints imposed by water deficit upon the cell components of one anhydrobiotic cell are equivalent to the constraints imposed upon those cell components of other anhydrobiotes. Simply put this means that any anhydrobiotic cell model can provide the means to understand desiccation tolerance. However, there are compelling reasons why anhydrobiotic bacteria offer considerable advantages for such studies. Bacteria grow rapidly, they are readily amenable to genetic manipulation, they have a comparatively simple cellular ultrastructure, their full complement of proteins is well characterised, and the size and complexities of their genomes are readily defined. A knowledge of the mechanisms of desiccation tolerance in bacteria provides a clear evolutionary perspective on the emergence of this cellular trait. One other advantage for the use of the bacterial anhydrobiotic cell model can be explained in the following terms. There are numerous cases where intriguing cellular phenomena, first observed in communities that constitute diverse

components of natural ecosystems, have been analysed at the ecological level, subsequently at the physical and biochemical level, and then finally at the molecular level. But, as pointed out and emphasized by Walsby[a], there are precious few instances where the information has been extended back, through the redesign of physiological studies, to the natural habitat where the organisms in question are found. The latter approach allows the fine analysis of natural selection and can, in the long run, permit the attenuation and manipulation of the activities of communities growing in situ. The widespread distributions of cyanobacteria, and their pervasive roles in microbial ecology, emphasize the unique utility of *N. commune* as the model with which to understand desiccation tolerance.

The Cyanobacteria

Of those organisms which express desiccation-, or water deficit-, tolerance, many cyanobacteria have a marked capacity to do so. These photosynthetic prokaryotes represent an ecologically-significant and phylogenetically-important assemblage within the Bacteria. They share both functional and structural affinities with the higher-plant chloroplast and offer model systems for the study of cell differentiation, signal transduction, photosynthesis and nitrogen fixation. As a group the Cyanobacteria epitomises the concept of hyperbradetely (*sensu* Schopf) - an inordinately slow, perhaps even a suspended, rate of evolutionary change over geological time. As such, cyanobacteria provide the means to study processes that may have changed little within the past 3.6 billion years. One form in particular, *Nostoc commune*, lends itself to the study of desiccation. The blackened, desiccated crusts of its colonies accumulate and become visually-conspicuous in terrestial limestone habitats of every continent on Earth where they often constitute the principal source of fixed nitrogen.

Nostoc commune, as is chronicled in this thesis, has been developed as the prokaryotic

[a] 1994 - Walsby, A.E.. Microbiol. Rev. 58:94-144.

model for the anhydrobiotic cell. This cyanobacterium elaborates abundant, visually conspicuous, and ecologically-significant communities in situ. One task, at the outset of this work, was to extend a knowledge and appreciation of the ecology of *N. commune* and to develop an understanding of its molecular cell biology.

THE STUDY OF DESICCATION

An Historical Perspective

In 1702 van Leeuwenhoek described the springing to life of dried objects - in this case rotifers - upon their rehydration. These observations generated little attention. Curiously, desiccation seems to continue to be a subject of little interest to the scientific community. During the 1960's Sydney Webb applied classical, yet rigorous, bacteriological techniques in the study of the effects of air-drying on the viabilities of bacteria. These studies provided considerable insight into the potential problems faced by cells subjected to desiccation but they tended to be concerned more with how cells lost viability upon air-drying, rather than how other bacteria, the desiccation-tolerant forms, withstood the same stress. Three decades have elapsed since the pioneering work of Webb. This period of time has witnessed the formal recognition of the prokaryote and the eukaryote, the rise of the Archaea, and the onset of the recombinant DNA revolution. Despite the fundamental importance of desiccation in bacterial physiology, microbial ecology, and clinical microbiology, and the renewed awarness of the role of water in cell biology, the anhydrobiotic cell continues to be ignored - in paraphrase of Albert Szent-Györgyi, Clegg[b] noted: "biology has forgotten the dried cell - or has never discovered it."

The studies chronicled in this thesis began in the late 1970's. At this time any studies of desiccation tolerance in bacteria relied, in large part, upon phenomenological and descriptive

[b]1986 - Clegg, J.S. p.169-187. *In* : A.C. Leopold (Ed.) Membranes, metabolism , and dry organisms. Comstock Publishing Associates, Ithaca and London.

observations - in many instances they still do. Molecular ecology, as a discipline, did not exist and the molecular biology of the Cyanobacteria had not yet been established -

> "cyanobacterial geneticists missed the boat in the early 1970s and failed to capitalize on the pickings that were then available."
>
> -WDP Stewart, FRS.
> (1980 - Ann. Rev. Microbiol. 34:497-536)

The majority of cyanobacterial strains then in current use were, and they remain, greenhouse weeds of undetermined origin and uncertain ecological significance. The decisison to focus attention on *N. commune* reflected the utility of the microorganism for the study of water stress, and the fact that this cyanobacterium represents a conspicous, readily identified, and tangible component of nitrogen-fixing microbial communities from the Tropics to the polar regions.

Scope of the Present Thesis

The collection of publications in this thesis provides a comprehensive account of the physiological, structural and biochemical properties of field materials of *Nostoc commune* and *N. commune* strain UTEX 584. These studies contribute to the molecular ecology of cyanobacteria, specifically they establish the molecular ecology of *N. commune*. The principal focus of the thesis is the dissection of the physiological and biochemical properties that define the cell biology of desiccated *N. commune*, and the identification of structural and molecular mechanisms that constitute components of desiccation tolerance. In essence the thesis documents the development of the prokaryotic model for the anhydrobiotic cell.

STATEMENT OF MAJOR CONTRIBUTION

Desiccation Tolerance of Prokaryotic Cells - A Critical Appraisal

A limited number of phylogenetically-diverse organisms have the capacity to tolerate the removal of the bulk of their intracellular water. In these anhydrobiotic cells the concentration of water is of the order of 0.02 g H_2O g cell solids^{-1}. For prokaryotes, it is widely accepted that bacterial spores, including eubacterial endospores and cyanobacterial akinetes etc., are highly tolerant of desiccation and that they represent examples of anhydrobiotic cells. Perhaps this assumption is responsible for the lack of information on the desiccation tolerance of prokaryotic cells because what is clear is that such spores or resting stages, by virtue of their (high) water contents, are not anhydrobiotic cells. Such misconceptions are considered in detail in a recent review - "Desiccation tolerance of prokaryotes[1]." The latter is the only appraisal of the topic to have appeared within the past three decades. The review defines the utility and importance of prokaryotic cells in the study of anhydrobiosis, and èstablishes the cyanobacterium *N. commune* - largely through the studies annotated in this thesis - as the prokaryotic model for the anhydrobiotic cell. The review considers why the role of water in cell structure and function continues to mystify and to confound, and it does so from the different perspectives that may be employed by biophysicists, protein chemists, biochemists, cell biologists, and microbial ecologists and physiologists. While the review considers much to do with desiccation tolerance, it is neither a summary of conclusions nor is it a wealth of answers rather, it is a consolidation of the many problems, some questions, and the inaccuracies and myths - it is the first crack in the opening of a large, and a ponderous, door.

The emerging phylogeny of the Bacteria, Archaea and Eucarya has provoked, and continues to sustain, an intense fascination with the nature of ancient cytoplasm and its paleophysiology. The deepest branches within the Prokaryota - the present roots of the Archaea and Bacteria - are defined by the nucleotide sequences of the small-subunit rRNA's of thermophilic microbes and it is thought, and it is argued compellingly, that "life arose in a very

warm environment." The seeking out of novel - hopefully old - prokaryotes, from the most inhospitable of our planet's environments, has taken on an impetus that can be rationalized in simple terms - there may exist representatives of old, unbroken lineages, that stretch from the earliest segment of the Archean. Today, the term extreme, or inhospitable, tends to be equated with ancient. Investigations of hot brines and salterns, caldera, sulfurous sea vents, boiling mud pots, and the like, have provided a source of organisms whose physiologies have come to shape our ideas as to the likely forms of primitive life. It is generally stated, and it is perhaps even widely assumed, that the new era of genome sequencing can answer all the nagging questions to do with the origins of cellular functions. Such optimism, and it is optimism, is at odds with the fact that even understanding the form and structure of cytoplasm in extant living cells remains one of the most intractable, and controversial, problems in cell biology. Certain of the ideas and questions presented in the recent review[1] have been used to speculate upon the role of desiccation tolerance in the evolution of primitive life, and to define the concept of the eoanhydrobiote[2]. It was argued that desiccation tolerance, as a cellular property, was consistent with the inordinately slow (hypobradytelic) rate of change (evolution) that is thought to have occurred within the Cyanobacteria. In this respect a completely new perspective has been provided with which to consider the constraints enjoyed by early life, the places to seek them out, and the means to study them.

[1] 1994 - Potts, M. Desiccation tolerance of prokaryotes. **Microbiol. Rev**. 58:755-805.
[2] 1995 - Potts, M. Ancient prokaryotes - water, water, everywhere? **ASM News** in press.

The studies that lead to the development of *N. commune* as a system to study desiccation tolerance, and the studies that established the molecular ecology and cell biology of this cyanobacterium are summarised below.

Identification of A Research Problem

In the early 1970's, any appreciation of desiccation tolerance that one derived from the current literature tended to rely upon a good deal of phenomenological and anectodal observations of microbial communities growing in situ. One publication is presented here[3]; it remains the only publication presented from my Ph.D. studies and it illustrates the rather superficial state of the subject at that time. During the late 1970's the significance of desiccation as an environmental stress parameter for marine microbial communities was documented, and these studies extended the earlier suggestions that desiccation was important in modulating in situ nitrogen fixation[4,5].

None of the books or reviews on cyanobacteria (blue-green algae) that appeared during the 1970's to 1990's make more than a passing reference to desiccation tolerance, if they do so at all. It was this singular lack of data on such an important biological problem topic that prompted the work effort that followed.

3 1979 - Whitton, B. A., A. Donaldson and M. Potts. Nitrogen fixation by *Nostoc* colonies in terrestial environments Aldabra Atoll, Indian Ocean. **Phycologia** 18:278-287.

4 1979 - Potts, M. Nitrogen fixation (acetylene reduction) associated with communities of heterocystous and non-heterocystous blue-green algae in mangrove forests of Sinai. **Oecologia (Berl).** 39:359-373.

5 1980 - Potts, M. Blue-green algae (Cyanobacteria) in marine coastal environments of the Sinai Peninsula; distribution, zonation, stratification and taxonomic diversity. **Phycologia** 19:60-73.

Development of the Procaryotic Model for the Anhydrobiotic Cell

The development of an isopiestic water equilibration method for clonal axenic isolates of bacteria, and its use in the measurement of physiologically-relevant properties of cells undergoing water stress, was first described for coccoid cyanobacteria[6]. Subsequently, three novel methods to achieve immobilisation and controlled desiccation of cell suspensions, including those of coccoid and filamentous forms, were developed[7-9]. These methods made it

possible to manipulate cells using air-drying - an environmentally-significant stress parameter - as a water stress. These studies identified the acute sensitivity of the nitrogenase complex to drying, as well as the relative insensitivity of the intracellular ATP pool to the same water stresses. These studies were the first to identify the discrete and stepwise lags in the recovery of metabolic functions which occur upon rehydration of desiccated cyanobacteria. These data provided the first indication that de novo protein synthesis accompanied rehydration of desiccated material and, in addition, showed that the cyanobacterial heterocyst is more sensitive to drying stress than vegetative cells[8]. However, the latter study also provided evidence that countered earlier suggestions in the literature that heterocysts (structurally- and biochemically-modified cells that provide an environment conducive to oxygen-sensitive nitrogenase activity) are unable to perform osmoregulation[8].

The application of methods that permitted immobilisation of cells and their subjection to water stress, together with the development of techniques to isolate polysomes from these same cells, provided evidence that the protein synthesizing complex remains intact during short-term matric water stress[9-11]. The first indications that protein stability may be a basic mechanisms of desiccation tolerance were obtained in experiments that failed to demonstrate any accumulation of labelled proteins in cells that were allowed to incorporate ^{35}S-labelled sulphate before and during their exposure to rapid air-drying[12]. These same experiments provided evidence of differential protein stability, namely the light-dependent sensitivity of phycobiliprotein complexes. A methodical approach to the characterization of drying-sensitive and drying-insensitive proteins lead to the characterization and description of the two-dimensional protein index of *N. commune* UTEX 584[13]. The concept of differential susceptibilites (stabilities) of proteins in anhydrobiotic cells is developed and discussed at length in ref. 1.

The response of cyanobacteria to desiccation, at least as far as lipid composition is involved, is different to that reported for higher plants and desiccated materials contain no detectable levels of cyclopropane fatty acids[14]. The latter accumulate in *E. coli* cells as they enter lag phase, and

it is thought that their synthesis may reflect a general strategy for responding to stress. Subsequent studies lead to the characterization of the fatty acid contents of the cell membrane and the measurement of the kinetics of fatty acid turnover upon cell rehydration (see below). Publication 14 describes the first attempts to use field materials of *N. commune* for the detailed biochemical analyses - and all the inherent problems that this meant - that subsequently provided such important information on the molecular ecology of this microorganism.

[6]1981 - Potts, M. and E. I. Friedmann. Effects of water stress on cryptoendolithic Cyanobacteria from hot desert rocks. **Arch. Microbiol.** 130:267-271.

[7]1984 - Potts, M., M. A. Bowman and N. S. Morrison. Control of matric water potential (ψ_m) in immobilized cultures of cyanobacteria. **FEMS Microbiol. Lett.** 24:351-354.

[8]1985 - Potts, M. and M. A. Bowman. Sensitivity of *Nostoc commune* UTEX 584 to water stress. **Arch. Microbiol.** 141:51-56.

[9]1986 - Potts, M. and N. S. Morrison. Shifts in the intracellular ATP pools of immobilized *Nostoc* cells (Cyanobacteria) induced by water stress. **Plant and Soil** 90:211-221.

[10]1986 - Angeloni, S. V. and M. Potts. Purification of polysomes from a lysozyme-resistant desiccation-tolerant cyanobacterium. **J. Microbiol. Methods** 6:61-69.

[11]1986 - Angeloni, S. V. and M. Potts. Polysome turnover in immobilized cells of *Nostoc commune* (Cyanobacteria) exposed to water stress. **J. Bacteriol.** 168:1036-1039.

[12]1985 - Potts, M. Protein synthesis and proteolysis in immobilized cells of *Nostoc commune* UTEX 584 (Cyanobacteria) subjected to water stress. **J. Bacteriol.** 164:1025-1031.

[13]1986 - Potts, M. The protein index of *Nostoc commune* UTEX 584 (Cyanobacteria): changes induced in immobilized cells by water stress. **Arch. Microbiol.** 146:87-95.

[14]1987 - Potts, M., J. J. Olie, J. S. Nickels, J. Parson and D. C. White. Variation in the phospholipid ester-linked fatty acids and carotenoids of desiccated *Nostoc commune* (Cyanobacteria) from different geographic locations. **Appl. Environ. Microbiol.** 53:4-9.

Molecular Biology of *Nostoc commune*

At the time work on the molecular biology of *N. commune* was initiated, detailed studies on the molecular biology of cyanobacteria were both meagre and restricted to only a few fast-growing strains. The studies presented here established the molecular biology of *N. commune* UTEX 584[15-17], specifically they identified the susceptibiities of DNA to light-induced desiccation[15] and changes in the extent of 6-methyladenine and 5-methylcytosine

modification[17], characterized one of the few systems available for the in vitro translation of cyanobacterial mRNA using an homologous system [16], and provided methods of widespread utility for the purification and analysis of extrachromosomal DNA's[18].

More specifically, two approaches were followed to identify molecular mechanisms involved in desiccation tolerance. The first was to employ *nif* probes and Nif antibodies as markers for water stress-induced changes in transcription and translation, respectively. Previous studies had shown that nitrogenase activity was rapidly curtailed upon subjection of cells to limited water deficit (see ref. 1 for example) therefore the *nif* system seemed to have the potential for use as a marker of a target of water stress. The objective of the second approach was to understand how the transcription of the genes encoding components of the DNA-dependent RNA polymerase (RNA-P) responded to water-deficit. RNA-P is the pivotal component of the transcription apparatus and it was felt that an understanding of the response of *rpo* transcription and *rpo* mRNA translation, in cells subjected to water deficit, could provide important information on the response of global gene regulation to desiccation.

Despite keen competition from the Chicago group our laboratory was the first to isolate, clone and sequence the genes (*rpoC1C2*) which encode the large subunits of RNA-P from any photosynthetic prokaryote[19]. Evidence was provided for the divergent evolution of the eubacterial RNA-P, and it was demonstrated that the organization of cyanobacterial *rpo* genes has been retained, with modifications, in the genomes of plant chloroplasts. Subsequently, it was shown that the *rpoBC1C2* gene cluster in *N. commune* UTEX 584 does not constitute an operon[20]. The latter is a particularly important finding - cotranscription of *rpoBC* genes and their counterparts is found in plant chloroplasts, Archaea and other eubacteria (*E. coli*). With respect to the latter, the cotranscription of *rpoBC* is a central feature of translational autoregulation of gene expression in *E. coli.* As such, these findings are of fundamental importance to the understanding of the evolution of transcription and its regulation. Furthermore these studies did achieve the insight into desiccation-induced changes in gene expression that was

hypothesized at the outset of the studies. Immobilization and short-term air-drying of *N. commune* UTEX 584 leads to a complete depletion of its cellular *rpoC1C2* mRNA pool[21]. This mRNA is required for the synthesis of the γ and β' subunits of the RNA-P. In contrast RNA-P remains stable in cells during long-term desiccation as judged from immunoblotting analyses of protein extracts using RNA-P core-specific antibodies, and the a subunit as a marker for the enzyme. It is the extant RNA-P holoenzyme in air-dried cells that drives the rapid de novo transcription of *rpoC1C2* which ensues in response to cell rehydration[21]. Parallel experiments utilizing *nif* probes, and antibodies directed against Fe protein of nitrogenase, have provided similar results. The Fe protein has been detected in a stable form in cells that have remained desiccated for more than a decade (see below) - in contrast note the sensitivity of the phycobiliprotein complexes (see above). More recently, evidence for structural and functional stability of the enzymes involved in lipid biosynthesis, as well as glycerol-, sulphate- and phosphate-uptake, has been obtained[22]. All of these data confirm the initial expectations that the ability to maintain protein stability is one mechanism that contributes to desiccation tolerance. Again, ref. 1 explores these considerations in greater depth.

One significant achievement of the studies described in this section is the development of an understanding of the molecular biology of an ecologically-significant, cosmopolitan prokaryote which is extremely difficult to manipulate under laboratory conditions.

15 1987 - Stulp, B. K. and M. Potts. Stability of nucleic acids in immobilized and desiccated *Nostoc commune* UTEX 584 (Cyanobacteria). **FEMS Microbiol. Lett.** 41:241-245.

16 1988 - Jäger, K. and M. Potts. In vitro translation of mRNA from *Nostoc commune* (Cyanobacteria). **Arch. Microbiol.** 149:225-231.

17 1988 - Jäger, K. and M. Potts. Distinct fractions of genomic DNA from the cyanobacterium *Nostoc commune* that differ in the degree of methylation. **Gene** 74:197-201.

18 1989 - Xie, W.-Q. and M. Potts. Quick screening of plasmid deletion clones carrying inserts of the desired sizes for DNA sequencing. **Gene Anal. Techn.** 6:17-20.

19 1989 - Xie, W.-Q., K. Jäger and M. Potts. Cyanobacterial RNA polymerase genes *rpoC1* and *rpoC2* correspond to *rpoC* of *Escherichia coli*. **J. Bacteriol.** 171:1967-1973.

20 1991 - Xie, W.-Q. and M. Potts. Gene cluster *rpoBC1C2* in cyanobacteria does not constitute an operon. **Arch Biochem. Biophys.** 284:22-25.

21 1995 - Xie, W.-Q., D. Tice and M. Potts. Cell water deficit regulates expression of *rpoC1C2* (RNA polymerase) at the level of mRNA in desiccation-tolerant *Nostoc commune* UTEX 584 (Cyanobacteria). **FEMS Microbiol. Lett.** in press.

22 1993- Taranto, P., T.W. Keenan and M. Potts. Rehydration induces a rapid onset of lipid biosynthesis in desiccated *Nostoc commune* (Cyanobacteria). **Biochim. Biophys. Acta** 1168:228-237.

Fundamental Contributions to Cell Biology

An availability of nitrogen and phosphate determines the distributions and activities of microbial communities and phosphate is known to regulate a wide and diverse range of cellular functions. Because *Nostoc commune* typically becomes abundant in nutrient-depleted habitats it was decided to examine whether this cyanobacterium synthesises any extracellular phosphatase enzymes. A totally novel approach was taken in this endeavour. A gene library of *N. commune* UTEX 584, that had been constructed in λgt 10, was plated in the presence of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) - a non-specific substrate for phosphomonoesterases that generates indigo-carmine (a coloured product) upon hydrolysis of the phosphoester bond in the presence of oxygen. One blue plaque was recovered from a plating of around 20,000 plaques. An *Eco* RI fragment was subseqently recovered, subcloned in a plasmid vector, and it was found that this fragment directed the synthesis of a secreted *i*ndole *p*hosphate *h*ydrolase (IphP)[23]. In retrospect this finding has all the elements of serendipity: λgt 10 is not an expression vector, and the fact that the gene product is correctly processed, secreted beyond the outer membrane of *E. coli,* and enzymatically-active, is remarkable. Upon DNA sequence analysis of *iphP* it was noted that a portion of the derived amino-acid sequence of IphP bore a striking resemblance to the consensus active site domain of the eukaryotic protein tyrosine phosphatases (PTPase's) - a group of enzymes implicated in the control of cell transformation and oncogenesis in humans. Upon purification and characterization of IphP it was found that the enzyme did indeed possess PTPase activity, as well as a secondary protein serine phosphatase activity[24] - such dual specificity is a property that

is shared with the VH1 gene product of vaccinia virus. In summary, IphP of *N. commune* UTEX 584 is the first example of a PTPase of genuine prokaryotic ancestory. This finding raises fundamental questions as to the origin and role of tyrosine phosphorylation - a fact that was discussed at length in a subseqent short review on protein phosphorylation in prokaryotic cells[25].

During the course of studies dealing with the effects of water stress on *nif* expression, the *nifUHD* gene cluster of *N. commune* UTEX 584 was isolated and a structural analysis was obtained[26]. The *nifU, nifH* and *nifD* genes have been isolated from a range of different organisms and, in the cyanobacterium *Anabaena* sp. PCC 7120 where the three genes are contiguous in the chromosome, the cluster and its divergent transcription have been studied in detail by the Chicago group. However, in *N. commune* UTEX 584 a novel ORF was identified between *nifU* and *nifH*.[27] The ORF had the potential to encode a protein of approximately 11 kDa and the derived amino acid sequence showed marked sequence similarity with the myoglobins of two lower eukaryotes, the cilliated protozoa *Paramecium caudatum* and *Tetrahymena pyriformis*. Subsequent purification and characterization of the recombinant protein confirmed that it was a hemoprotein and it was given the trivial name cyanoglobin[28]. The fact that *glbN* is located between two *nif* operons raised the possibility that the gene product may play a role in nitrogen fixation. Experiments did demonstrate that cyanoglobin synthesis was induced only in cells that had been starved of nitrogen, under microaerobic conditions. Recently, it has been demonstrated that cyanoglobin synthesis occurs only in the heterocyst (unpublished data). At present, the role of cyanoglobin remains cryptic. For many years there has been some speculation that certain proteins may provide some protection to nitrogenase and, more specifically, that particular hemoproteins may be involved in the maintenance of the oxygen tension in heterocysts. Our data provide the first, and at this time, the only definitive indication of a potential role for a specific hemoprotein, a hemoglobin, in nitrogen fixation by a cyanobacterium.

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Mechanisms of Desiccation Tolerance

Cell Structure

The first description of the structural properties of desiccated cyanobacteria was for those isolated from hot-desert rocks[29]. The thoroughness of that characterization permitted the data to be used as the type description of the genus *Chroococcus* in the recent edition of Bergey's Manual of Determinative Bacteriology - the international standard reference text for bacterial taxonomy, systematics and nomenclature. Subsequent studies with *Nostoc commune* described techniques to permit the fixation and preparation of desiccated cells that avoided artifacts and permitted immunocytochemistry[30-32]. Application of the latter technique confirmed that the water stress proteins (Wsp) of *N. commune* were secreted proteins, intimately associated with the extracellular glycan. The finding that Fe protein of nitrogenase was stable in cells desiccated for more than a decade complemented studies directed at the analysis of protein stability[31]. The recent characterization of the extracellular glycan of *N. commune* is a thorough and definitive description of the structure of a cyanobacterial sheath complex[32]. The latter, as described in ref. 1, may represent a central mechanism for desiccation tolerance. Ref 32 has provided the framework for recent studies that have since demonstrated a glass transition in water-plasticized

colonies of *N. commune* (unpublished data).

A purification of the cytoplasmic membrane of *Nostoc commune* UTEX 584 through subcellular fractionation and its characterization is one of only a few such studies of cell membranes from cyanobacteria[33]. The cytoplasmic membrane of *N. commune* UTEX 584 contains an unusually high concentration of 20:3ω3 fatty acid (some 58%) - a feature that would be expected to contribute susbstantially to membrane fluidity during desiccation. The significance of these data is reviewed in ref 1.

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Water Stress Proteins (Wsp)

Characterization of desiccated colonies of *Nostoc commune* identified a novel class of acidic polypeptides - these were termed water stress proteins (Wsp) because they constitute some 70% of the total soluble protein of desiccated cells. Wsp polypeptides are highly stable in desiccated cells, and they are induced in liquid grown cultures if those cells are subjected to multiple cycles of drying[34]. Initially, it was not appreciated that Wsp proteins are secreted by *N. commune*, but it was noted that the accumulations of Wsp in laboratory grown cultures never acheved those present in field materials. Ref. 34 provides a preliminary characterization of Wsp. Recently, it has been shown that Wsp polypeptides are secreted, - a totally unexpected finding

given the concentrations of these proteins, - they associate with a 1,4-β-D-xylanxylanohydrolase activity that is also secreted from the cells, they form complexes with secreted UV-absorbing pigments through salt-dependent ionic interactions, and they are immunologically-related to several carbohydrate-modifying enzymes including beta endogalactosidase[35]. At this point it can be noted that there are conspicuously few characterizations of those proteins that are secreted by cells of cyanobacteria (see also the discussion of IphP above). The solving of the structure of the E_{335} carboydrate-containing chromophore of the UV-absorbing pigment, completed in the laboratory of S. Scherer, has shown that it contains xylose. In contrast, xylose is absent from the purified extracellular glycan of *N. commune*. These data, and those provided in ref. 35, offer the first indications that the role of Wsp may be to do with the synthesis and/or modification of carbohydrate-containing components which are secreted from the cells. Further evidence has been obtained to substantiate this idea. An 8-kb *Eco* RI fragment was isolated recently from a library of *N. commune* UTEX 584 DNA in pTrc 99A. The fragment was contained in a clone that showed intense cross-reaction with Wsp antibodies. Of the 5 ORF's that have been identified, one shows conspicuous derived amino acid sequence similarity to proteins involved in the secretion of cyclic glucans by *Agrobacterium tumefaciens* (as well as HetA from *Anabaena* PCC 7120 - a protein involved in the development of the heterocyst carbohydrate wall layer). The other ORF's all show correspondence with carbohydrate-modifying enzymes (unpublished data).

The emerging picture is that the capacity to maintain proteins in a functionally-stable state is a central feature of the desiccation tolerance of *N. commune* [35,36] - a point discussed in detail in ref.1 For example, the onset of the synthesis of all classes of lipid in desiccated cells resumes upon rehydration, instantaneously[22]. The further characterization of the Wsp proteins of *N. commune*, and their corresponding genes, will shed further light on the molecular basis for desiccation tolerance in the cyanobacterial, anhydrobiotic cell.

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EPILOGUE

The anhydrobiotic cell is characterized by its singular lack of water - with contents as low as 0.02 g H_2O g dry wt-1. These levels are orders of magnitude lower than those which are found either in bacterial spores or in cells subject to acute salt stress. At such low levels the monolayer coverage by water of macromolecules, including DNA and proteins, is disturbed and depleted. As a consequence the mechanisms that confer desiccation tolerance upon air-dried bacteria are markedly different from those, such as the mechanism of preferential exclusion of compatible solutes, that preserve the integrity of salt-, osmotically, and freeze-thaw-stressed cells. Desiccation tolerance reflects a complex array of interactions at the structural, physiological and molecular levels. Many of the mechanisms remain cryptic, but it is clear that they involve interactions, such as those between proteins and co-solvents, that derive from the unique properties of the water molecule. A water-replacement hypothesis accounts for how the non-reducing disaccharides trehalose and sucrose preserve the integrity of membranes and proteins. Nevertheless, we have virtually no insight as to the state of the cytoplasm of an air-dried cell. There is no evidence for any obvious adaptations of proteins that can counter the effects of air-drying, nor is there any indication of the occurrence of any proteins that provide a direct and a tangible contribution to cell stability. The long term goal of this research is to uncover the mechanisms for desiccation tolerance. That goal will be realised in large measure through the accumulation of information on *Nostoc commune* and derivative strains. The approach is not without obstacles - a transformation system for these strains has yet to be developed. The task of finding such a system has not proved to be an easy one, but it is also not an impossible one, and current progress suggests that that task will be accomplished. The need to develop such a system is obvious - *N. commune* provides a unique opportunity to resolve a long-standing problem in cell biology.

NOTE ON AUTHORSHIP

Authors on all publications have been identified (graduate student, undergraduate student, technician etc.) in the list of publications provided in the curriculum vitae. First authors either took full responsibility for the writing of the primary manuscript, provided the bulk of the data upon which the manuscript was written and/or made the discovery that permitted the further progress of the research. In most cases two or all three of these classifications apply to the primary publications that follow this text.

PUBLICATIONS BY CATEGORIES SPECIFIED IN THE TEXT

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Desiccation Tolerance of Prokaryotes

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INTRODUCTION

—they began to fit into one picture with the missing piece that small but plentiful entity—water.

Sydney J. Webb
Bound Water in Biological Integrity (403)

There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable. There is another theory which states that this has already happened.

Douglas Adams
The Restaurant at the End of the Universe (3)

The removal of water from cells, the storage of cells in the air-dried state, and the rewetting of air-dried cells impose physiological constraints that few organisms can tolerate. No single group of organisms has monopolized the capacity to withstand air drying, and desiccation tolerance has been recorded for bacteria, higher and lower plants (including their seeds), insects, yeasts, fungi and their spores, and crustacea (67, 81, 83). Our present understanding of the anhydrobiotic cell derives, in large part, from studies with mycophagous nematodes, rotifers, tardigrades, the star moss *Tortula ruralis*, parasitic protozoa including microsporidia, the anostracan crustacean *Artemia salina*, plant seeds and pollen, the resurrection plants *Selaginella lepidophylla* and *Craterostigma plantagineum*, and the cyanobacterium *Nostoc commune* (74, 75, 106, 171, 239, 266, 267). Among the invertebrates, only the echinoderms appear to lack representatives, while none of the reports of desiccation tolerance in vertebrates has withstood critical scrutiny (68). The role of water in the structure-function relationships of anhydrobiotic cells has been inferred, in some cases, from biophysical studies with single purified proteins (53, 54, 56), while ecological studies, for the most part, include a good deal of phenomenological and anectodal observations that shed little light on the mechanisms of desiccation tolerance. The selective pressure of a water deficit is likely to have impinged upon prokaryotes at a very early stage of their evolution. More so than any other boundary/interface effect, the removal of cell-bound water through air drying and the addition of water to air-dried cells are the predominant forces that influence the distribution and activities of bacterial communities. Some bacteria can cope with these water problems, but many cannot. Why? The question has attracted surprisingly little attention. Considerations of the water relations of bacteria have been confined almost exclusively to osmotic systems where cells are immersed in solvent-solute mixtures (58, 85, 86). Curiously, despite its intrinsic importance and ecological significance, desiccation of bacteria as a major stress parameter seems to continually escape the critical attention of bacteriologists (e.g., see references 62, 150, and 330). As a consequence, there has been no review of the topic of desiccation tolerance in bacteria within the past quarter century—a period that has witnessed the formal recognition of the dichotomy between prokaryotes and eukaryotes (369) and the emergence of the archaebacteria (98). This review considers how and why air drying can induce water stress in prokaryotic cells and how and why some prokaryotic cells tolerate that stress. Vegetative cells are the primary focus of these considerations for reasons that will become apparent. The resilience of bacterial spores (111, 149) is referred to in comparative terms only. Osmotic stress in bacterial cells is the topic of recent reviews (85, 86) and is considered here only from the perspective that an osmotic stress is one consequence of the initial stages of the air drying of cells. To aid our understanding of the nature of dry bacterial cells, some reliance has been placed upon the data available for other anhydrobiotic cell models. If there is an emphasis in this present appraisal, it is with the cyanobacteria and with one form in particular, the terrestial nitrogen-fixing form *Nostoc commune*, which has become a very useful model with which to study the desiccation tolerance of prokaryotic cells (see reference 296 and references therein).

WATER RELATIONS AND THEORETICAL CONSIDERATIONS

At first glance, then, the topic of this review seems complex in view of the numerous biophysical and physiological components that contribute to, interact in, and require consideration with respect to desiccation tolerance. But the true complexity is this—everything must be explained from the perspective of one component, water, and the analysis of single-component systems is notoriously difficult. Water molecules are critical components of reaction mechanisms; they contribute to the stabilities of proteins, DNA, and lipids; and they confer a structural order upon cells. What properties of water make it so uniquely suited to the diverse roles it plays in cell processes? In large part, the properties of water reflect the dipole that results from the greater electronegativity of the single oxygen atom over the two hydrogen atoms in each molecule. Other molecules have electronic structures related to water, but water is singular as a liquid because of its ability to form three-dimensional networks of molecules that are mutually hydrogen bonded (420). Inelastic incoherent neutron-scattering spectra have provided evidence for the existence of two different kinds of hydrogen bond, of different strengths, in ice (226). The fact that electrons in sp^3 orbitals of oxygen atoms can easily be rehybridized to respond to the relative configurations of adjacent molecules may account for the two types of hydrogen bond. Spectra of thin films of water suggest that only hydrogen bonds of the strong variety are present, and these strongly hydrogen-bonded clusters should have all the properties reported for vicinal water (226). The formation and breakage of hydrogen bonds between different water molecules occurs on a timescale of approximately 1 ps—this is almost three times faster than the rotational relaxation time of the water molecule. As a consequence, molecules of water continually and rapidly shift positions through electrostatic and hydrogen bond interactions (214, 323). For model solutions of small sugars at room temperature, nuclear magnetic resonance and dielectric relaxation measurements have shown that the residence time of a given water molecule at a solvation site (e.g., a hydroxyl group on a sugar) is extremely short, i.e., 1 ns (see reference 357 and references therein). "Bound" water molecules are, in fact, highly mobile. Within the bulk phase, molecules separated by approximately 10 Å (1 nm) have no statistical interaction. Even at temperatures as low as −40°C (the homogeneous nucleation temperature for ice in pure water), a minimum of around 200 water molecules must associate within a domain of about 40 Å (4 nm) to form a critical nucleus that will grow spontaneously into an ice crystal. Thus, smaller numbers of water molecules would require temperatures much below −40°C (or heterogeneous catalysts) for ice crystals to grow (see reference 357 and references therein).

It has not escaped attention that water may have played a determinative role in the origin and evolution of the genetic code (425). The physical properties of water are discussed at length by Nobel (259) and elsewhere (159, 214, 323), and the implications of some of these properties for a number of cell processes have been considered critically by Wiggins (420). A brief discussion of some of these properties is provided here to give some perspective to the causes and consequences of a cell water deficit.

Properties of Water

The energy required to free a population of water molecules from a liquid phase and to move those molecules to an adjacent vapor (gas) phase, without a change in temperature, is the heat of vaporization (H_{vap}). Water has the highest H_{vap} value for any known liquid and, as such, is the only nonvolatile solvent found in cells in appreciable amounts (236). At 25°C, the evaporation of 1 mol of water requires 44.0 kJ. Most of this

energy is needed to disrupt hydrogen bonds—much of the remainder is needed to overcome van der Waals forces and to account for the relative expansion upon the transition from a liquid to a gas. For bacterial cells far removed from the air-water interface, the effects of vaporization are probably negligible. However, H_{vap} is of significance to cells or colonies in contact with films of water or in microdroplets, because a substantial heat loss accompanies the evaporation of water. Consider a single bacterial rod (2 by 1 μm) entrapped upon leaving an air-water interface in a 10-μm-diameter spherical droplet of pure water. The droplet contains approximately 30 μmol of free water. The amount of energy required to evaporate this water, over a physiological range of temperatures (20 to 50°C), is 1.27 J or approximately 0.3 cal. Clearly, the bacterium not only is dried upon movement of the droplet into the gas phase but also is cooled, and, what is more, these events take place extremely rapidly (see below). Aerophytic, epiphytic, and epidermal bacteria and those bacteria of communities in association with solid substrata are, to a greater or lesser degree, subject to these effects of H_{vap}.

As water molecules are brought from the interior of an aqueous phase to the air-water interface, energy is required to increase the surface area and to disrupt hydrogen bonds. The amount of energy required to expand a surface by unit area is the surface tension. The surface tension, or surface free energy σ_w, at an air-water interface is 0.0712 N m^{-1} or 7.12×10^{-8} MPa m (at 30°C). Surface tension is responsible for keeping liquid water (but not water vapor) out of bacterial intracellular protein gas vesicles (397), responds to the quantities of dissolved solutes, and therefore contributes to cell surface hydrophobicity. The surface tension of aqueous solutions is slightly influenced by the composition of the adjacent gas phase but is markedly affected by solutes. Certain solutes, such as sucrose or KCl, do not preferentially collect at air-liquid interfaces and consequently have little affect on σ_w. Fatty acids and lipids, however, may concentrate at interfaces and thus can markedly reduce σ_w. Salts of fatty acids (soaps) or denatured proteins with hydrophobic side chains may collect nearly exclusively at interfaces and reduce σ_w to less than one-third of the value for pure water. The biosurfactants of bacteria are usually complex lipids that participate in the solubilization of hydrophobic substances to aid in their assimilation (245).

Chemical Potential

The activity of water (a_w) is related to its concentration through an activity coefficient (γ_w), where $a_w = \gamma_w N_w$ (N_w is the mole fraction of water in the system). The chemical potential of water (μ_w) in a system is expressed according to the following equation:

$$\mu_w = \mu_w^* + RT \ln a_w + \overline{V}_w P + z_w FE + m_w gh \quad (1)$$

In equation 1, the term $RT \ln a_w$—the activity term (where R is the gas constant)—gives the water activity term the units of energy per mole. $\overline{V}_w$ is also the partial molal volume of water, i.e., in a bacterial cell, in contrast to μ_w, which is the partial molal Gibbs free energy ($\delta G/\delta n_w$). $\overline{V}_w$ is the differential increase or decrease in the volume of a bacterial cell when a differential amount of water is added or removed, respectively, and it is expressed as the volume per mole. Pure water, or a very dilute solution, has a value of $\overline{V}_w$ equal to 18×10^{-6} m^3 mol^{-1}. P is the hydrostatic pressure in excess of the atmospheric pressure, so that the $\overline{V}_w P$ term in equation 1 reflects the effects of pressure on the chemical potential of water and is expressed, therefore, in energy per mole. $z_w FE$ is the electrochemical potential, and because water is uncharged ($z_w = 0$), the electrical term $z_w FE$ can be ignored. The gravitational term, $m_w gh$, represents the work needed to move a given mass per mole of water, m_w (18.016 g mol^{-1}), to a given height (h) under gravitational acceleration (g). Only under circumstances when cells are distributed at high altitudes throughout a water vapor can the term contribute significantly to the overall μ_w of the cell. μ_w^* is an additive constant and represents the chemical potential of water in a standard (ideal) reference state where $RT \ln a_w = 0$, $\overline{V}_w P = 0$, $z_w FE = 0$, and $m_w gh = 0$. For practical purposes, one compares the chemical potentials of cells with different intermediate water contents, say those of a dried bacterial cell (μ_w^D) and a cell at some stage of rehydration (μ_w^R). During comparison of these two chemical potentials, the two μ_w^* terms cancel out.

Osmotic Pressure and Osmolarity

Bacterial cells contain finite amounts of dissolved solutes, with exceptions (the cytoplasm of *Halobacterium* spp. accumulates KCl to 5 M [327]), and any consideration of transitions in the water potentials of these cells requires an appreciation of osmotic pressure. The addition of a solute to a pool of water causes a net displacement of the water molecules. The decrease in the partial molal volume depends on the amounts of solute that go into solution and on the extent to which they do so. The lowering of μ_w causes a_w to decrease, and the $RT \ln a_w$ term in equation 1 becomes more negative. Concomitant with this decrease in μ_w, there is also an increase in the osmotic pressure (Π), which is attributable to the addition of a species of solute (j). As bacterial cells undergo changes in the amounts of water (and thus in the net concentrations of solutes) that they contain, the equilibrium is continuously shifted to one of higher or lower osmotic pressure with concomitant changes in a_w. Equation 2 relates water activity to osmotic pressure:

$$RT \ln a_w = \overline{V}_w \Pi \quad (2)$$

The osmotic potential, Ψ_Π, is one component that contributes to the overall water potential (Ψ; see below) of the system and is numerically equal to the prevailing osmotic pressure (Π) but has a negative sign (in units of bars, kilopascals, or megapascals; 1 bar = 100 kPa = 0.1 MPa). The two terms "osmotic pressure" and "osmotic potential" are often a source of some confusion, and the reader is directed to the more-detailed discussions and appraisals by Nobel (259) and Wiggins (420).

A more familiar relationship used to define osmotic pressure due to solutes (Π_s) combines equations 1 and 2 and is referred to as the Van't Hoff relation:

$$\Pi_s \approx RT \Sigma_j c_j \quad (3)$$

At low concentrations of solute (0.1 M) molarity and molality are virtually equivalent for a given solute, whereas at high solute concentrations the molarity is numerically less than the molality. Osmotic pressure increases linearly with increasing concentrations of a solute, but different slopes of Π_s versus dissociation are obtained for different electrolytes (e.g., NaCl) and different nonelectrolytes (e.g., sucrose). Osmolality refers to the mole fraction of active particles of the solute per kilogram of water and differs for given salts depending upon their degree of dissociation in water. The osmotic pressure of a 0.1 osmolal (i.e., 100 mmolal) solution can be calculated as follows: $R = 8.3 \times 10^{-6}$ m^3 MPa mol^{-1} K^{-1}, and T = temperature on the Kelvin scale, e.g., 37°C = 310 K; thus, RT at 37°C = 0.002573 m^3 MPa mol^{-1}. According to equation 3, $\Pi_s = (0.002573)(100 \text{ mol } m^{-3}) = +0.26$ MPa. Note that the

osmotic pressure (which results from the addition of species j) has a positive value. The osmotic potential of that same solution ($\Psi_{\Pi s}$) = −0.26 MPa. The chemical potential of water can therefore be rewritten as

$$\mu_w = \mu_w^* - \overline{V}_w\Pi + \overline{V}_wP + m_wgh$$

and, by rearranging, we find

$$(\mu_w - \mu_w^*)/\overline{V}_w = P - \Pi + m_wgh = \Psi$$

Water Potential

The water potential of a system (Ψ) is proportional to $\mu_w - \mu_w^*$, so that the term $\mu_w - \mu_w^*$ has considerable utility when the water relations of bacterial cells are compared. The term represents the work involved in moving 1 mol of water from some point in a system (at constant pressure and temperature) to a pool of pure water at atmospheric pressure and at the same temperature as the system under consideration (the gravitational term is ignored for reasons described above). A difference between two locations in the value of $\mu_w - \mu_w^*$ indicates that water is not in equilibrium, so there will be a net tendency for water to flow toward a region where $\mu_w - \mu_w^*$ is lower.

Matric Water Potential

A term frequently included, and often ignored, when defining the chemical potential of water is $\overline{V}_w\tau$, where τ is the matric water potential, or matric water pressure (259). This term generally is applied to considerations of water interactions at surfaces and interfaces and is therefore of prime importance in the present review. When water molecules are associated with interfaces such as the surfaces of colloidal particles (solid particles that range from ~0.002 to 1 μm in diameter, e.g., proteins, ribosomes, some bacteria, and viruses) in an aqueous solution, they have less tendency to react chemically in bulk solution or to escape to the surrounding vapor phase. Interfaces thus lower the thermodynamic activity of the water (a_w), especially near the surface of the colloid. Wiggins (420) noted that water equilibrates by increasing its density where the concentration of solutes is high and decreases its density where the concentration of solutes is low. Solutes, of course, also lower the a_w (they also influence surface tension [see above]). As an approximation, it is possible to consider these two effects that lower water activity as being additive in solutions containing solutes and colloids.

Osmotic pressure (Π) depends on the activity of water regardless of the reason for the departure of a_w from unity. Therefore, we can consider that $\Pi = \Pi_s + \tau$; hence, P is strongly affected by proteins and other colloids present in the solution. Although Π and a_w may be the same throughout a system, both Π_s and τ may vary. For example, water activity in the bulk of the solution may be predominantly lowered by the pressure of the solutes, whereas at or near the surface of colloids the main factor decreasing the a_w from unity could be the interfacial attraction and binding of water. Such interfacial interactions do not change the mole fraction of water, but they do reduce its activity coefficient, γ_w.

Water Vapor

Water molecules in aqueous solution continually escape into the surrounding gas phase for reasons discussed above. Water vapor is the third (generally) most prevalent gas in air, although its mole fraction is relatively low compared with the levels of O_2 and N_2 (259). The partial pressure exerted by the water vapor (in equilibrium) is the saturation vapor pressure. Vapor pressure at equilibrium depends on the temperature and the amount of solutes in solution. The water vapor content of air is markedly dependent on temperature, and at saturation it decreases nearly exponentially with temperature. For example, the relative humidity (RH) of air saturated at 20°C (100% RH) drops to 50% when the air is heated to 32°C at constant pressure. Changes of temperature are significant because they often lead to a condensation of water. For example, early-morning mists often occur in desert localities, and their associated water may be sufficient to trigger nitrogen fixation by bacterial communities in the locality (291). Heating of air at constant pressure therefore causes the RH to drop dramatically. As solutes are added to the liquid phase, the activity of water is lowered; therefore, fewer water molecules have a tendency to leave the solution to the vapor phase. Concentrated solutions of solutes are therefore useful for controlling the vapor pressure of the air with which they are in contact. Raoult's law states that for dilute solutions, the actual partial pressure of water vapor (P_{wv}) at equilibrium depends linearly on the mole fraction of water (N_w) in the liquid phase. For pure water $N_w = 1$ and P_{wv} has its maximum value P_{wv}^*, which is the saturation vapor pressure. The chemical potential of water vapor is μ_{wv}. In consideration of equation 1, then,

$$\mu_{wv} = \mu_{wv}^* + RT \ln (P_{wv}/P_{wv}^*) + m_{wv}gh \quad (4)$$

where P_{wv}^* is the saturation vapor pressure in equilibrium with pure liquid water at atmospheric pressure and at the same temperature as the system under consideration and m_{wv} is the mass per mole of water vapor, which is the same as the mass per mole of water, m_w. Therefore, the water potential of water vapor in a gas phase such as air is Ψ_{wv}, which is expressed as

$$\Psi_{wv} = (RT/\overline{V}_w) \ln (\%\text{RH}/100) + \rho_wgh \quad (5)$$

where ρ_w is the density of water. A convenient relationship used for the estimation of matric water potentials in such systems can be derived from equation 4:

$$\Psi_m = 1{,}065T \log P/P_0$$

where P/P_0 is simply a_w (%RH/100) and 1,065 is a derived constant (301).

Glasses

In intermediate-moisture systems, such as bacterial cells, most physical and chemical processes, perhaps with the exception of free radical-induced reactions, are under kinetic control; i.e., they are diffusion limited. As a consequence, the system, or the cell, within which these processes occur may be in a stationary state but not in equilibrium. To appreciate the inherent complexity of the aqueous cytoplasm in a bacterial cell, it is necessary to begin by considering "simple" systems. A solute, in water, can achieve a continuum of hydration states that range from the anhydrous solute to a solution of infinite dilution (pure water). Each of these hydration states has a characteristic temperature that defines the point of a kinetic (time- or frequency-dependent) material-specific change in physical state, from a glassy mechanical solid that is capable of supporting its own weight against flow due to gravity to a rubbery viscous fluid that can flow in real time (357). At temperatures below this glass transition temperature, $T < T_g$, diffusion-limited processes are inhibited by the extremely high local melt viscosity (η) and elastic modulus so that water is, in essence, immobilized and unavailable. In such a glass, molecular diffusion periods are greater than 10^5 s for rotation or

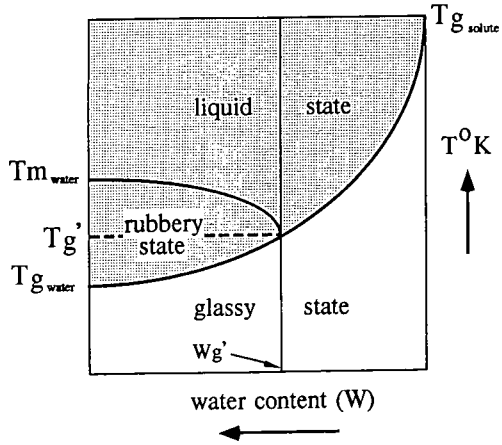


FIG. 1. Idealized phase diagram for a polymeric solute. See the text for details. Reproduced from reference 357 with the permission of authors and publisher.

translation through one molecular distance, i.e. 10^6 s nm^{-1} or around 300,000 years cm^{-1} (see reference 44 and references therein). Unlike a crystalline solid, however, a glass has surface characteristics that may lead to much lower water vapor pressures. The singular importance of water is that it acts as a mobility enhancer and serves to both increase the free volume and decrease the viscosity of a given solute system. The net effect of increasing the moisture content, W, at a constant temperature is equivalent to the net effect of increasing the temperature at a constant value of W. In each case there is a lowering of T_g (Fig. 1). The significance of this point is discussed further below. Different solutes and polymers have comparatively high values of T_g in their undiluted, anhydrous state. For example, starch and gelatin have T_g values around 200°C, while the T_g of anhydrous sucrose is 67°C. In contrast, the T_g of water is −137°C (44, 233), and this temperature marks the lower limit of state diagrams that describe the characteristics of aqueous glasses (Fig. 1). One further qualification required is that solute systems, including those encompassed by a cell membrane, typically are heterogeneous and may contain both crystalline and amorphous solid phases. If the crystalline phase is anhydrous, the water is physically confined to the amorphous domains. When the crystalline phase is also hydrated, the water may be distributed, in a nonuniform manner, between each of the different domains. The ease of migration of water through such a multiphase material depends in part on the properties of the crystalline-amorphous interface (357). At atmospheric pressure, those regions of the system which are in equilibrium can be described solely with respect to the two dimensions of temperature and composition. The description of regions which are not in equilibrium requires the consideration of a third dimension, time, expressed as t/τ, where τ is a relaxation time.

T_g' is an invariant point on the continuum (curve) of T_g values and represents the state-specific subzero T_g of the maximally freeze-concentrated, amorphous solute/unfrozen water matrix surrounding the ice crystals in a frozen solution. T_g' corresponds to, and is determined by, the point of intersection of the glass curve and the nonequilibrium extension of the equilibrium liquidus curve for the T_m of ice (T_m is the crystal-melting temperature). This solute-specific location defines the composition of the glass that contains the maximum practical amount of plasticizing moisture (W_g', with units of grams of unfrozen H_2O per gram of solute). The term "collapse" refers to the microscopic and macroscopic consequences that are manifest at around 20°C (note the difference between 20°C [a fixed temperature] and 20C° [a temperature range]) above the glass transition T_g, i.e., at T_c, the collapse transition temperature.

For nonequilibrium glassy and rubbery systems, mobility transitions can be described in terms of a dynamics map with axes of temperature, pressure, concentration, and time (Fig. 1). The glass transition is a second-order transition and is characterized by a change in the slope of the volume of expansion (the first-order derivative of free energy), a discontinuity in the thermal expansion coefficient, and a discontinuity in the heat capacity (a second-order derivative of the free energy [357]). In the low-viscosity highly fluid region, the coefficient of temperature dependence (activation energy) is a constant and a plot of log relaxation rate (viscosity) versus $1/T$ is a straight line. If the relaxation rate is viewed as the velocity of a reaction, this relationship is described by Arrhenius kinetics:

$$v = Se^{-\Delta E^*/RT} \quad (6)$$

where v is velocity (in this case relaxation rate or change in viscosity, η), S is a constant (collision or frequency factor [A] when chemical reactions are considered), E^* is the activation energy, R is the gas constant, and T is temperature in Kelvin. Taking the logarithm,

$$\ln v = (-\Delta E^*/R)(1/T) + \text{constant}$$

The logarithm of the velocity of a chemical reaction is a linear function of the reciprocal of the absolute temperature. In the glassy state, rates are, of course, much lower but Arrhenius kinetics nevertheless still apply. A quite different situation applies for the region between T_g to a point approximately 100C° higher than T_g or the crystalline melting temperature (T_m) for partially crystalline polymers. In this region, the dependence of viscoelastic properties on temperature is described by Williams-Landen-Ferry theory (WLF kinetics [357]):

$$\log_{10}[(\eta/\rho T)/(\eta_g/\rho_g T_g)] = -C_1(T - T_g)/[C_2 + (T - T_g)]$$

where η is the viscosity or another diffusion-limited relaxation process, ρ is the density, and C_1 and C_2 are coefficients that describe the temperature dependence of the relaxation process at temperatures above the reference temperature, T_g. C_1 is proportional to the inverse of the free volume of the system at T_g, while C_2 is proportional to the ratio of the free volume at T_g over the increase in free volume due to thermal expansion above T_g (i.e., a ratio of free volume at T_g to the difference between the volumes of the rubbery liquid and glassy solid state as a function of temperature above T_g). The significance of the WLF expression is that activation energy is, itself, temperature dependent such that a plot of log relaxation rate (or velocity) versus $1/T$ is curvilinear. Simply stated, this means that as ΔT increases, the faster the system is able to move, the greater is its mobility, and the shorter is the relaxation time. One consequence of this is that there is a change of 5 orders of magnitude in the rates of relaxation processes (such as viscosity) over a 20C° interval near T_g. A factor of 10 change in the rate of a diffusion-limited process above T_m, where $Q_{10} = 2$ (Arrhenius behavior), would require a 33C° change in temperature, in comparison with only a 3C° change for WLF behavior near T_g of a partially crystalline polymer (where $T_g/T_m = 0.67$). It is important to note that both synthetic and naturally occurring solute systems have been shown to follow WLF kinetics.

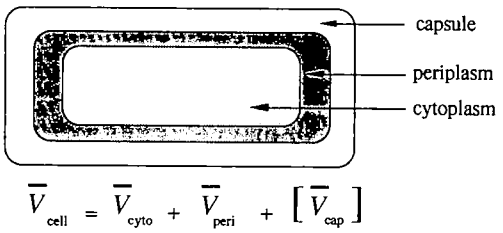


FIG. 2. Physical compartments of a prokaryotic cell. The capsule (or EPS) is a source of interstitial water that can be considered an additional compartment. Methods to calculate the volumes (V) of the different compartments are described in the text.

Glasses are expected to have lower water vapor pressures than the corresponding crystalline solid, and therefore they may add resistance to further dehydration of the system. The consequences of glass theory and WLF kinetics in desiccation kinetics will be discussed in a later section.

WATER INSIDE AND OUTSIDE CELLS

Living cells achieve a dynamic state that is characterized by temporal and transitory shifts in the net concentrations of intracellular water, salts, lipids, macromolecules, trace metals, and cofactors. These changes may be so subtle that they are virtually immeasurable or so extreme as to cloud any considerations of their ultimate cause. From a biophysical perspective it can be argued that bacterial cells, while not bags of enzymes, are membrane-bound bags of water. These bags of water also happen to contain on the average some 2,000 different proteins (some of which are present in up to 100,000 copies) (285), one or more chromosomes each of the order of several millimeters in length when uncoiled (104), around 600 different mRNAs, and 10×10^3 to 20×10^3 ribosomes (255). We have no notion how these components are dispersed throughout the aqueous solvent that represents some 70% of the wet weight of the cell. The question whether there is an inherent organization, an underlying structure, or a chaos inside cells has caught the attention of a number of groups with quite opposing views (65, 66, 235, 420). The topic is by no means resolved. In an attempt to visualize the interior of an *Escherichia coli* cell, "water (was) omitted, to clarify the distribution of macromolecules" (145)!

Water in Different Cell Compartments

How much water is in a bacterial cell? Independent measurements made since the 1950s on cells of heterotrophic and phototrophic forms have all provided rather similar values (255, 398, 403). One widely quoted assumption is that the protoplasm of *E. coli* contains 70% water and 30% solids (255), and recent studies by Cayley et al. (57) lend biophysical data to support this assumption. The water-accessible volume of the cytoplasm of *E. coli* K-12 strain MG 1655 ($\overline{V}_{cell}$) grown at 37°C in MBM medium was measured as approximately 2.5 μl of H_2O mg (dry wt)$^{-1}$ (72% water and 28% dry weight) (57). Intracellular water in the cyanobacterium *Anabena flos-aquae* was measured as some 88% of the cell mass (12% solids) (398).

Cell water is distributed among several structurally distinct cell compartments: the cytoplasm, the periplasm, and, if present, the capsule or extracellular investments (Fig. 2). The term "structurally distinct" is emphasized here, as there is now some indication of additional physiological compartments such as the one speculated to house the 10,000 or so copies of thioredoxin in *E. coli* cells (258). It is unknown whether the properties of water may cause discrete subpartitioning of physiological zones within the cell compartment. There are few data available for the turgor pressures generated in bacterial cells—largely because of technical difficulties—and all measurements have been made with cells from liquid cultures. A value of 187 kPa was determined in *Ancyclobacter aquaticus*. The turgor pressure varied considerably from cell to cell but nevertheless was independent of cell size (207, 286). A similar value of 2 to 4.5 bars (200 to 450 kPa) was determined in *Anabaena flos-aquae* (398).

Interstitial Water

Measurements of cell volume and the amounts of water in cells are more complex than they would otherwise seem, in large part because of the variable amounts of interstitial (extracellular) water. Substantial amounts of interstitial water may be associated with bacterial cells. Fibrils and polymers may form strong links between the cell and a solid surface and encourage film development. A film provides the largest surface area available for rewetting, and a film with a clay envelope, especially monmorillonite, may protect bacteria from excessive desiccation (390). The presence of a water-retaining structure poses problems if that structure is physically attached to the cell wall, in view of the torsional and elastic forces that may be conducted to the cell as the structure changes its water content. If the structure is also stress bearing, any addition of new material to the structure must take place as far from the cell surface as possible (364). Measurements involving extracellular investments from some cells suggest that the cells hold in excess of 95% of their own weight as water (381). Most of that water, of course, may be removed upon air drying.

Water in Enzymatic Reactions

Water formally acts as any allosteric ligand, but, unlike any other ligand, it is the only one that is always active (70). It is now being recognized that water and solvation play crucial roles in membrane function and protein regulation (231, 284, 315, 316, 318, 433). When a protein undergoes a conformational change, the partial specific volume of the structure changes. Often this change, ΔV, is small and cannot be easily detected; sometimes the change is large, the equilibrium between the forms is in a reasonable range, and ΔV can readily be evaluated at pressures below 300 MPa. In recent work, Kornblatt and Hoa (211) have determined that some 10 molecules of water are directly involved in the reaction mechanism of cytochrome *c* oxidase and enter and exit the protein during every turnover (Fig. 3). The role of these water molecules is not clear. One suggestion is that the water movements may be part of the channel-gating processes of the oxidase and that these movement can lead to the pumping of protons across the membrane. It is clear that perturbations of the water structure at and around the oxidase, especially $\overline{V}_b$, during dehydration can be expected to significantly influence not only the structure but also the function of the protein. Water plays a critical role during the catalytic cycle of cytochrome P-450$_{cam}$, in which at one level, movements of water molecules in and out of the active site regulate enzymatic activity, and at the second, during dissociation of putidaredoxin with cytochrome P-450$_{cam}$, roughly 28 molecules of water are involved in the catalytic cycle (91). Some 50 to 70 solute-excluding water molecules become part of the hemoglobin tetramer in its transition from the full deoxgenated (tense) T state to the fully oxygenated R (relaxed) state. The osmotic work required for the binding of these 60 water molecules in

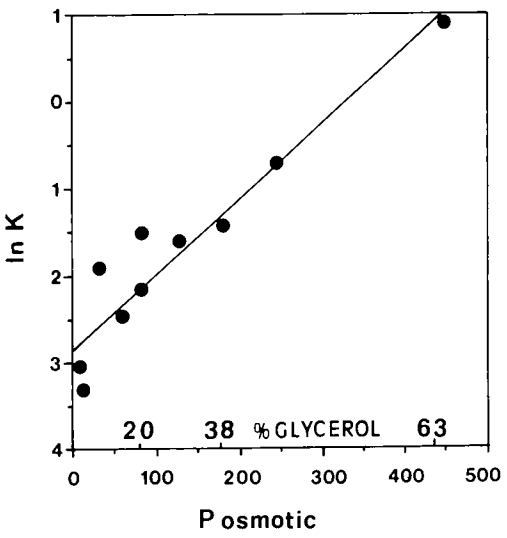


FIG. 3. Relationship between ln K and osmotic pressure (P). By treating values of the ratio of reduced cytochrome a to pulsed oxidase as equilibrium constants (K)—each indicating the extent of inhibition of electron transfer between the cyt a Cu_A pair and the cyt a_3 Cu_B pair of the oxidase, at a given water potential—ln K scales as a linear function of the osmotic pressure of the system at a hydrostatic pressure of 1 bar (0.1 MPa). The slope of the line extrapolates the magnitude of the ΔV as 190 = 22 ml mol^{-1}. At 90% glycerol, the cytochrome oxidase maintains the majority of its spectral character but there are noticeable shifts of the protein toward low-spin forms. Both oxidized and reduced proteins show an invariant spectrum up to 60% glycerol. It is postulated that cytochrome c oxidase contains an osmotically active compartment when the protein is turning over, which is not detectable when the protein is in either the fully oxidized or fully reduced static state. Reproduced from reference 211 with permission of the authors and publisher.

the change from the T to the R state is about 0.2 kcal mol^{-1} (0.84 kJ mol^{-1}) at 0.28 osmol ($\psi_{\pi s}$ of −0.73 MPa [70]). Both binding and kinetic studies show that approximately 65 water molecules are released when hexokinase binds glucose (315). Dehydration and rehydration reactions contribute far more to the energetics of protein and membrane conformation than was previously thought. Considerable progress has been made in understanding the structure of water and its role in physiological processes. These studies emphasize the important role of water in the structure and function of macromolecules, but they also seem to only emphasize the complexity of the whole cell system. Are there discrete fluxes in the cycling of water molecules across membranes? How many water molecules are required to sustain the translational efficiency of a ribosome, the fidelity of RNA polymerase, or the secretion of a protein as it traverses a membrane? More importantly, how are these waters influenced as a cell is dried and then rewetted?

How Much Water Is in a Desiccated Cell, and Where Is It?

Webb's measurements were in agreement with those from earlier studies on a range of desiccation-sensitive and -tolerant bacteria and viruses (see reference 403 and references therein). When dried at RH 40 and 30%, bacterial cells contain around 10 g of H_2O (per 100 g of solids) and 3 g of H_2O, respectively. The lower value, 0.03 g g (dry weight)$^{-1}$, is comparable to those measured for other anhydrobiotic cell types, such as *Artemia* cysts, plant seeds, etc., that have water contents of about 0.02 g g^{-1} under extreme desiccation (64, 392). The amount of water removed through freeze-drying of desiccated colonies of *N. commune* CHEN was equivalent to that which was lost after heating them, i.e., about 0.06 g g (dry weight)$^{-1}$ (170). All these examples clearly involve very low water contents, much lower than the level (0.3 to 0.4 g g^{-1}) at which there is monolayer coverage of proteins by water, for example. For proteins, water contents at and below 0.05 g of H_2O g of protein^{-1} are the minimum needed to hydrate charged and polar groups and are the minimum needed to form clusters of water. Acids are not saturated below values of 0.1 g g^{-1}, nor are polar side chains or peptide -NH bonds. The low water contents of desiccated cells imply that the cell proteins have been subject to a transition in proton distribution. For purified proteins, such transitions lead to a reordering of disulfides, side-chain and backbone conformational shifts are prevented, and the proteins are "tense" as opposed to being "loose."

The water contents of bacterial spores are considered to be difficult to measure but appear to be lower than those of their corresponding vegetative cells. The spore, spore coat, cortex, core, and protoplasm of *Bacillus stearothermophilus* had values in the range of 0.21 to 0.58 g of H_2O g (dry weight)$^{-1}$, and the a_w of the cortex was 0.83 (5). Akinetes (Dauerzellen) are resting stages (differentiated cells) that are produced by certain species of heterocystous cyanobacteria (136). Calculations made with optical measurements derived from analysis by light microscopy indicated that in *Anabaena variabilis* the akinetes, heterocysts, and vegetative cells contained 2.06×10^{-10} g of solids and 63% water, 0.46×10^{-10} g of solids and 85% water, and 0.31×10^{-10} g of solids and 77% water, respectively. Akinetes thus had more dry matter and the lowest water content, and it was also demonstrated that both their formation and their germination were associated with changes in their hydration level (36). But what is the significance of the numbers presented above? It seems that anhydrobiotic cells have such low water contents that their major constituents must lack a monolayer of water molecules. However, one other very curious fact emerges after considering these values. If we can believe the values quoted for bacterial spores (5; also see reference 149 for a review) and cyanobacterial akinetes (36), these structures do not belong in the class of anhydrobiotic cells—they contain too much water! Spores and akinetes must belong to the physiological group of cells that respond to water deficit through osmotic adjustment; i.e., the mechanism of tolerance is the use of compatible solutes (see below).

REMOVAL OF WATER FROM CELLS

The removal of almost all or some of the water from a cell can occur slowly or rapidly. The removal of that water can, depending on the quantity removed, cause mild, moderate, severe, or extreme water deficit. The early literature has described bacterial distribution in terms of the prescribed limits of water activity within which the cells function. Although this view is now the subject of considerable criticism (357), it is clear that bacteria may respond to water deficits through a number of different physiological responses.

Methods To Remove Cell Water

The addition of variable amounts of a solute to growth media has become the method of choice in studies concerned with the subjection of microorganisms to water stress. The advantages of this approach are that physiological processes such as the uptake of radioisotopes and secretion of metabolites are easy to measure and, more significantly, that the microorganisms can be readily harvested and the cell mass can

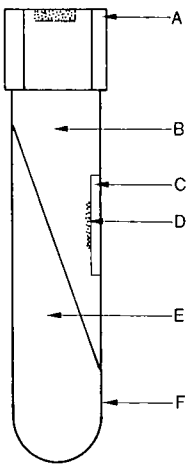


FIG. 4. Isopiestic control of water potential. Cells (D) are immobilized on an inert support such as a filter (C) that is curled around the inner surface of a glass tube (F). The tube contains salt-amended agar (E) and is sealed with a cap (A) that can permit sampling of, or additions to, the gas phase of controlled water potential (B). See reference 301.

be readily recovered. The limitations of this approach are that concentrated solutions tend to retard gas exchange and that high ion concentrations may impair membrane function and prove toxic to cells. Furthermore, this approach can never reproduce the extreme water deficit characteristic of anhydrobiotic cells. As a result, only a comparatively restricted range of water potentials can be achieved without compromising cell viability. The desiccation of microorganisms and the exposure of cells to various degrees of matric water stress require different approaches. One method for controlling the water status of a solid microbial substrate (community) is to bring the substrate into water vapor equilibrium with a solution of known water potential. The exposed surface of a bacterial colony growing on an agar surface is a good example of a community subjected to such isopiestic control of water potential. An experimentally convenient system can be constructed by placing the cells in an enclosure in close proximity to agar that has been amended with an appropriate concentration of a solute (160). Comprehensive lists of saturated solutions and their vapor pressures and humidities at different temperatures are provided by Winston and Bates (423). One isopiestic system, designed to measure $^{14}CO_2$ uptake by chasmoendolithic cyanobacteria (301), is shown in Fig. 4. A more elaborate system that achieved isopiestic equilibration through the control of temperature differentials has been described by Palmer et al. (272). The immobilization of cells in the form of pastes, slurries, and thick suspensions on various inert supports such as sterile sand, paper and nylon filters, cotton gauze, and even curtain material also affords the means to achieve comparatively rapid drying (and storage) of microorganisms (92, 93, 294, 300–302).

The studies of Webb focused on many aspects of the desiccation tolerance of bacteria (401–409). Using a system based on one devised originally by L. Goldberg, Webb built a drum system that, when operated at 28 lb/in^2, generated a collision spray of bacteria with liquid droplets with a mean diameter of 10 μm. Air was cleaned by passage through spin filters and charcoal and was dried in a column of silica gel. Samples were removed from the aerosol by means of a critical orifice with a liquid impinger operating at 12.5 liters min^{-1}. With this system, it was possible to study the survival of airborne bacteria atomized from various media. This latter approach is being used extensively in studies directed at understanding the dispersal of bacterial aerosols.

Preferential Exclusion Hypothesis

Consider a bacterial cell as it undergoes a transition between two intermediate-moisture states, x and y (with y at a lower water activity than x), in response to a change in the prevailing matric water potential.

$$\begin{array}{c} k_{\text{matric}} \\ \text{(minimum } E \text{ required)} \\ \text{State } x \rightleftharpoons \text{State } y \\ \text{(maximum } E \text{ derived)} \end{array}$$

In such a system it is the change in the chemical potential of water ($\mu_w{}^x$ to $\mu_w{}^y$) that shifts the equilibrium. The most extreme case would be for a change when $\mu_w{}^x = \mu_w{}^R$ (the rehydrated state) and $\mu_w{}^y = \mu_w{}^D$ (the desiccated state) (see equation 1 and the accompanying discussion). These changes in the chemical potential of water involve net free energy changes, and the magnitude of these changes will differ depending on factors such as the time of drying and the temperature changes.

The following discussion considers the removal of water from a bacterial cell with a somewhat rigid wall and an elastic cytoplasmic membrane appressed to the cell wall and encompassing the cell compartment. At this point, let us assume that the cell has no sheath or outer investments. There are several ways in which such a population of cells may be subjected to a water deficit ($\mu_w{}^x$ to $\mu_w{}^y$) with respect to the environment. The one most often considered is an "osmotic stress" in which the cells are suspended in an aqueous solution of some solute that cannot enter them (Fig. 5a). In accordance with Gibbs-Donnan equilibria, there is a net efflux of water molecules until a state is reached at which there is a balance between the water activities of the two compartments. Such osmotic adjustment, which may occur rapidly, can be viewed as being a passive alteration of cell volume (Fig. 5a). A similar balance in water activity can be achieved if the cells accumulate compatible solutes or osmoprotectants (Fig. 5a). The latter include K^+ ions, glutamate, glutamine, proline, quaternary amines (glycine betaine), and the sugars trehalose and glucosylglycerol (58, 85, 86). An accumulation of one or more intracellular solutes can be achieved either by transport from the environment or through de novo synthesis or by both means.

What do these "compatible" solutes do, or, rather, why are they compatible? The value $\Delta G_{N \rightarrow U}$ (N = native, U = unfolded) defines the equilibrium that determines the conformational state of a protein, and it is a tenuous one. The half-life of a protein in an aqueous environment may be increased or decreased by a range of different chemical agents. Agents that stabilize proteins include sugars, amino acids, glycerol, polyols, amines, and salts (13). There is considerable experimental evidence that in a three-component system composed of a protein, water, and a cosolvent (solute), the solute may have a stabilizing effect on the protein. The mechanism is, in principle, a simple one—a stabilizing solute is excluded from the immediate vicinity of the protein. The solute is held at bay, albeit at some finite distance from the surface of the protein, such that the protein is, in effect, preferentially hydrated in time and space. Such preferential exclusion is thermodynamically unfavorable (entropically unfavorable) but would become even more unfavorable if the protein unfolded to provide yet more exclusion domains. As a consequence, preferential exclusion drives the equilibrium between the native and unfolded

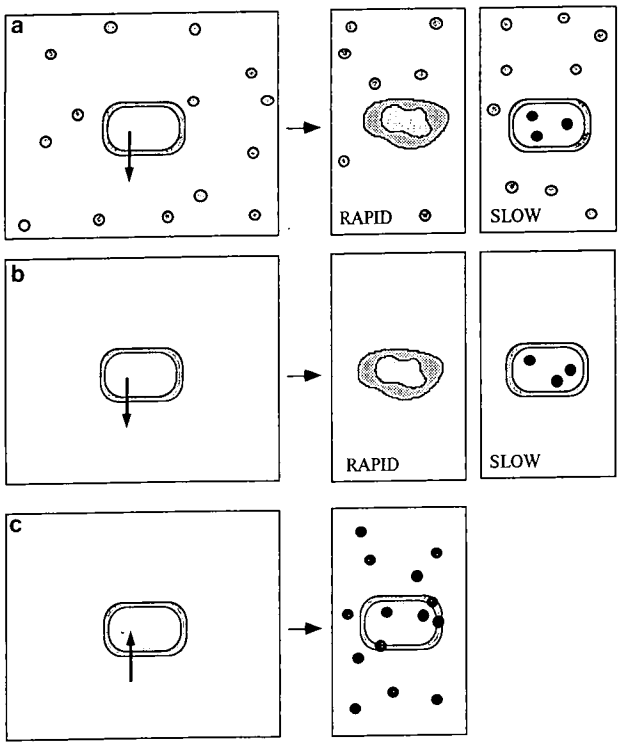


FIG. 5. Consequences of the removal of cell water. (a) Hyperosmotic stress. (b) Matric stress (air drying). (c) Hypoosmotic stress. The outer and inner membranes of a hypothetical cell are shown. Movement of water (vertical arrows) is either rapid or slow. Hatched circles represent a cosolvent (e.g., a salt). Cross-hatched circles represent a compatible solute (e.g., a carbohydrate).

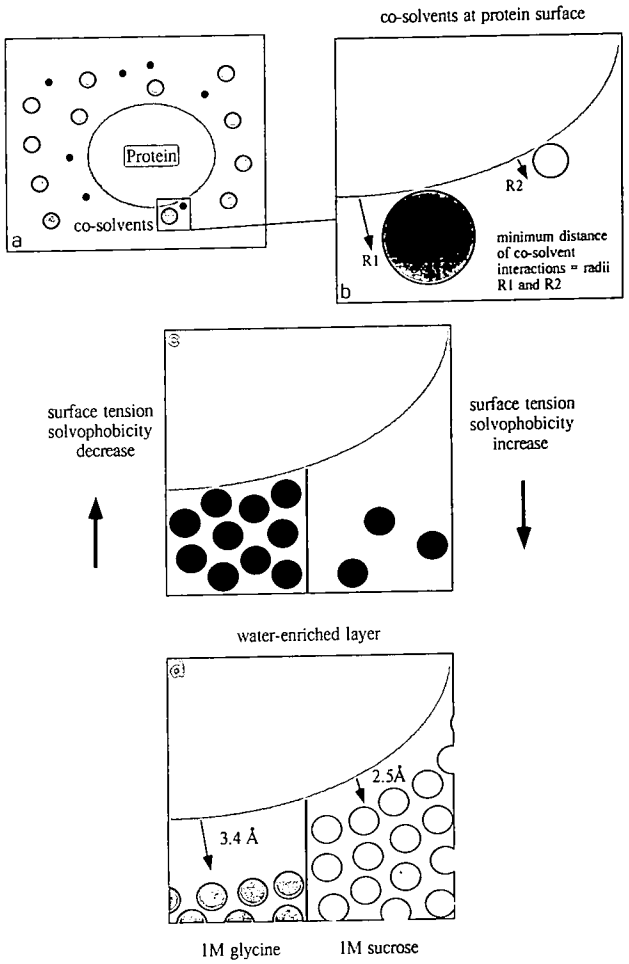


FIG. 6. Principles of water and solute exclusion by proteins. (a) A protein in water that contains different cosolvents (circles). (b) The minimum distance of cosolvent interaction is determined by the effective radius of the cosolvent molecule; in the steric mechanism, the cosolvent cannot penetrate the outer shell of the protein but water can. Thus, the solvent is enriched in water in the immediate vicinity of the protein. (c) Influence of cosolvents on surface tension and solvophobicity, and the effect on the water layer at the protein surface; sugars, nonhydrophobic amino acids, and most salts increase the surface tension of water. Therefore, their concentration in the surface layer must be reduced relative to that of the bulk solvent and they must raise the chemical potential of the protein, leading to its stabilization. (d) The size of the zone of exclusion at the protein surface differs with different cosolvents. See reference 383 for more details.

state toward that of protein stabilization. In contrast, destabilizing agents, which include ethylene glycol, polyethylene glycol, 2-methyl-2,4-pentanediol, dimethyl sulfoxide, urea, and guanidine HCl, bind preferentially to proteins. Here, there is an excess of solute in the immediate vicinity of the protein relative to the bulk solution, the equilibrium is thus shifted towards denaturation, and the unfolded form of the protein is favored.

Solutes that interact with proteins belong to two classes: the kosmotropes (water structure builders) and the chaotropes (water structure breakers). The former can be characterized through Hofmeister series considerations. Compatible solutes, for the most part, are kosmotropes and include sugars, other polyols, amino acids and amino acid derivatives, methylamines, and urea (a chaotrope), frequently in combination (13, 383, 384). All are electrically neutral, and all, except urea, are compatible solutes. Potassium, the exception, is used as a compatible solute by some bacteria (56). The charged amino acids Arg and Lys are not used, and neither are amino acids with large hydrophobic side chains.

Timasheff has described how, in accordance with the preferential exclusion hypothesis, there may be three consequences of the immersion of a protein in an aqueous solution of a given solute. There may be a preferential hydration of the protein, a preferential binding of the solute to the protein, or no preferential interaction (383, 384) (Fig. 6). In reality, however, preferential exclusion and binding are not all-or-nothing events. Some salts that are considered to be stabilizers will, at sufficiently low concentration, bind to charged regions of a protein. The preferential exclusion mechanism represents the additive properties of a given solute, and these must reflect a continuum of specific, and in some cases opposed and quite different, interactions with the protein.

The binding of cosolvent by the protein can be described in simple Scatchard notation (ligand binding) with designations where 1 is water, 2 is protein, 3 is cosolvent, m is the molal concentration, μ is the chemical potential, g is gram of component per gram of water, and T is the temperature in Kelvin:

$$\nu_3 = [\delta m_3/\delta m_2]_{T,\mu_1,\mu_3}$$

When ν_3 is positive, there is preferential binding of component 3 (the cosolvent); when ν_3 is negative, there is preferential hydration of the protein (preferential exclusion of the ligand). Stabilizing compounds all have negative binding values. As the

chemical potential increases, the derivative is positive and the measured binding is therefore negative. Equilibrium constants for the native and unfolded states can be presented as follows (note that I have introduced a hypothetical constant K_2 here to emphasize that an unfolded protein is not necessarily a denatured [dead] protein; Finney (117) made the wry point, "dry proteins are dead, or at best asleep."):

$$\mathrm{N} \overset{K_1}{\rightleftharpoons} \mathrm{U} \text{ and } \mathrm{U} \overset{K_2}{\rightleftharpoons} \mathrm{D}$$

It is seen that these K values will vary with the concentration of the cosolvent:

$$[\delta \ln K/\delta \ln a_3]_{T,P} = \nu_3{}^D - \nu_3{}^N = \Delta\nu_3$$

were $\Delta\nu_3$ is the difference between the preferential binding of component 3 (cosolvent) in the denatured and native states. If the preferential exclusion is greater in the denatured than the native state, $\Delta\nu_3$ is negative, the equilibrium is shifted to the left, and there is protein stabilization.

The mechanisms of stabilization include steric exclusion, increase of the surface tension of water by the cosolvent, and the so-called solvophobic effect (Fig. 6). Solvophobicity is a consequence of the reinforcement of the hydrophobic effect by a solvent additive as a result of water structure enhancement by the additive. Contact between nonpolar residues on the protein surface and water molecules which are constantly fluctuating in and out of structure clusters is thermodynamically unfavorable. A redistribution of solvent molecules is thought to occur at the protein surface, but current opinion does not favor the idea that there is an impenetrable hydration layer consisting of water molecules ordered on the protein surface ("iceberg" hypothesis). The range in osmotic pressure gradients that can derive from different concentrations of different cosolvents leads to different extents of cosolvent exclusion from protein surfaces. As a consequence, the extent of cosolvent exclusion, and hence the degree of stabilization, varies (Fig. 6). A positive deviation of the osmotic pressure from ideal reduces the redistribution of solvent components in a cellular compartment.

Not every protein will have the same zone of exclusion for a given cosolvent. Many solutes that stabilize or destabilize proteins in solution by preferential exclusion or binding may also be excluded from, or be bound to, membranes. However, such effects are hard to assess, although many, such as the destabilization of the phospholipid bilayer, the formation of the H_{II} phase at low temperature, and leakage from phosphoethanolamine/phosphatidylcholine liposomes, are clearly deleterious (78). Many molecules that stabilize proteins in solution will prevent this dissociation when excess water is present, but when the hydration shell of the protein is removed, as occurs in the desiccated cell, the specificity for the stabilization becomes very high (383, 384).

Drying versus Salting (or Sugaring)—What Is the Difference?

Water efflux occurs when bacterial cells are exposed to a gas phase with a water activity that is lower than the cell compartment. If there is a considerable difference between the water activities of the two compartments, exposure of the cells for a limited time may lead to rapid shrinkage of the cytoplasm (Fig. 5b). However, if the water activity of the gas phase is sufficient to permit growth, albeit slow growth, the cells may achieve a water balance through de novo synthesis of a compatible solute as described above. These drying stresses are often termed matric water stresses. The removal of a substantial fraction of the bulk water from cells through a matric water stress is termed desiccation, and such desiccation can be achieved through either rapid or slow drying. There is one distinction between matric and osmotic systems and one that is evident from a comparison of Fig. 5a and b. The immediate environment of a cell under matric stress is the atmosphere; i.e., the surfaces of their cell walls are exposed to a gas phase, while cells under osmotic stress are bathed in an aqueous solution, albeit it one of diminished water activity. As such, considerations of osmotic stress (even when that stress is administered with 5 M KCl) deal with restricted, and high, water activities. What this really means, and it is the crux of an understanding of desiccation, is that a cell that has been subjected to an extreme of air drying, say incubation at −400 MPa (see, e.g., reference 92), has no capacity to rely on preferential exclusion mechanisms for its protection—virtually all water is gone. And if the water is gone, what of our single-component system—how can emphasis be placed upon the properties of water in a discussion about desiccation tolerance and the anhydrobiotic cell? This is a misleading argument, of course, but it has lead to the development by John and Lois Crowe, their students, and colleagues of a water replacement hypothesis to account for the preservation of biological integrity in the absence of liquid water (67, 81). The essential elements of this hypothesis and its consequences for understanding mechanisms of desiccation tolerance are reviewed in detail later in this review.

To account for either survival under more moderate matric stress or the attenuation of the effects of rapid drying, the discussion presented above requires certain qualifications. First, our cell was assumed to have no sheath or outer investments that may contribute a substantial fraction of interstitial water (Fig. 2). Second, although a balance in the water activities of different compartments is implied by osmoregulation, it should be noted that water is never at equilibrium throughout a given system "at balance" as a consequence of local changes in density (420). Third, osmotic and matric water stresses are generally regarded as being quite distinct and are treated as such in discussions of water stress. Such distinctions are rarely easy to make in microbial ecology. For example, an intertidal microbial mat may be submerged by seawater for part of the tidal cycle (Fig. 5a), yet, for many days of the year, the mat may remain "high and dry"—desiccated and salted (Fig. 5b). In this dry state the mat community is prone to rehydration stresses imposed by transitory rainfall and/or hypersaline tidal waters.

The different methods available to remove water from cells have been used with a wide range of bacteria. Some of the general consequences of drying cells are summarized in Table 1. Are the initial consequences of water removal under matric (drying) and osmotic conditions really similar? The cost of dehydrating or the benefit of fully rehydrating any hydrophilic surface is remarkably uniform and high at 1.5 to 15 kcal mol^{-1} (6.3 to 63 kJ mol^{-1}) per 100 $Å^2$ (1 nm^2) of surface area (316). Theoretically the energy gain or loss upon rehydration or drying of a bacterial cell of 1 by 2 μm (6.28 μm^2) would be on the order of 10^8 kcal mol^{-1} (4.2×10^8 kJ mol^{-1})! The removal of water itself should result in a positive entropy change (403). Upon drying bacterial cells, the magnitude of the activation energy (ΔH) was found to be small in comparison with that required to denature protein, and ΔS was negative (403). The studies of Webb (401–409) provide comprehensive measurements on the responses of bacterial cells to the stress of air drying. In these experiments cells were dispersed in aerosols at different relative humidities or were immobilized and dried by

TABLE 1. Gross responses of cells to air drying

Level of effect	Response
Community	Change (usually increase) in surface area
	Shrinkage
	Salt precipitation
	Change in texture
	Change in shape
	Change in color (oxidation of pigments)
Cell	Shrinkage of capsular layers
	Increase in intracellular salt levels
	Crowding of macromolecules
	Changes in volumes of cell compartments
	Changes in biophysical properties (e.g., surface tension)
	Reduced fluidity (increased viscosity)
	Damage to external layers (e.g., pili, membranes)
	Change in physiological processes (e.g., growth arrest)

isopiestic equilibration. These studies indicated that all evaporation, as far as free water ($\overline{V}_f$) is concerned, takes place almost instantaneously upon aerosolization, and an equilibrium between cell-bound water ($\overline{V}_b$) and the environmental water vapor pressure is reached very quickly. Under these conditions, then, the principal consideration is cell survival—the drying event is too rapid to permit either growth adjustment or de novo regulated synthesis of osmoprotectants. Webb calculated death rate constants (K) for bacterial strains from the plots of $\ln N_t/N_0$ against time for the period up to 1 h and then from 1 to 5 h, following aerosolization. Highly reproducible death curves were obtained for sensitive bacteria such as *E. coli* and for tolerant bacteria such as *Staphylococcus* spp. Typical data obtained in these experiments are presented in Fig. 7. Webb (403) estimated that sensitive bacteria such as *E. coli* and *Serratia marcescens* had a $\overline{V}_{cell}$ of ~4 (80% water, 20% solids), and cell death upon drying occurred only at or below $\overline{V}_{cell} = 0.3$. This value was reached when the cells were dried at or below a relative humidity of 80% ($a_w = 0.80$; equivalent to a Ψ_{Π_s} of some −31 MPa). Arrhenius plots ($\ln K$ versus $1/T$) indicated that the activation energy (slope) associated with cell death appeared to increase as the time of storage in air

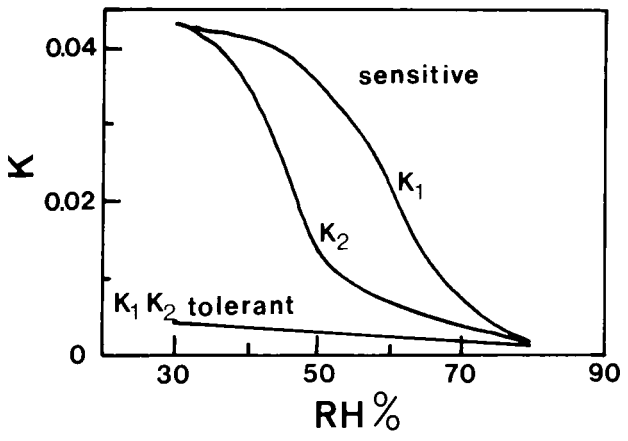


FIG. 7. Relationship of estimated death constants (K) to RH. Constants were measured during the first 1 h (K_1) and subsequent 1 to 5 h of drying, for sensitive bacteria (e.g., *Serratia marcescens*) and drying-tolerant bacteria (e.g., *Staphylococcus aureus*). Adapted and redrawn from reference 403.

lengthened (see equation 6). The calculated E values were thought to represent the strengths and/or numbers of the new inter- or intramolecular hydrogen bonds that formed either when the remaining water molecules reorientated themselves or as dehydrated groups interacted. To examine these events further, Webb calculated the entropy (ΔS) changes associated with time of storage of cells in air, taking into consideration the Boltzmann energy distribution:

$$K = (K_b/h)e^{\Delta S/R}e^{-\Delta H/RT}$$

where K is the death factor, K_b is the Boltzmann constant, h is Planck's constant, and ΔH is the heat of activation. Although the removal of water itself should result in a positive entropy change, comparisons for various viruses and bacteria indicated that the values of ΔS were negative when microorganisms were inactivated in the dry state, with energy values of 9 to 12 kcal mol^{-1} (37.7 to 50.2 kJ mol^{-1}) for *S. marcescens* ($\Delta S = -40$ to -18) and 8.5 to 10 (35.6 to 41.8 kJ mol^{-1}) ($\Delta S = -38$ to -19) for *E. coli*. The ΔS and ΔH values which Webb estimated and which were thought to be responsible for the reactions leading to cell death were small, suggesting very subtle changes. As the entropy change was not thought to be related to the size of the organism, it was concluded that inactivation due to drying takes place in relatively small regions of the cell. Webb concluded that only "bound" water was involved in the mechanism by which cells died when they were desiccated, and the negative entropies of aerosol deaths were interpreted to reflect a "tightening" (crowding) of molecules (note the previous discussion referring to tight and loose states of proteins). The two-phase death rate was explained in terms of the removal of water molecules first from —N, =N-H, or —OH groups and then, upon further drying of cells, from =C=O or =P=O groups.

There has been virtually no appraisal of Webb's findings within the past 30 years. Webb considered much about why bacterial cells are sensitive to drying and desiccation but less so to why some cells are tolerant. The air drying of cells clearly leads to a very rapid curtailment of cell growth. What of osmotic stress? If the osmolarity of the suspending medium is increased, cells of *E. coli* respond by decreasing their growth rate in a linear fashion (57). The cells also show changes in behavioral responses (225). Growth of the cells was found to effectively cease at an extrapolated value (not determined experimentally) of 1.8 osm ($\Psi_{\Pi_s} = -4.64$ MPa). The final arrest of growth due to this osmotic shock was thought not to be due to energy limitations (178). One must question, therefore, what the cells respond to in this situation if water removal does not compromise their survival but simply modulates their growth rate. While the steady-state water-accessible volumes for the cytoplasm ($\overline{V}_{cyto}$) and the cell ($\overline{V}_{cell}$) decreased linearly with increasing osmolarity, $\overline{V}_{peri}$ (equal to $\overline{V}_{cell} - \overline{V}_{cyto}$) increased linearly with increasing external osmolarity. Extrapolation of the data suggested that growth ceased when the steady state value for $\overline{V}_{cyto} = 0.5 \pm 0.2$ μl mg (cell dry weight)$^{-1}$ or, more significantly, when $\overline{V}_{cyto} = \overline{V}_b$, the quantity of "bound" water ($\overline{V}_{cyto} = \overline{V}_f + \overline{V}_b$, where f is free water and b is bound water). This limiting value of cytoplasmic water is very similar to that calculated to cause loss of viability of *Serratia marcescens* and *E. coli* when these bacteria were air dried (see above) and is equivalent to the value for the amount of bound water associated with protein. In a three-component system containing water, lysozyme, and 1 M glycine as a cosolvent, the value of bound water is around 0.54 g of H_2O g of protein^{-1} (384). In the absence of cosolvent, the value is closer to around 0.2 g g^{-1}. What do these measurements tell

us? For *E. coli*, the mean cell volume is related to growth rate such that, at 37°C,

$$\overline{V}_{\mu} = 0.4 \times 2^{\mu}\ \mu m^3$$

where $\overline{V}_{\mu}$ is the average volume per cell at a growth rate of μ. More generally, $\overline{V} = \overline{V}_{u} \times 2^{\mu}$ where $\overline{V}_{u}$ is a constant and is equal to the average cell volume in a population growing at a rate μ approaching 0 doubling per h (96). In these respects, one can argue that the reduced growth rate of *E. coli* at higher osmolarities and the linear dependence of the growth rate on external osmolarity are responses to a change in the space available ($\overline{V}_{cell}$) for cell functions (growth) and not a lack of water per se. Parallel observations that the relationship of $\overline{V}_{cyto}$ and growth rate at a given osmolarity changes when the carbon source is changed are consistent with this conclusion. If only a small amount of $\overline{V}_f$ is required for growth, then the growth of cells and different growth rates can be accommodated within a wide range of $\overline{V}_f$ values. The real water stress therefore is the perturbation of $\overline{V}_{cyto}$ at levels at which $\overline{V}_{cyto}$ becomes equivalent to or less than $\overline{V}_b$. The factors that lead to perturbation of $\overline{V}_b$, and the mechanisms used to restrict that perturbation, may be of relevance to considerations of the consequences of the initial stages of desiccation. An understanding of desiccation tolerance requires an appreciation of the function of $\overline{V}_b$, the means by which $\overline{V}_b$ is perturbed, and the mechanisms used to prevent and/or survive that perturbation. One conclusion of the study of Cayley et al. (57) was that the mechanism by which osmotic stress controls growth rate is fundamentally different from the means by which nutrient limitation controls it, even though the patterns of gene expression under stationary-phase growth and under osmotic upshift are regarded, superficially, as being rather similar (62, 354). Should we assume that the mechanism by which the initial stages of desiccation control growth rate is also different?

The results from drying and salting (and sugaring) of cells emphasize the critical role of bound water. Only a small amount of free cytoplasmic water is required for cell growth, or, simply stated, growth depends on the presence (or absence) of only a small fraction of $\overline{V}_{cyto}$. For enzymatic activity, only a monolayer coverage of a protein with water molecules is required for activity. In the air-dried cell, however, even monolayer aggregations of water molecules have been perturbed and diminished.

Hypertonicity and Hypotonicity

As cells generally have elastic cell walls, the removal of water may lead to plasmolysis but not necessarily to cell death. Even though cells of *E. coli* are relatively sensitive to osmotic stress, their suspension in 3 M NaCl for 60 min does not lead to a decrease in viability (57). One bacterial cell that does appear to plasmolyze readily, however, is the cyanobacterial heterocyst (398). The heterocyst is a structurally and biochemically modified cell that accommodates active nitrogenase (426). Heterocysts tend to be larger than vegetative cells, they have an inelastic cell wall, and, through physical connections with adjacent vegetative cells, they receive supplies of carbohydrate that sustain their high respiratory rate (426). Evidence suggests that plasmolysis of the heterocyst in hypertonic solution is due to the separation of the cell wall from the enclosing envelope. In one study, heterocysts of *Nostoc muscorum* were found to collapse in 0.3 M mannitol ($\Psi_0 = -0.46$ MPa) (29) and the authors concluded that heterocysts are unable to perform osmoregulation. This is an interesting proposal—particularly in view of the fluxes in reductant (carbohydrate) supply that take place between the vegetative cell and heterocysts—but is the proposal true? The heterocysts of *N. commune* UTEX 584, but not the vegetative cells, collapsed when cells were dried, desiccated, and then rehydrated in nonionic osmotica (1% [wt/vol] glucose; $\Psi_0 = -0.14$ MPa) but not when they were rehydrated in a minimal-salts medium of equivalent osmotic strength ($\Psi_0 = -0.18$ MPa) (298). Heterocystous cyanobacteria are a conspicuous feature of the marine shore (290, 293, 308), an environment where the rates of evaporation, the rainfall, and repeated inundation with salt waters would seem to guarantee a rather miserable life for a nonosmoregulating heterocyst. On the other hand, a survey of a range of hypersaline environments along the coast of the Sinai, Israel, that support highly abundant communities of nitrogen-fixing cyanobacteria failed to identify one heterocystous form (292, 293), and heterocysts of laboratory-grown strains (in logarithmic growth) do appear to be particularly prone to drying damage, as judged from analyses at the ultrastructural level (278). Curiously, a nonheterocystous form, *Trichodesmium*, is the dominant nitrogen-fixing cyanobacterial genus in the open ocean (118). Why? No one has looked at the volume water relationships of heterocysts as thoroughly as the water relationships of vegetative cells (398). Perhaps such studies would be very informative.

Paradoxically, cells may also be placed under a water stress through dispersal under hypoosmotic conditions, and in this case the bacteria are faced with the prospect of continually bailing out their water (57). To counteract this hypoosmotic stress, the cells may secrete osmotically active macromolecules such as glucans (Fig. 5c). In a MOPS (morpholinepropanesulfonate)-buffered glucose minimal medium supplemented with various amounts of NaCl (nonpenetrating), optimum growth of *E. coli* K-12 strain MG 1655 occurred at 0.28 osm (57). From equation 3, this value is equivalent to an osmotic pressure (Π_s, at 37°C) of +0.72 MPa ($\Psi_{\Pi_s} = -0.72$ MPa). These data suggest that the cells were hypotonically stressed in this growth medium and that the 0.28-osm value must represent an equilibrium value. Under these hypoosmotic conditions, *E. coli* cells accumulate oligosaccharides (membrane derived [see below]) in the periplasm (201) and replace cytoplasmic K^+ with putrescine (154).

Although it is ultimately the singular removal of water that is to blame, the exposure of a cell to an extreme osmotic stress and the exposure of the same cell to dry air result in fundamentally different changes that affect growth potential and physiology.

Sensitivities of Prokaryotes to Air Drying

Priestley summarized much of what is published on the longevity of plant seeds, including the results of a survey conducted by the British Association for the Advancement of Science in the 19th century (310). While the data suggest that seeds may survive many tens of years, perhaps even centuries, of desiccation, Priestley dealt with many of the reports of extreme longevity in a balanced and critical manner. It appears that the best-substantiated incident of longevity, a record of six centuries, is for a seed of *Canna compacta* (Cannaceae) found within a walnut shell rattle, at a pre-Hispanic archeological site in northwestern Argentina. Critical summaries of the longevities of desiccated bacteria are harder to come by, for several reasons. A plant seed represents the culmination of a prescribed and readily observed series of developmental events, while the history of dried bacterial cells, certainly those in nature and often those dried under laboratory conditions, is cryptic if not virtually impossible to assess. It is difficult to compare the sensitivities of different groups or genera of

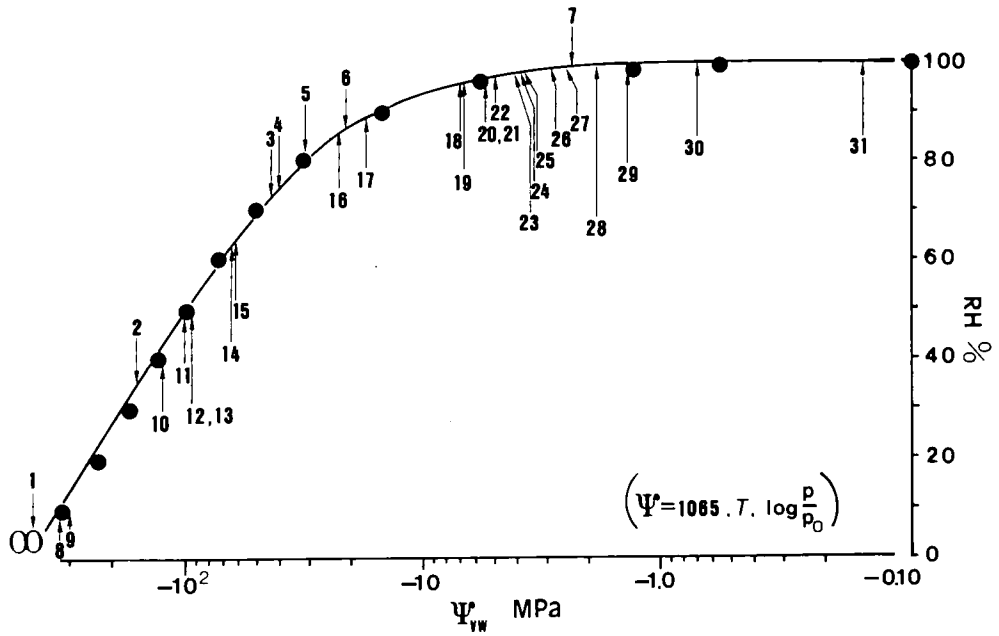


FIG. 8. Relationship between water potential (Ψ_w) and relative humidity (RH %). The scale on the x axis is logarithmic. Solid circles represent the values of Ψ calculated with the equation shown (301) from values of RH/100. Numbers refer to data presented in Table 2. Numbers above the curve relate to physical constants, and those below the curve relate to physiological processes (see Table 2).

bacteria in view of the many different techniques used to grow cells and to dry cells (see, e.g., reference 339) and the fact that some cells have an inherent ability to form resting stages, cysts, and spores (see below). Nevertheless, many studies have attempted to describe the values of a_w at which cells maintain or lose viability and grow or cease growing or the a_w values at which particular physiological properties or activities are modulated (61, 159, 180, 222, 277, 289) (Fig. 8; Table 2). However, the utility of such ranges of water activity for the assessment of bacterial growth relationships has come under question (357).

The rate at which cells are dried in air is critical to cell survival. The survival rates of *Arthrobacter*, *Pseudomonas*, *Mycobacterium*, *Escherichia*, *Micrococcus*, and *Saccharomyces* cells were rather similar when the cells were counted immediately after fast or slow drying (20 min and 24 h, respectively). After prolonged storage, viabilities were higher for slow-dried, as opposed to fast-dried, cells (11, 12). These observations were true for *E. coli* only when more than about 8 mg of water per 10^8 cells was present on paper filters. To obtain optimum resistance to water loss, it was estimated that the drying period for a 25-μl aliquot of such a cell suspension of *E. coli* should be greater than 13 h. The times of survival following air drying of representatives from the major groups of prokaryotes are summarized in Fig. 9. To achieve some degree of consistency, only data relating to the immobilization and drying of suspensions of bacteria on a hydrophobic surface, i.e., glass, are included. Even so, the times summarized in Fig. 8 and 9 should be viewed as being very approximate, even artificial, given that in many instances the descriptions of the physiological status of the cells were lacking and experimental conditions were variable (see, e.g., reference 358). However, these data do indicate that a capacity for a certain degree of tolerance to drying is associated with all bacteria, to greater or lesser extents, and the range of time during which bacteria may remain viable in the air-dried state is extreme. There is no obvious trend here, other than the seeming resistance of spore-forming bacteria. For perspective, the estimated ages of what may be the oldest bacterial populations on Earth, those in Antarctic rocks and Siberian permafrost, are included.

When distributed in air, in association with mucus or slimes, cells may settle out and adhere to a surface or otherwise remain in liquid droplets for finite periods (Fig. 10). Evaporation of water in aerosols is essentially instantaneous (403). Following desiccation, the same cells may also remain suspended in air as components of dust. These different stages of drying may influence the viability of cells considerably. Cells of *Mycobacterium tuberculosis* remain viable for around 1 week when they dried as an aerosol on glass in physiological saline. In dust, tubercle bacilli remain viable for up to 120 days, and if they are stored under various vegetable oils the viability is extended to around 2 years (241). Solid media are generally used to sample the populations of bacteria present in air. It is thought that lengthy periods of sampling cause desiccation of the growth media, which leads to loss of viability of the airborne bacteria. In fact, it appears that bacteria commonly found in room air are little affected (less than a 10% drop in viability) by substantial (13%) reductions in the water content of agar. Therefore, exposure times can be extended considerably to permit accurate sampling, for example to over 1 h in a 30-liter min^{-1} bacterial slit sampler (419). Viabilities increase if cells are dried in the presence of certain sugars, blood, serum, or complex media, and survival times increase if these dried cells are kept in the dark, under an inert-gas phase (291, 299). Optimum growth of *E. coli*, generally assumed to be a desiccation-sensitive bacterium, occurs at −0.72 MPa, cessation of growth occurs at −4.6 MPa, and cell death occurs at or below relative humidities of around 70% (−51 MPa). How do the responses of other bacteria respond to drying? The survival rates of *Clostridium mangenoti* (spores), *Halobacterium halobium*, *Bacillus subtilis* (spores), and *E. coli* were tested by applying droplets of cell suspensions to aluminum foil and then drying them at 10^{-8} torr (1.3×10^{-6} Pa) for 24 h at 77 K (209). Only spores survived under these conditions (55 and 75% survival of *Bacillus* and *Clostridium* spores, respectively). Fol-

TABLE 2. Water deficits that limit growth or physiological activity

No.[a]	Pressure (MPa)	Comment	Reference
1	0	Atmosphere over fuming P_2O_5	423
8	−400	*Crinalium epipsammum* and *Tychonema* spp. survive	92
9	−300	*Enterobacter cloacae* and *Alcaligenes eutrophus* become nonculturable	281
2	−168	Saturated solution of $CaCl_2$	423
10	−129	Limit for survival of *Rhizobium meliloti* in alginate beads	212
11	−100	Typical exposure of *Nostoc* colonies in situ	290
12	−99.5	Nitrogenase activity lost in 30 min in *Nostoc* strain UTEX 584	300
13	−99.5	Polysomes of *Nostoc* strain UTEX 584 intact after 2 h	7
14	−66	Mean lowest value in Antarctic rocks	256
15	−62.5	Ambient values above marine *Scytonema* mats	290
3	−44	Saturated solution of urea	423
4	−41	Saturated solution of NaCl	423
5	−31	Nucleic acids and proteins fully hydrated	405
16	−26	Cortex and core of *Bacillus* spores	5
6	−22	Saturated solution of sucrose	423
17	−17	Lower limit for growth of *Arthrobacter* spp.	61
18	−7	Lower limit for growth of *Bacillus subtilis*	422
19	−6.9	Minimum required for photosynthesis by *Chroococcidiopsis* in hot-desert rocks	271
20	−5.6	Nitrogenase active for at least 3 h	298
21	−5.6	Minimum for growth of *Flavobacterium*, *Pseudomonas*, and *Rhizobium* spp.	61
22	−5	Bacterial respiration ceases	422
23	−4.6	Growth of *E. coli* ceases	57
24	−4.4	Competitive bacterial growth ceases	159
25	−4.2	Nitrification and sulfur oxidation cease	159
26	−2.8	Inhibition of photosynthesis in desert crusts of *Microcoleus* spp.	41
27	−2.7	35‰ seawater at 34°C	301
28	−1.8	Inhibition of growth of *Microcoleus* spp.	41
29	−1.5	*E. coli* MM294(pEMR1) nonviable	317
30	−0.7	Mean of minimum value that supports bacterial growth (see no. 21)	61
31	−0.14	Motility of bacteria ceases	159

[a] For the relationship between RH and water potential, see Fig. 8. Note that examples are presented from most to least extreme water deficit.

lowing the exposure of dried cells to 200 mC (a barrage corresponding to about 250 years of irradiation in near-Earth orbit), the survival rates for *Bacillus* and *Clostridium* spores were 45 and 25%, respectively.

The vegetative cells of many different species of bacteria can undergo some degree of differentiation into structurally and physiologically distinct forms classified variously as endospores, exospores, myxospores, cysts, akinetes, and resting stages (24, 116, 261, 370, 389). While highly diverse, common features of these growth forms are that they possess thickened, structurally characteristic extracellular cell wall layers and are desiccation resistant. More significant, however, is the fact that these forms develop during the time the parent vegetative cell is growing in an aqueous environment, i.e., desiccation and drying are not cues for the synthesis of spores, etc.

Drying rarely occurs without being superimposed upon some other stress. In fact, the combined effects of desiccation, or osmotic stress, and pH stress (2% acetic acid) provide a useful sanitization procedure for *Salmonella typhimurium* and *Listeria monocytogenes* (95). The factors that appear to influence the upper and lower limits of the range of longevity are the additive effects of cold, enhancing survival to perhaps millions of years, and oxygen and radiation (sunlight), decreasing survival times to seconds. It is unclear from the data summarized in Fig. 8 and 9 whether other factors, such as the possession of capsules and sheaths, influenced the reported times of survival. One characteristic of desiccation-tolerant cells is that while they are tolerant of desiccation, they tend to be physiologically active only at comparatively high water potentials. A *Microcoleus* sp. in desert crusts was partially inactive at −7 bars (−0.7 MPa) and completely inactive at −18 bars (−1.8 MPa) (41), and isolates of coccoid cyanobacteria from hot-desert rocks cease to photosynthesize below a matric water potential of approximately −7 MPa (205, 234) (Fig. 8; Table 2).

Desiccation plays a determinative role in the ecophysiology of bacterial communities that are found in aerophytic environments, on and inside rocks, on and in soils (276), in crusts and accretions, in soils and sediments, in the phyllosphere, in dusts and aerosols, and on the skins of animals and humans. At high matric potentials, water retention in soils and sediments is dependent on capilliary effects and is therefore strongly influenced by soil structure (274). At lower potentials, the effects of structure are much less pronounced and the texture and specific surface are of greater consequence. In terms of the distribution of water in soils, a soil water potential, Ψ_m, of 0.1 bar (−10 kPa) is normally associated with water saturation of soil capilliaries less than 30 μm in diameter; a Ψ_m of −0.3 bar (−30 kPa) is associated with saturation of capilliaries less than 4 μm in diameter; and at a Ψ_m of less than −5 bars (less than −0.5 MPa), the soil water is thought to be distributed as a film only a few water molecules thick. Matric water potential can also markedly affect gas diffusion characteristics of sediments. The drying of soils is clearly a problem if water is required for locomotion. Such effects are expected to influence the types of communities that colonize the surfaces of sediments where water loss is greatest, including the surface sands that support a Sandkörnerflora (epipsammic bacteria on sand grains [136]), the surfaces of sand dunes (290, 415, 416), and the surface sediments of Farbstreifen-SandWatt—laminated and colored sands and muds in intertidal localities that support a varied community of photosynthetic and nonphotosynthetic bacteria

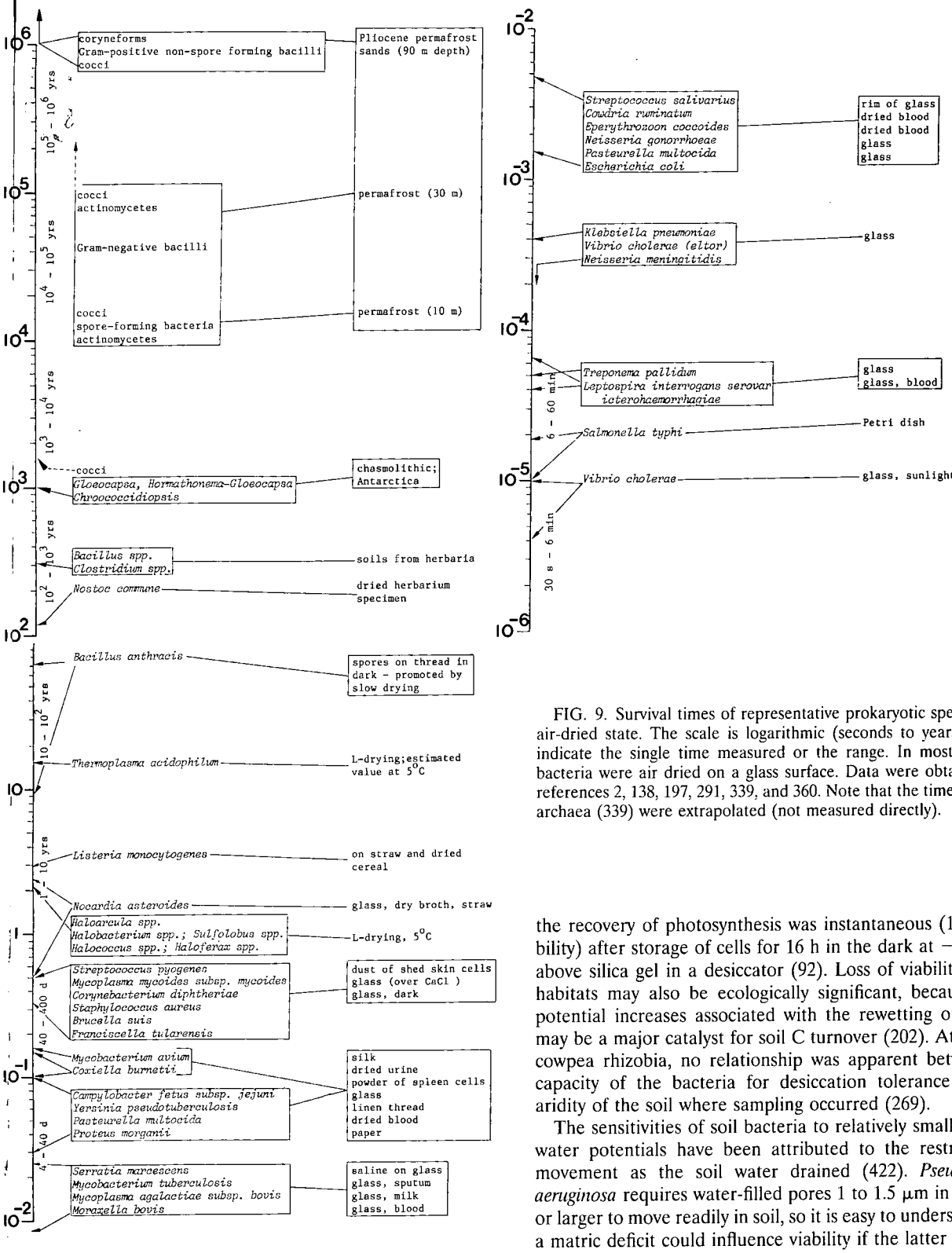


FIG. 9. Survival times of representative prokaryotic species in the air-dried state. The scale is logarithmic (seconds to years). Arrows indicate the single time measured or the range. In most cases the bacteria were air dried on a glass surface. Data were obtained from references 2, 138, 197, 291, 339, and 360. Note that the times for some archaea (339) were extrapolated (not measured directly).

(307) (Fig. 8; Table 2). The importance of soil structure and water retention characteristics can be emphasized by noting the quite different results obtained from drying cells under identical conditions on different immobilization supports (92, 93). Here, rehydration enabled the recovery of photosynthesis of desiccated cyanobacteria only on filters with good water retention. In an *Oscillatoria* strain isolated from sand dunes, the recovery of photosynthesis was instantaneous (100% viability) after storage of cells for 16 h in the dark at −400 MPa above silica gel in a desiccator (92). Loss of viability in such habitats may also be ecologically significant, because water potential increases associated with the rewetting of dry soil may be a major catalyst for soil C turnover (202). At least for cowpea rhizobia, no relationship was apparent between the capacity of the bacteria for desiccation tolerance and the aridity of the soil where sampling occurred (269).

The sensitivities of soil bacteria to relatively small negative water potentials have been attributed to the restriction of movement as the soil water drained (422). *Pseudomonas aeruginosa* requires water-filled pores 1 to 1.5 μm in diameter or larger to move readily in soil, so it is easy to understand how a matric deficit could influence viability if the latter is dependent on motility. Because the drainage characteristics of sediments differ, the optimum matric water potentials for movement and motility must vary between different sediments (274). Strains of cyanobacteria were found to have quite different survival times when liquid-grown cultures were used to inoculate dry soils (385). In this case, the different sensitivities were thought to be due to the ability of some strains to form akinetes and/or extracellular polysaccharide sheaths.

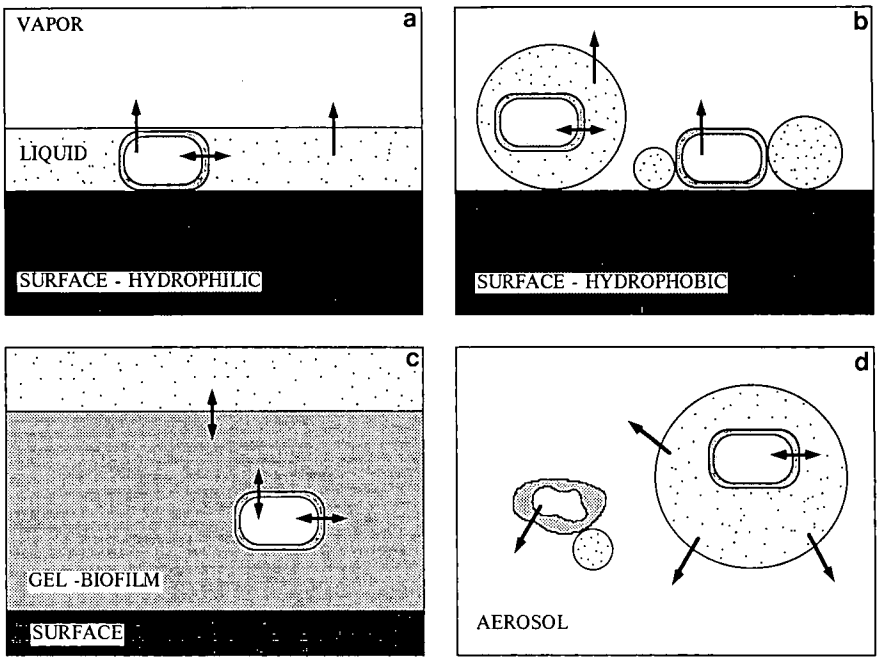


FIG. 10. Interactions of bacterial cells, water, and surfaces. (a) Bacteria in a thin film of water; (b) hydrophobic interactions at the air/surface interface; (c) bacteria in an extracellular biopolymer; (d) bacteria in an aerosol. Arrows indicate the direction of potential water movement.

Matric water potential in soils and sediments has become a topic of greater interest with the increasing attempts to introduce genetically engineered bacteria into the environment (212, 280, 282). *Enterobacter cloacae* and *Alcaligenes eutrophus* AE106(pR0101)—an engineered strain that degrades 2,4-dichlorophenoxyacetic acid—remain as intact DNA-carrying units but lose viability within 2 h of inoculation into air-dried soils (−300 MPa) (281). Light output from cells of *E. coli* MM294(pEMR1) (Lux^+) following soil inoculation decreased with time of exposure at different matric water potentials, whereas substrate-amended respiration remained constant (317). Relatively recent studies have measured the survival in aerosols of *E. coli* strains which contain Tn*5* derivatives of ColE1 (234). The plasmid-containing strains showed enhanced survival relative to the wild type, and survival was markedly dependent on the surfaces colonized.

The most extreme xerophytic environments are found in the Antarctic. Bacteria have been isolated from ice cores taken from sediments between 10,000 and 13,000 years old at Vostok station (2). Here, the bacteria can be considered to be freeze-dried. There was a general correlation between the drop in the number of viable bacteria and the depth of the sediment (below 114 m, i.e., 3,000 years old), with *Streptomyces* and *Nocardia* species being found in these deeper cores. A non-spore-forming *Pseudomonas* strain represented 7% of the biomass, and *a* and *b* variants, with two distinct morphologies, arose during culture of this bacterium. Such dissociation is known to occur when bacteria are kept for long periods under laboratory conditions, and increased storage under anaerobiosis also promotes dissociation. In deeper ice (2,450 m) only a few samples were able to generate growths in liquid culture, and all contained spore formers (*Bacillus* spp.). Moisture limitation caused by rapidly changing low temperature is thought to be the main cause of death of these cells in situ. The additive effects of drying and freezing contribute to the survival times of bacteria in ice sediments (Fig. 9). In the comparatively warmer regions of the Antarctic continent, the soils are frigic, ahumic, and are largely aerobic (2, 393). Seasonal changes in the numbers of bacteria found in these soils, including chromogenic forms, generally correlate with water availability, but in general it seems that there is a poor understanding of the distribution and activities of bacteria in Antarctic soils. Low temperatures make free water extremely scarce, and the rapid wetting of dried soil may cause cell lysis and release of a substantial fraction (17 to 70%) of the biomass carbon. In the colder regions of the continent the water potential of soils is lowered by a high mineral salt burden characteristic of soils in which evaporation exceeds leaching. Matric forces are usually unimportant, because the sediments typically consist of sandy gravel. Here, the soils are effectively sterile and the scattered populations of bacteria sustain their existence inside rocks. The rock-dwelling prokaryotes of the Dry Valleys of the Ross Desert provide a unique model for the study of desiccation tolerance (see below).

Since (i) desiccation in nature involves air drying, (ii) one consequence of desiccation is damage caused by reactive oxygen species, and (iii) the drying of bacteria is accomplished under laboratory conditions in the presence of oxygen, it is not surprising that studies of desiccation tolerance in bacteria have focused on aerobes. Do anaerobic bacteria show a greater susceptibility to air drying than aerobic forms? Probably not. Oxygen may lead to a cessation of growth of strict anaerobes, but their spore-forming representatives appear to include some of the most desiccation tolerant of bacteria (Fig. 9). No spore formers have been identified among the *Archaebacteria*, yet some archaeal strains withstand air drying and comparatively long-term storage under aerobic conditions (Fig. 9).

Freeze-Thawing, Freeze-Drying, and Air Drying—the Differences

For many research laboratories, the method of choice for storage of bacterial cultures, such as those of *E. coli*, is to add glycerol to a final concentration of anywhere between 10 and

30% (vol/vol) and to freeze the mixture, usually rapidly in liquid nitrogen, for storage at −70°C. Upon thawing, the slush can be used to inoculate fresh media. Such "permanents" can be stored for many years, and they provide a useful means to recover cell lines. Culture collections, such as the American Type Culture Collection, and industrial firms that specialize in the distribution of cultures, for example starter cultures for use in the manufacture of yoghurt and cheese, rely on freeze-drying of bacteria, fungi, algae, and viruses. Here, the culture may be mixed with some cryoprotectant, such as double-strength skim milk or growth media supplemented with 12% (vol/vol) sucrose, before being subjected to a programmed rate of cooling and drying under vacuum (45). For long-term storage, the freeze-dried pellets can be sealed in borosilicate glass vials that contain a plug of desiccant or in metal cans that are sealed under vacuum. In each case the cells are reconstituted through the addition of fresh media. To dry cells in air, cultures may be applied to some inert support which can be held, until dry, under a vapor phase of controlled humidity or a stream of air. Immobilization of cells on dry sand or dry paper has been used to preserve the viability of beta-hemolytic streptococci and other bacteria for more than 4 years (12, 213). As the research community is aware, post offices worldwide are unwittingly in the business of distributing samples of bacteria spotted on paper filters and enclosed within envelopes after preparation using either the first or the last of the three methods described above.

The same types of compounds that act as compatible solutes for osmotically stressed bacteria offer protection during freeze-thawing of proteins (13, 55). For freeze-thawing, the cryprotection afforded to isolated proteins can be accounted for by the same mechanism, the preferential exclusion hypothesis, that is thought to stabilize proteins in nonfrozen systems (53). In this respect, we can view osmotic stress and freeze-thawing as being similar in that they impinge upon systems where there is still plenty of water around. In contrast, anhydrobiotic cells appear to rely upon only disaccharides, either trehalose or sucrose, to achieve their stabilization and that of their components through a mechanism accounted for by the water replacement hypothesis (81; see reference 64 and references therein). At subzero temperatures, the increase in solute concentration due to ice formation, alterations in pH, and other changes that may occur are solution-related phenomena. Despite ice formation, then, interactions in the aqueous phase permit the exclusion mechanism to occur (55). So what about freeze-drying? Evidence shows that a compatible solute, polyethylene glycol, can protect enzymes during freeze-thawing but cannot do so when the same enzymes are freeze-dried. The same enzymes can be stabilized during freeze-drying, however, if a sugar is added together with the polyethylene glycol. Under these conditions, it was observed that the polyethylene glycol was crystalline while the sugar remained amorphous. In instances when the sugar underwent devitrification, the proteins degraded. The conclusions from these studies were that the carbohydrate forms hydrogen bonds with the protein in the dry state and serves as a water substitute and that to do so it must remain in the amorphous state. Freeze-thawing (cryprotection) and freeze-drying are fundamentally different for both intact cells and purified macromolecules (53).

There is an extensive literature on the freeze-drying of microorganisms including bacteria (249). Freeze-drying and desiccation of *E. coli* both lead to the induction of mutations (16). Recent work has focused on the survival differences among freeze-dried genetically engineered and wild-type bacteria (185). Early stationary-phase cultures of wild-type and engineered strains of *Pseudomonas syringae* and *E. coli* were flash-frozen at −80°C and then dried in a freeze-drier under vacuum. The cultures were then exposed to air of 60% RH for different time periods under other experimental variables. The engineered strains of *P. syringae* (cit7del1b [Ice$^-$] and cit#17 [Ice$^-$]) showed more sensitivity to air exposure than did the wild-type cit7 wild-type (Ice$^+$). The latter contains the IceC product in its membrane. All of these *Pseudomonas* strains showed survival during 4 h of exposure, while all *E. coli* strains tested showed survival of less than 0.01% within the first 30 min. Survival rates with a second-order decay pattern increased with dark exposure and were greater at 60% rather than at 56% RH. In the light, comparable survival rates were obtained for cells but the survival curves generated were first order. The conclusions of the study were that (i) plasmid constructs and expressed recombinant gene products represent an energy demand that can induce higher death rates upon drying and (ii) an open-air factor, identified as cyclohexane, may enhance death rates outside of the laboratory setting. This study did not provide details of the growth characteristics of the strains in liquid culture that may have permitted a more critical appraisal of the "DNA load" suggestion. The data obtained by these workers were consistent with predictions of oxygen-induced death for *Serratia marcescens* and other dehydrated bacteria (72).

TARGETS OF DESICCATION DAMAGE

At the level of the bacterial community, air drying may lead to a change in the surface area of a community, its shrinkage, a change in texture, precipitation of salts, and color changes as pigments are oxidized (Table 1). In the cells themselves, the changes may be more subtle. Discoloration may be due to condensation reactions between lysine and methionine residues of proteins and reducing sugars—the Maillard (browning) reaction. It seems that the energy changes involved in the loss of cell viability upon drying are small and that even desiccation-sensitive cells can cope with the removal of a substantial fraction of their $\bar{V}_f$ but the perturbation of a restricted number of cell water molecules leads to growth rate adjustment and/or cessation of growth. The fact that reliable viability measurements can be made at all on air-dried bacteria tells us that not all cells in a population respond in a similar fashion to a water deficit. The physiological status of the cell at the time of drying, and the time of the drying event, seem to be potential sources of this variation in sensitivity (Fig. 11). In these respects, the numbers and activities of cell macromolecules must markedly influence the physiological status of cells, and, in the long-term, macromolecules must represent the ultimate targets for desiccation-induced damage.

Proteins

Proteins may be present in cells in a few copies (e.g., regulatory proteins such as LacI) or in as many as 100,000 or so copies (e.g., ribosomal translation factors such as Ef-Tu [40]). These proteins are distributed throughout the cell compartment in an aqueous environment, whose properties remain poorly understood. Again, it must be emphasized that the organization and function of water in protein crystals and in polymers and the concept of "bound" water are topics of some controversy (117, 264). A global model of the protein-solvent interface has recently been provided (232). The essential features of the model are that there is a persistence of water structure beyond the primary layer of hydration. Second, the three-dimensional network distribution of water is strongly correlated to the protein surface topography on both local and

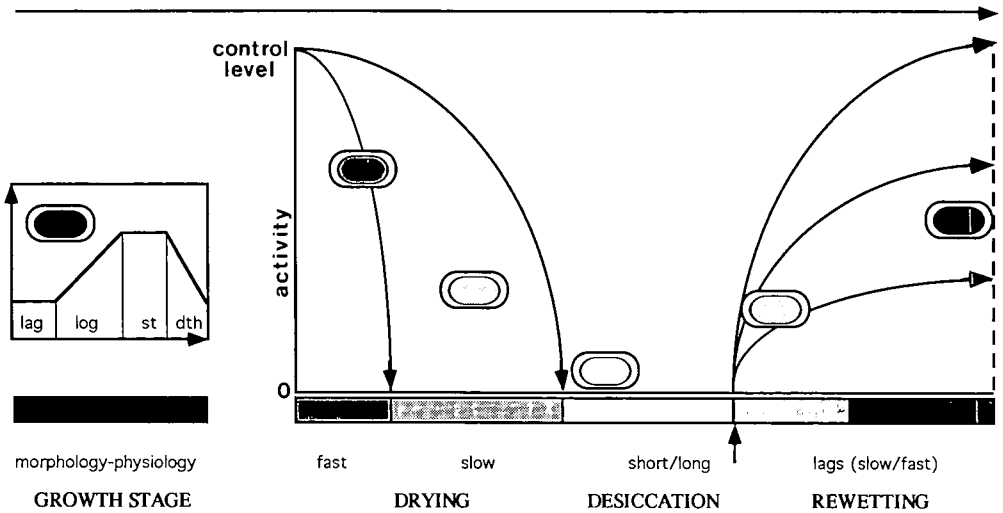


FIG. 11. Schematic time course of events during the change in water status of a prokaryotic cell following its removal from a liquid culture at a given stage of growth (extreme left) (st, stationary phase; dth, death phase). Straight horizontal arrows indicate time. A small vertical arrow indicates the time of rewetting. Shading indicates relative water content, and curved arrows indicate changes in cell activities. The *y* axis represents any measurable activity—the control level is the activity measured at the time the cell is removed from liquid culture and immobilized.

global scales. Specifically, the local solvent mobility is greatly enhanced for certain locations at the protein surface and in its interior. The immediate environment of each molecule in a cell must be vastly different. Are these differences maintained after the cells are dried, or is the state of the dried cell more homogeneous? As a cell is completely dried, are proteins left in an inanimate state ready to resume activity simply at the onset of rehydration, or do they change their state? These are important considerations because microenvironments will influence the equilibria between the different intermediates of folding pathways for each protein. For actively growing bacterial cells in liquid culture, the turnover of individual proteins can be characterized in terms of k^{cat} values which have magnitudes of seconds or minutes such that aberrant or otherwise damaged proteins do not accumulate. As cells are dried and then desiccated, these k^{cat} values tend to have less meaning as cells enter a period of quiescence that may last for years. At the time a cell is rehydrated, a considerable fraction of its protein pool may be represented by modified (damaged) proteins. Fractions of this pool may be degraded rapidly and turned over upon cell rehydration (295). How do rehydrating cells discriminate between damaged and undamaged proteins?

The normal degree of hydration of proteins (bound water) is around 0.25 g of H_2O g of protein^{-1} (189), and the ordering of these water molecules contributes directly to the properties of the proteins by influencing their interactions with ligands (24, 314). There appear to be two qualitatively different types of hydration sites. A well-defined, small number of water molecules in the interior of the protein are in identical locations in the crystal structure and in solution, and their residence times are in the range from about 10^{-2} to 10^{-8} s. Hydration of the protein surface in solution is carried out by water molecules with residence times in the subnanosecond range (270). Anhydrous lysozyme, exposed to atmospheres of different RH, has been used to obtain information on the effects of water on protein structure (see reference 357 and references therein). Franks has summarized how the protein responds to a sequential stripping of its water (120, 121). These studies have used measurements of infrared amide bonds (peptide bonds), infrared COO- bands (to monitor acidic residues; D, E), infrared -OD stretch (to monitor perturbations of water molecules), specific heat (to monitor internal degrees of freedom of macromolecules), electron paramagnetic resonance (or nuclear magnetic resonance) that can estimate rotational correlation time (freedom of rotational diffusion of macromolecules), and enzyme activity.

Both osmotic and matric methods show that cessation of bacterial growth and the onset of cell deaths occur after $\overline{V}_f$ is removed, i.e., at some compensation point when $\overline{V}_{cyto}$ approaches $\overline{V}_b$ (34, 322). When cells are dried, this value is reached at a comparatively high RH (80%). Cells that express desiccation tolerance undergo drying at much lower water potentials, and it would appear that they must withstand the most extensive perturbations of $\overline{V}_b$ for their various cellular constituents over the long term—the absolute lower limit beyond which no reactivation can occur is unknown—and/or ensure that such perturbations do not occur or at least minimize them. There is a major problem when attempting to consider these alternatives. The free energy of stabilization of globular proteins in aqueous solution is marginal, and $\Delta G_{N\rightarrow U}$ is no more than 24 kJ mol^{-1} (189). $\Delta G_{N\rightarrow U}$ is equivalent to the energy required to break a maximum of five hydrogen bonds, corresponding to about 1% of the total number of bonds in the folded structures of proteins taken from crystallographic data (189). This means that a marginal difference in hydrogen bond strengths in contacts between water-water and water-protein will lead to an energy change which may exceed $\Delta G_{N\rightarrow U}$ by an order of magnitude.

$$\underset{\text{Wet}}{\overset{\Delta G_{N\rightarrow U}}{\rightleftharpoons}}\ \text{Dry}$$

During drying, will there be a tendency for the equilibrium to shift to the right? This equilibrium is controlled directly by the degree of solvation of polypeptides and has a specific effect on protein folding (107). The relationship between amino acid sequence, folding pathways and kinetics, and the functional spatial arrangement of the polypeptide chain, is presently the least well understood step in the "central dogma" that relates storage of genetic information with its expression by protein functions (187–191, 270). Faced with these facts, the prospect for explaining how a single cell with a complement of some

3,000 proteins can have the bulk of its water removed, remain desiccated for perhaps tens of years at water potentials (assuming cell equilibrium) where $\overline{V}_b$ has been drastically perturbed, and then resume coordinated metabolic activities within seconds of rehydration is daunting at best!

In considerations of the adaptation of microorganism to extremes of temperature, pH, and pressure, it is generally assumed that evolution of protein structure is driven toward the achievement of optimum function rather than maximum stability (191). Adaptation to desiccation can be viewed as being quite different for one important reason, namely, a desiccated cell does not grow, and the time the cell remains desiccated may represent the greater part of the life (the time the cell remains viable) of that cell and its component proteins. Unless desiccation-tolerant cells accumulate proteins that serve some structural or protective role (and no evidence for this has been forthcoming), the consideration of protein "function" during desiccation is irrelevant. This fact places the question of function versus stability in a quite different context. However, there may be many ways to accumulate the ΔG_{stab} required for stabilization of a protein during desiccation and, the potential for optimized function may be of some consequence at the critical time the cells emerge from desiccation upon rehydration. If the proteins of desiccation-tolerant cells do differ from those of desiccation-sensitive cells (again, no evidence for this has been forthcoming), the features that contribute to increased protein stability are unlikely to be apparent from an inspection of the primary sequence. The proteins of desiccation tolerant cells may be either (i) inherently more stable than their counterparts in other sensitive cells, (ii) no different from those of sensitive cells but able to remain in a stable state because of one or more extrinsic factors (including other cellular components) exclusive to desiccation-tolerant cells, or (iii) no different from those of sensitive cells and equally sensitive to inactivation during desiccation (i.e., protein stability is not a feature of desiccated cells). What is known? Studies with field materials of the desiccation-tolerant cyanobacterium *N. commune* and a clonal axenic culture of *Nostoc* strain UTEX 584 have provided clues which hint that some, but not all, proteins remain stable despite extended desiccation (168, 345). The commencement of de novo lipid biosynthesis upon the rewetting of desiccated cells is instantaneous (379). With the experimental conditions used to measure this biosynthesis, it must be concluded that the lipid biosynthetic machinery, as well as the proteins required for the uptake and phosphorylation of glycerol (presumably GlpF- and GlpK-like proteins, respectively [412]) and the uptake of sulfate (presumably a permease complex [217]) and phosphate (399), remains functional during desiccation. Although the drying of *Nostoc* cells leads to a rapid cessation of nitrogenase activity (298, 300), no evidence was obtained for hydrolysis of at least one structural component of nitrogenase, Fe protein, which was present in cells following 10 years of desiccation (279). The intracellular ATP pool, as well as the protein-biosynthetic machinery, of desiccated cells remained unperturbed during 30 min and 2 h, respectively, after rapid drying at −99.5 MPa (7, 298, 300). In contrast, even short-term drying leads to structural changes in the pigment antenna complexes of cyanobacteria, the phycobilisomes. In the light, the phycobiliproteins are degraded, and even in the dark, short-term drying leads to subtle changes in the polydispersity of the complexes when analyzed in sucrose gradients (294, 345). Long-term desiccation leads to destruction of phycobiliproteins (345). In view of the short half-lives of mRNA and the susceptibility of nucleic acids to desiccation-induced damage (see below), the need to protect RNA-binding proteins (ribosomal proteins, transcription factors, DNA-dependent RNA polymerase) may be crucial. The immobilization and rapid drying of *Nostoc* strain UTEX 584 cells lead to a rapid loss of the *rpoC1C2* transcripts that encode two subunits of the RNA polymerase (γ and β′). These transcripts accumulated to control levels within 60 min of rewetting of cells that had been kept dry for 24 h, at −99.5 MPa. Immunoblotting confirmed the presence of Rpo proteins in the same dried cells (see above). De novo transcription in rehydrated cells of *Nostoc* strain UTEX 584 is therefore directed by extant RNA polymerase holoenzyme that maintains its stability during desiccation and at least during the time of rehydration when some transcripts accumulate to control (predrying) levels (430). Are DNA- or RNA-binding proteins any more stable than other cellular proteins, or does the stability of RNA polymerase in the example mentioned here simply reflect the fact that there are numerous copies per cell and a finite pool escapes damage? The work needed to overcome frictional drag under turgid conditions in *E. coli* can be calculated as roughly 0.2 kcal (0.84 kJ) (432). One consequence of drying cells must be that it imposes some restriction upon the efficiency of enzymes, such as RNA and DNA polymerases, by increasing the work needed to overcome the frictional drag against the viscous solvent—remember the discussion on glasses and WLF kinetics. Over the long term (millions of years), catalase is another enzyme that has the capacity to maintain its stability (138). Yet, although it seems clear that many proteins remain stable in air-dried cells of desiccation-tolerant bacteria, it should be noted that even in desiccation-sensitive cells of *E. coli* the enzymes required for productive T2 infection remain active in dried, nonviable (dead) cells (403). The anhydrobiotic cell is more than simply a collection of dried components.

Nucleic Acids

Nucleic acids represent prime targets for desiccation-induced damage. In large part the damage reflects the accumulation of mutations during the time when there is no cell growth (during desiccation). It is unlikely that repair mechanisms operate in air-dried cells, and this damage will become manifest only upon rehydration (351). Mutation of an arginine auxotroph to the prototroph was induced in *E. coli* strains dried to an a_w of 0.53 and below but not when dried to an a_w of 0.75 and above (15). Because significant mutation on drying occurred in wild-type strains and in strains carrying *uvrA* and *polA*, but not in *recA* strains, it was concluded that the mutation is caused by errors in *rec*-dependent repair of the drying-induced breakage of the DNA. Damage to DNA may arise through chemical modifications (alkylation or oxidation), cross-linking, base removal such as depurination, or ionizing and nonionizing radiation. For bacterial spores, it is thought that the reduced water content retards and/or alters reactions that affect DNA; however, it is quite clear that even with reduced water contents, damage sufficient to compromise viability over even short periods occurs. For example, the measurement of more than 10 breaks per single-stranded genome in cells of *E. coli* K-12 strain AB 1157 (*uvrA*$^+$ *recA*$^+$), caused by drying them for only 12 min, emphasises the sensitivity of DNA to drying (28). The genome of a *Bacillus* spore suffers around 50 strand breaks during exposure to vacuum for 3 weeks (28) and becomes progressively altered upon more prolonged storage. One feature of the alteration may be an increase in the number of cross-links between protein and DNA that accumulate continuously during dry storage. Even the DNA of radiation-resistant *Deinococcus* strains suffers single-strand breaks during a few days of vacuum

exposure (28). Single-strand breaks and other DNA lesions are readily repaired during the germination phase of *B. subtilis* spores (101). In a study that compared the survival of different mutants, the survival of one mutant (*ssp uvr*) was about 20% less than that of other strains. The conclusion from this study was that single-strand breaks are probably repaired by the same ligases that are required for DNA replication and therefore that special "repair-ligase"-deficient mutants may not exist (101). A substance has been identified that degrades both DNA and RNA in cells of the anaerobe *Roseburia cecicola* when they are exposed to air (263). The substance has an M_r of 2,800 and requires a reducing agent for activity. The spectrum of factors that can contribute to cell death upon drying is clearly very broad.

An important factor in the accumulation of damage must be the number of chromosome copies present per cell, and this in fact may be one mechanism of desiccation tolerance. Fifty strand breaks may be critical for a sensitive cell that carries one chromosome equivalent, such as *E. coli*, and for other bacteria that may partition different sets of genes on different chromosomal elements but much less so for bacteria, such as cyanobacteria, which may have multiple copies of their genome present in each cell (355).

For *Nostoc* DNA, a rate of depurination at 37°C of ~20 per genome per day can be estimated from the median genome size measured for several *Nostoc* spp. (5×10^3 MDa = 7.6×10^6 bp [166]), assuming an internal pH of 7.4 for the cytoplasm (note that the thylakoid space may have a different pH and that the pHs of the two compartments vary with light intensity [398]), and an in vitro rate of depurination of $k = 2.58 \times 10^{-6}$ day^{-1} (227). The rate of depurination of a desiccated *Nostoc* cell would achieve a 1% depurination of the genome after storage of the cells at 37°C for 10 years. However, desiccated crusts in situ are exposed to temperatures far in excess of 37°C, and depurination rates would be expected to increase; furthermore, the genomes of *N. commune* contain significant amounts of 6-methyladenine (193) and these residues are lost from DNA at a rate approximately two to three times higher than for other purines. Yet herbarium specimens remain viable after more than a century in the desiccated state (49)! These considerations again lead back to the question of the state of dried cytoplasm. The DNA in laboratory-grown cells of *Nostoc* strain UTEX 584 undergoes appreciable light-dependent nicking after only short periods when the cells are dried in air (375); however, it lacks the conspicuous glycan of field material. The mRNAs isolated from desiccated *N. commune* supported low rates of translation in vitro in either homologous or heterologous translation systems, suggesting that they may be modified (192). It is interesting that the activation energy for depurination (31 ± 2 kcal mol^{-1} [130 kJ mol^{-1}]) is around sixfold greater than that needed to unfold a protein. Superficially, then, it seems that DNA damage would be expected to follow, rather than precede, protein damage if only the removal of water is considered and no extrinsic factors are taken into account. Mechanisms must be present to retard the rate of depurination and other DNA damage in cells growing in situ.

It is expected that lesions in DNA may be accentuated in organisms that are dried in situ where incident UV fluxes may be appreciable, yet such communities may have a range of induced systems, not present in laboratory-grown strains, to prevent damage. Studies suggest that induction of DNA damage by UV in dried cells is a multistep process (186). Photon energy is initially absorbed in a sugar-phosphate group, and destruction of the sugar follows, accompanied by breakage of the ester bond between the 3′ C of the sugar and the phosphate, leaving a 5′ phosphate terminal. A base attached to the destroyed sugar moiety may then be released. The other end of the DNA strand may have a sugar or a 3′ OH residue (P_i may also be released during this process), and in polynucleotides 5′ deoxynucleoside triphosphate is lost at the site of the strand break. Drying of cells certainly leads to an accumulation of free radical and reactive species, and these may participate in further DNA damage, as indicated in Fig. 10. The kind of UV damage to DNA appears to depend on the secondary structure of the double helix—which is further influenced by the amount of bound water (228)—and apparently the availability of water (238). In mature spores of *B. subtilis*, the A form of DNA prevails, and one must presume that it does so also in desiccated vegetative cells of other bacterial species. Irradiation with UV results in the formation of 5-thyminyl-5,6-dihydrothymine, the so-called spore photoproduct. Only when cells are irradiated in vacuo ($a_w = 0$) do the *cis,syn* and *trans,syn* isomers of the thymine dimer accumulate, and these appear to be responsible for the lethal effects of acute drying. The degree of hydration around forespore DNA is reduced by the presence of dipicolinic acid, which influences photoproduct formation without causing a change in the conformational state of DNA (229). Desiccated field materials of *N. commune* contain two discrete fractions of genomic DNA, which are readily separated by cesium chloride density ultracentrifugation (193). One fraction is hypermethylated, and the other is hypomethylated. The two fractions associate with different types of carbohydrate complexes, a feature that probably permits the separation of the DNA fractions in gradients. The functional significance of these two fractions remains unknown.

Lipids and Membranes

The concept of lipid fluidity in membranes has its origin in the studies of Chapman and colleagues (59). The surface tension of water holds membranes together. To deform (stretch) a membrane, or to make a hole in it, requires an increase in the water/membrane interface. The latter requires that hydrogen bonds be broken and that the water molecules reorientate; these requirements contribute positive enthalpy, and negative entropy, respectively, to the large surface free energy of water (424). However, this does not mean that if a membrane is dried it will simply fall apart. Because of a decrease of the lateral spacing of the polar head groups and the subsequent lining up of the hydrocarbon chains, the transition temperature (T_m) of membranes increases considerably when they are dried (48, 172). As such, during drying T_m surpasses ambient temperature and the gel and liquid-crystal phases transiently coexist. As the permeability is very high in such two-phase systems, this event will be catastrophic for a cell if free water is available for solute transport (76, 77). Desiccation-tolerant cells must have mechanisms to suppress the dehydration-induced rise in T_m in order to postpone phase changes that take place during drying to moisture contents at which no free water is left for solute transport and leakage. The polar head groups of phospholipids, by influencing Fe^{2+} autooxidation, generate dangerous oxygen species which may play a role in the oxidation of the hydrophobic components of the lipids (246). Considerations of the effects of drying on membranes are extensive (76–80, 82, 84, 172, 173, 175). For *Rhizobium* spp., studies indicate that death of these bacteria in response to desiccation and rehydration is caused by changes in membrane permeability (47). Differences in the polar/nonpolar nature of the outer membrane may account for the variabilities in susceptibilities to desiccation of different strains.

The freeze-drying of *E. coli* and exposure of the dried cells

to oxygen cause damage to the bacterial cytoplasmic membrane so as to make it leaky to potassium. The damage is most extensive, and becomes irreversible, if the cells are not held under vacuum (when dried) prior to rehydration (183). The damage is localized in the cell membrane, seems to involve the DNA initiation complex, and appears to occur in two stages. The primary damage is due to freeze-drying, but this damage can be repaired upon subsequent rehydration of the cells and following a period of incubation that requires protein synthesis. Second, after exposure of the freeze-dried cells to oxygen, the injury becomes irreversible and the cells die. The lethal effects can be countered by engaging the DNA initiation sites, for example with colicin E1, or by arresting the activity of the initiation complex. Such arrest occurs in temperature-sensitive mutants at the nonpermissive temperature or in auxotrophic mutants starved of amino acids or thymine (184).

Mechanisms of Damage

Damage to both DNA and proteins is mediated through reactive oxygen species (37). Although molecular oxygen is strongly oxidative with respect to its fully reduced form, water, its oxidative potential is normally held in check by kinetic restrictions imposed by its two unpaired spin-parallel electrons (179). Consecutive univalent reductions of oxygen produce superoxide ($O_2^{\cdot -}$), H_2O_2, and hydroxyl radical (HO·), with the following reduction potentials (158):

$$O_2 \xrightarrow{-0.33\ V} O_2^{\cdot -} \xrightarrow{+0.94\ V} H_2O_2 \xrightarrow{+0.38\ V} HO\cdot + H_2O \xrightarrow{+2.33\ V} H_2O$$

DNA breaks accumulate during exposure of bacterial cells to $O_2^{\cdot -}$ and H_2O_2. It is expected that free radicals will accumulate during drying, especially in the light, when cells are subjected to high incident solar radiation. Electron spin resonance spectra suggest that desiccated cells of cyanobacteria do, in fact, contain appreciable amounts of free radicals (291). Oxidative damage will be manifest in protein damage and lipid peroxidation, leading to a loss of a diffusion barrier to membrane-impermeable markers and ultimately to cell lysis. Some indications of the type of damage that may occur come from studies in which bacterial cells have been exposed to H_2O_2. Such exposure of *E. coli* cells leads to two types of cell killing. Mode 1 killing is pronounced at low concentrations of H_2O_2, and mode 2 killing is pronounced at high concentrations. It seems likely that mode 1 is of most relevance to desiccated cells, and the acute sensitivity of *Treponema pallidum* (Fig. 9) to air drying may be a reflection of its acute sensitivity to H_2O_2 (371). One proposed mechanism for single-strand nicking of DNA catalyzed by reactive hydroxyl groups is shown in Fig. 12. Single-strand DNA breaks that contribute to mode 1 killing in DNA repair-deficient strains result from the collapse of the deoxyribose ring after abstraction of a hydrogen atom (179). Abstraction of the hydrogen proceeds through the Fenton reaction and is catalyzed by transition metals, such as cellular iron, and affected by the ferryl radical. To maintain an ongoing Fenton reaction, an electron source must be available to regenerate the reduced metal. Evidence suggests that the Fenton-active metal is reduced on the surface of DNA—where it is probably chelated to the phosphodiester backbone—and the metal reductant is a small diffusible molecule such as NAD(P)H. Lines of defense against such Fenton reactions include DNA repair mechanisms, synthesis of scavenging enzymes such as catalase, and the depletion of intracellular NADH. As such, these reactions would represent three potential responses to drying-induced damage. Thiols such as glutathione (H donation) may either prevent or enhance damage, depending on the prevailing physiological conditions (216).

Conformational changes in DNA can be influenced by hydrophobic interactions in the major groove of the DNA as a result of base methylation (196). It is not clear to what extent methylated and nonmethylated DNAs are subject to damage. Damage may be prevented by lowering the intracellular pool of reduced pyridine dinucleotide. It is interesting that the cyanobacterial heterocyst has a very high level of NAD(P)H (161) and this may contribute to the sensitivity of heterocysts to drying, despite their capacity to synthesize superoxide dismutase (SOD) and catalase (51, 153, 426). Small, α/β-type acid-soluble proteins influence the structure and photoreactivity of the spore DNA in *Bacillus* and *Clostridium* species (113). The binding of the proteins to DNA prevents formation of cyclobutane-type thymine dimers upon UV irradiation and promotes formation of the spore photoproduct. These DNA-binding proteins require that the DNA be able to form A-type DNA, and they prevent depurination of more than 20-fold (112, 351). Although the affinity of the proteins for DNA is not great, they are present in such large amounts that virtually all of the DNA is likely to be coated. The repair systems in spores include an excision repair system which can repair spore photoproduct and cyclobutane dimers and an error-free spore photoproduct-specific system (characterized by the *spe* locus) that requires energy but not light (351). Because depurination is water catalyzed at neutral pH, the low a_w of desiccated cells may slow strand scission but will not prevent a certain proportion from occurring. The low a_w will cause conformational changes in DNA, converting it from the B form to the A form and, as shown for dried spore DNA, may decrease the accumulation of T<>T. No T<>T is formed in dormant spores. Curiously, the drying of spores in a vacuum, where the atmospheric a_w is effectively at infinity, does lead to the accumulation of T<>T dimers (228).

Oxygen itself can act as a free-radical scavenger! When oxygen is present, it rapidly forms $O_2^{\cdot -}$ from Fe^{2+}, and the following reactions become physiologically relevant:

$$Fe^{2+}\ (\text{chelated}) + O_2 \rightleftharpoons Fe^{3+} + O_2^{\cdot -}$$

$$NAD\cdot + O_2 \rightarrow NAD^+ + O_2^{\cdot -}$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

These reactions suggest how SOD could prevent the Fenton reaction-catalyzed damage to DNA. Interestingly, there is some argument whether inactivation of bacteria by singlet oxygen occurs in a lipid or an aqueous environment (275).

Dehydration of proteins induces significant and measurable conformational changes, as deduced from analyses by Fourier transform infrared spectroscopy (309; see above). The changes observed are protein dependent, the removal of water has no effect on the amide I vibration, and the observed spectral changes upon dehydration are conformational. These changes may be prevented in large part through the addition of sugars and polyols (see below). The very fact that water is removed at all may be the reason why some proteins remain stable when they are dry (asleep).

Metal-catalyzed oxidation of proteins is an additional likely consequence of drying cells. The oxygen-dependent reaction proceeds in a manner that is rather similar to the one described

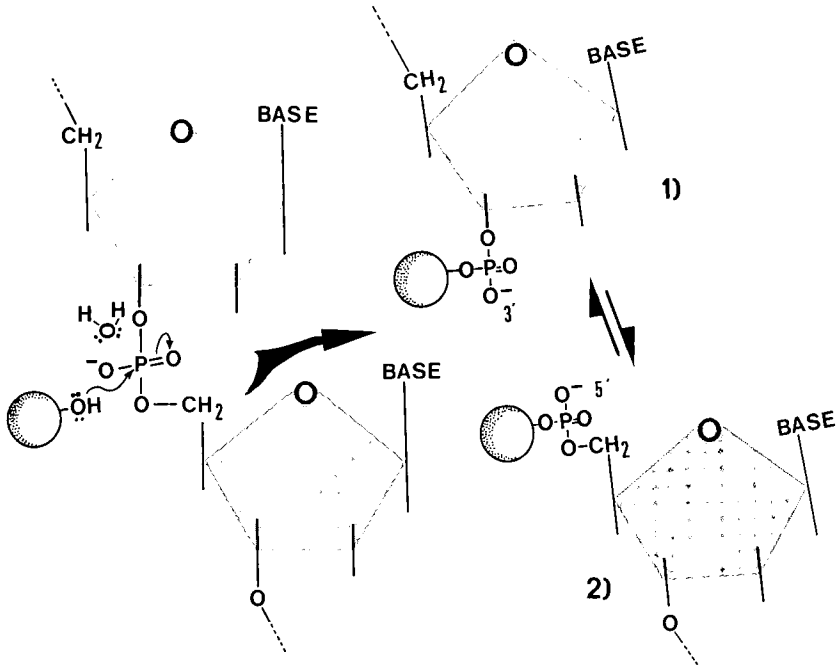


FIG. 12. Possible mechanism for the introduction of single-strand breaks into desiccated DNA. Shown is a molecule (sphere) with a reactive hydroxyl group that nucleophilically attacks the P atom of the phosphodiester backbone. The reaction depends on the availability of water molecules. The formation of DNA strands with molecules covalently attached at their 3′ or 5′ positions is in equilibrium.

above that leads to DNA damage, in that the reaction is mediated through [Fe^{3+} OH·]. Reduction of oxygen yields H_2O_2 directly or yields superoxide as an intermediate, which reacts with Fe^{3+} to give Fe^{2+} and oxygen. The Fe^{2+} binds a metal-binding site in the protein, and the Fe^{2+} complex reacts with the H_2O_2 to generate ferryl ion, leading to reactions with protein side chains. After oxidative modification, the proteins become sensitive to proteolysis and/or may be inactivated, or they may show reduced activity. Histidyl, prolyl, lysyl, and methionyl residues are converted to aspariginyl, pyroglutamyl, aldehyde, and methionyl sulfate residues, respectively (367). Some residues are converted to carbonyl derivatives. Carbonyl derivatives accumulate in proteins as they age (4, 361), and it seems that they may accumulate in desiccated cells, although, again, it is unclear to what extent the very low a_w will diminish the effects of these reactions. Protein damage is clearly of some consequence to cells that remain desiccated for long periods with very low turnover rates, such as the Antarctic chasmolithic communities described below.

Cells stored for long periods also may become subject to the effects of ionizing radiation (360). Apart from any intentional exposure, only the natural abundance of ^{40}K in cells requires consideration. The intracellular K content of *E. coli* grown between 148 and 938 mosm varies between 150 and 495 mmol liter of cell water^{-1} and is 0.6 M when cells are grown at 1.2 osm (181). Similar values have been measured for *Anacystis nidulans* (398). Assuming that a bacterial cell contains approximately 6.7×10^{-16} liter of water (255), at 1.2 osm a single cell will contain 4×10^{17} atoms of K, of which 4×10^3 atoms are ^{40}K, each with a half-life of 10^9 years and a single decay energy of 1.3 to 1.5 MeV. It seems that the contribution of ^{40}K can be considered negligible on the scale of desiccation discussed here, although it is true that the form of this decay is not well understood and short-term effects may still occur.

RESPONSES TO DESICCATION—MECHANISMS OF TOLERANCE

Fulton (128) has drawn an analogy between the contents of a cell (cytoplasm) and a protein crystal. In the latter, the solvent component represents between 20 and 90% of the total weight (an average ratio of 40:60 of H_2O to protein; or 0.66 g of H_2O g of protein^{-1}). Using this analogy, Fulton considered that "either size variations in the bulk phase are accompanied by exquisitely balanced reciprocal variations in enzyme activities with changes in concentration or, the precise volume of the bulk water phase is of minor importance because the major site of regulation is the water of hydration." Certain of the discussions presented so far would seem to favor the latter opinion.

Certain advantages may be gained from a juxtaposition (chaneling) of groups of enzymes that catalyze sequential reactions—so-called metabolons (235)—and one advantage may be to protect the solvation capacity of cell water. One feature of the recovery of desiccated cells upon rehydration is a very rapid onset of cellular metabolism in conjunction with a stepwise and stringent recovery of metabolic processes (379). Perhaps desiccated cells have evolved such that they make good use of global metabolon organization with a limited amount of water. The inherent structural organization inside air-dried cells, the most critical consideration, is the least understood feature of desiccation tolerance.

The Anhydrobiotic Cell and a Water Replacement Hypothesis

The anhydrobiotic cell is characterized by its singular deficiency in water. The water deficit is far greater than can arise if a cell is immersed in a concentrated solution of solute (osmotic stress) and far greater than can arise in freeze-tolerant cells in the presence of extracellular ice. There is also

another important distinction. Some desiccation-tolerant cells accumulate large amounts (sometimes in excess of 20% of their dry weight) of either one or both of the disaccharides trehalose and sucrose (81). Such observations have led to the realization that such disaccharides are effective at protecting enzymes during both freeze-drying and air drying. The important point is that the preferential exclusion mechanism described for systems of intermediate water content cannot be applied to the desiccated cell in which the proteins have been stripped of their solvent monolayer. That is, trehalose and sucrose are not acting as compatible solutes here. How can we reconcile the functions of oligosaccharides such as trehalose and cell survival without water? John and Lois Crowe and colleagues have developed a water replacement hypothesis to explain how components may be protected during extreme drying (64). Essentially, the hypothesis is that polyhydroxyl compounds, such as trehalose, replace the shell of water around macromolecules, circumventing damaging effects during drying. Webb alluded to a similar effect, due to polyhydroxyl compounds such as inositol (but see below), in dried bacterial cells (403, 404). Experimental evidence for a water replacement hypothesis has, indeed, been forthcoming (see, e.g., reference 388). When carbohydrates are dried in the presence of proteins, the capacity for the carbohydrate molecules to form intermolecular hydrogen bonds between themselves is diminished. Significantly, not only does hydrogen bonding occur between stabilizing carbohydrate and protein but also the binding of the solute is requisite for preservation of labile proteins (54). Dehydration-induced alterations in the vibrational spectrum of lysozyme were reversed, in part, when the protein was dried in the presence of trehalose and lactose but much less so when it was dried with *myo*-inositol. The data indicate that carbohydrates appear to be more effective at reversing changes in the infrared spectrum of lysozyme that derive directly from from removal of water (shift of amide II to a lower frequency and loss of the carboxylate band at 1,583 cm^{-1}). Recent evidence for this includes the observation that the P=O stretch of the phospholipid increases in frequency by about 30 cm^{-1} when the protein was dried without trehalose but is decreased to or below the frequency of hydrated P=O when the protein was dried with trehalose. Europium, which binds phosphate, competitively inhibits the stabilizing effect of trehalose, and molecular modeling shows that trehalose can fit between the phosphates of adjacent phospholipids (334). Dextran is a noncompetitive inhibitor for stabilization by trehalose. At low trehalose/lipid ratios, the trehalose is not available to bind water; this is taken as evidence for a direct interaction between the sugar and lipid. Stabilization of liposomes is achieved in the region where the water content remains low, suggesting that residual water in dry samples is not involved in stabilization (78, 82, 84).

As far as we know, the properties of water are immutable. With a leap of faith, we could assume that every type of anhydrobiotic cell must conform to the same constraints imposed by the removal of water. The state of one particular anhydrobiotic cell may therefore be representative of other types of dried cells. In this context, Clegg has provided a most lucid and illuminating discussion of the state and content of water in *Artemia* cysts and the nature of the cytoplasm in an air-dried cell (64, 67). The most significant finding is that if cysts contain any water that exhibits the dielectric properties of water in aqueous solutions, it can only be a tiny fraction of that present. Only a fraction of the water detected at low water contents had the properties ascribable to "bound" water as deduced through measurements of quasielastic neutron scattering, and, in the initial stages of rehydration (unlike anhydrous protein), cysts exhibited no initial independence of e' (dielectric permittivity). Such an independence is taken as a measure of "bound" water. At this point the reader may review, again, recent discussions on some of the controversy surrounding the nature and existence of "bound" water (357). An additional feature of *Artemia* cysts was, perhaps as expected, the conclusion that their internal viscosity must be enormous. In short, Clegg's study of whole cells provided data that supported, and in some cases were consistent with, the water replacement hypothesis. This work provided some hints as to how dried cell components remain viable. What this study and others cannot explain fully is how these components can be redirected, instantaneously and in perhaps an ordered and stringent fashion, to resume integrated metabolism (and all that it means) upon rehydration of the dried cell.

Physiological Mechanisms

In isolated membranes, free radicals cause fatty acid deesterification from phospholipid. Free fatty acids typically accumulate in desiccation-sensitive cells during aging and are a cause of reduced membrane integrity. The respiratory rate prior to desiccation correlates well with the number of free radicals in the dry state, which suggests that the curtailment of respiratory metabolism prior to rehydration may be essential for the retention of membrane integrity and desiccation tolerance in general (172). Of course, cells will not know when they are due to be dried—or will they? There is recent evidence for a circadian rhythm in a prokaryotic cell, a cyanobacterium (210), and there are many cases in which the desiccation of bacterial cells is subject to an environmental rhythm, e.g., communities in the upper intertidal of maritime zones.

Imbibition of viable, dry cells may result in extensive leakage and death, particularly when it occurs at low temperature, because rehydration may involve a reverse phase change of membrane lipids from the gel to the liquid-crystal phase—this phase change occurs in the presence of ample water (82). Two methods can circumvent this effect: humidifying with water vapor prior to imbibition, and heating at imbibition (172).

Studies suggest that cells in the stationary phase are structurally, physiologically, and functionally distinct from those in the log phase. Stationary-phase cells of *E. coli* show a marked enhancement of resistance to air drying (Fig. 13), and the genes expressed in response to osmotic shock also appear to be involved in the adaptation to stationary-phase growth (354). Some of the properties of stationary-phase cells may therefore contribute to this enhanced resistance and deserve consideration here (Fig. 11). Table 3 summarizes the major events that occur when bacterial cells enter stationary-phase growth (354). The decrease in overall translation activity occurring with the transition from exponential to stationary-phase growth is accompanied by the appearance of 100S (dimers) ribosomes. A protein (protein E) is thought to be a ribosome modulation factor responsible for the dimerization. Interestingly, the gene encoding protein E, *rmf*, maps next to *fabA* (395). FabA is required for the introduction of double bonds into growing fatty acid chains of membrane phospholipids (73). The role of the double bond is to decrease the phase transition temperature of the phospholipid that contains the fatty acid (see below). As cells enter the stationary phase, however, cyclopropane fatty acid synthase modifies the phospholipids of the inner and outer membranes to cyclopropane fatty acids, a modification of *cis* double bonds with CH_2 (using CH_3 from *S*-adenosylmethionine). Cyclopropane fatty acids thus accumulate in stationary-phase cells. Although the function of this modification seems unclear, it has been observed that "they

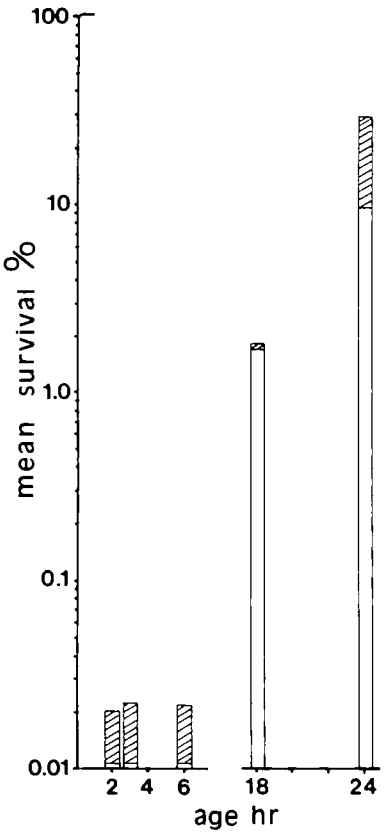


FIG. 13. Mean survival of air-dried *E. coli* cells according to the age of the culture at the time of immobilization (hours). Viability was measured after either 2 h (▨) or 24 h (□) of drying. The scale on the *y* axis is logarithmic. Data from reference 241.

play a vital role in the natural environment that has not been duplicated in the laboratory (73)." No accumulations of cyclopropane fatty acids could be detected in desiccated samples of *N. commune* collected from a range of different geographical locations (304).

Drying of cells may lead to a change in ribosome structure, because water is involved in the maintenance of the integrity of the ribosome (88). The translational machinery of *Nostoc* strain UTEX 584 was maintained for 2 h following drying of cells at −99.5 MPa, but longer periods of drying led to a loss of polysomes (7).

Bacterial Glasses

What do "glass theory" and WLF kinetics really mean in the context of desiccation tolerance? Three questions must be considered here. First, do bacterial glasses exist? Second, if bacterial glasses do exist, could they represent a mechanism for the desiccation tolerance of the cell in question? And third, but not least, how could a bacterial cell enter (and leave!) the glassy state through the corridor controlled by WLF kinetics?

Bacterial cells may accumulate significant amounts of a diverse collection of solutes, including sucrose and trehalose, that behave as glasses, as well as other polymers such as polyglucosyl granules that may also have the capacity to do so. Some sugars that replace bound water, e.g., trehalose, sorbitol, fructose, sucrose, and glucose, all can form aqueous glasses, and some of these are stable at 90°C. The glass transition temperatures and phase relations for several saccharide-water systems have been reported (151). The trehalose-water system is distinguished from the others by a significantly higher T_g at all water contents, with a particularly large advantage near the stoichiometry of 1 water molecule per glucose ring. The significance of glasses is that, in principle, the complete dehydration of bacterial cells may be avoided at temperatures below the melting point of the glass. The thermal transition characteristics of glasses is such that they could provide protection between 0 K and 90°C (44). However, the temperature range over which glasses form is unclear for complex biological systems such as bacterial cells. Yet, some bacterial cells elaborate conspicuous amounts of complex extracellular polysaccharides (EPS) (biopolymers) that are so varied in their gel and sol properties to suggest that they may have properties that mirror those described for other natural and synthetic glass-forming polymers. The characterization of naturally occurring bacterial EPS may in fact shed light on the development of new synthetic polymers. The extracellular glycan of *N. commune* has been characterized in detail. When dry, colonies are brittle. In the initial stages of wetting, the colonies become leathery and then achieve the consistency of a semirigid, malleable gel. Liquid cultures derived from these colonies secrete a viscous polysaccharide (169). How could we identify bacterial glasses? The interpretation of differential scanning calorimetry isotherms of purified components has become a controversial subject (see reference 1 and references therein). The interpretation of transitions in cell systems would be even more complex.

In the glassy state, reactions are slowed to periods that are more than sufficient for the times some bacterial cells may remain viable in the desiccated state. All water in a bacterial glass would, in effect, be immobilized, and the properties of this extremely crowded cytoplasmic glass would appear to be consistent with the requirements for long-term stability. Now the problem. If a bacterial cell enters into a glassy state upon the removal of a large fraction of its intracellular water (Fig. 1), it must pass through the glass transition state. An equivalent transition (different direction) would occur upon the arrival of water (rehydration = plasticization). These transitions may be accompanied by a change in T (ΔT), and the cells during these transtions will then be subject to the effects of WLF kinetics. The special case when a transition occurs at constant temperature in response to ΔW is considered further below. What could be the effects of WLF kinetics on the rehydrating or drying cell? The rates of biological reactions, like those of chemical reactions, are limited by temperature, but they are also limited by the rates at which diffusion can bring the reactants together (394). The maximum rate is thus set by the encounter probabilities of the components:

$$k_{\text{encounter}} = 4\pi(D_A + D_B)(r_A + r_B)N_0/1{,}000$$

the so-called Smoluchowski limit where D_A and D_B are the diffusion constants and r_A and r_B are the hydronamic radii of molecules A and B, respectively. N_0 is Avogadro's number, and the 1,000 factor normalizes units of $k_{\text{encounter}}$ to reciprocal molar seconds. The net rate at which A and B (assumed to be spheres) diffuse together depends on their sizes, the temperature (T), and the solvent viscosity (η), as defined by the Stokes-Einstein relation:

$$D_A = kT/6\pi\eta r_A \text{ and } D_B = kT/6\pi\eta r_B$$

It is important to emphasize that the problem under consideration here is the consequence, say during rehydration, of the rapid and abrupt change in the rate of a reaction within a prescribed range of temperature. The substrate and its catalyst

TABLE 3. Characteristics of the transition from log phase to stationary phase[a]

Physiological target	Consequences
Cell morphology	Cells become smaller (almost spherical); cytoplasm condenses; volume of periplasm increases
Surface properties	More hydrophobic, adhesive, may make fimbriae
Fatty acid composition	All unsaturated fatty acids converted to cyclopropane derivatives
DNA	Remains stable but the nucleoid(s) condenses
Metabolic rate	Rate decreases, and ΔpH and ΔΨ contribute to a change in the membrane proton motive force; trehalose synthesis increases in response to C starvation (osmotic challenge); starvation-specific thermotolerance and osmoprotection induced
Gene regulation	*katF* expression increased
Proteins	Turnover increases fivefold, enhanced proteolysis; 30–50 new proteins are synthesized; expression of catalase, HPII
RNA	Stability diminishes; 20–40% of total RNA lost during first several hours of starvation; tRNAs may be hypermodified
Ribosomes	Dimerization
Spontaneous mutation rate	Rate increases

[a] Data are for cells of *E. coli*; data are taken from reference 354.

are, however, also under mutual constraints with respect to reaction specificity. In the crowded cytoplasm of a desiccated cell, reactants will certainly be in high concentration initially. Upon rehydration, there will be an instantaneous appearance of moisture gradients, a rapid dilution of reactants, and an activation of enzymes. The speed at which these processes occur and the time during which WLF kinetics may apply appear to be very important considerations, yet they are hard to gauge. While chemical reactions can be interpreted in terms of Arrhenius kinetics, the sum of a collection of reactions, in the context of bacterial growth, shows a somewhat different response. In the midrange of growth, Arrhenius kinetics do appear to operate. Above and below this range, however, the growth rate is less than would be predicted through extrapolation (181). Furthermore, the binding of *E. coli lac* repressor to its target (operator) DNA site occurs at a rate that is almost 3 orders of magnitude greater than the upper limit estimated for a diffusion-controlled process involving macromolecules of equivalent size (see reference 394 and references therein). We really have little idea of how desiccation and rehydration affect the whole cell, and it is clearly more complex than simply a sum of a collection of reactions in, or out of, equilibrium. It is interesting that for anhydrous (0.01 g of H_2O g of protein^{-1} up to 0.1 g g^{-1}) samples of lysozyme, hemoglobin, and myoglobin, their heat capacity slowly increases with increasing temperature without showing an abrupt increase characteristic of glass-liquid transitions (342).

WLF theory predicts the viscoelastic properties of solutes and polymers in terms of ΔT above T_g. Can an analogous equation be used to predict those same properties on the basis of water content changes such as those associated with desiccation and rehydration, i.e., ΔH_2O? This is not a trivial question, because glass transitions in biological systems, if they occur, may occur in response to large fluctuations in W at or close to constant temperature. Consider the following hypothetical example. Cell$_x$ of intermediate water content (W_{g_x}) is in the fluid low-viscosity state at temperature T_{ambient}. As shown in the hypothesized state diagram in Fig. 14, T_{ambient} is above T_{g_x}. If the cell is now dried, such that it achieves a lower water content, W_{g_y}, a glass transition occurs. Now, because of the dependence of T_g on the mass fraction of water, T_{g_y} is above T_{ambient} (the latter is assumed to have remained constant). If cell$_y$ is now rehydrated, such that W_{g_y} returns to the intermediate value W_{g_x}, a glass transition occurs again as T_{g_y} falls below T_{ambient}, and cell$_y$ enters the fluid phase and returns to the state of cell$_x$. The question of interest here is whether, under this set of conditions where T_{ambient} remains constant, cell$_x$ and cell$_y$ undergo their respective transitions under the influence of WLF kinetics. It seems that the transformation from ΔT to ΔH_2O might be possible but only under quite restricted conditions that appear to have limited biological significance. One such condition includes the plasticization of a linear polymer with different organic solvents. In any event, the conditions are not met when water is the plasticizer, not even with small sugars or starch as the solute. Furthermore, it seems that it would be essentially impossible to verify this under experimental conditions (357). Of course, a number of different permutations are possible with the system described in Fig. 14. For example, another way for cell$_x$ to enter the glassy state would be for T_{ambient} to be lowered, at a constant value of W_{g_x}, to $T < T_{g_x}$. Note also that dried cell$_y$ in the glassy state could, in principle, enter the active fluid state at $T > T_{g_y}$ without an external input of water. Any interpretation of the events that could occur in situ becomes difficult, largely because the water content and temperature will fluctuate simultaneously. This simple example does, however, indicate why any consideration of desiccation must involve a consideration of thermal stress, be it heating or freezing. As such, it seems that if bacterial glasses do exist, and the case for one good candidate is presented in the latter part of the review, it is likely that some sets of environmental conditions will guarantee that the bacterial cytoplasm must march to the tune of WLF kinetics.

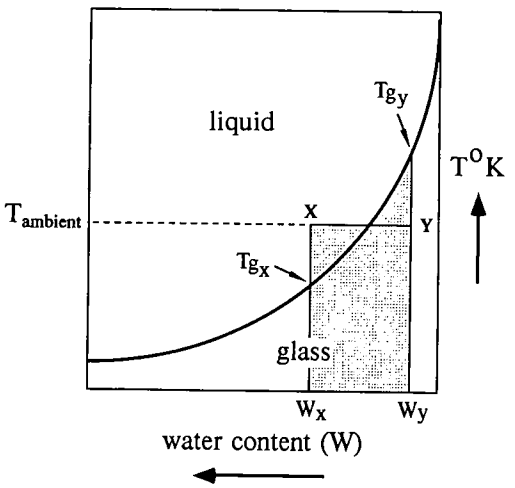


FIG. 14. Phase diagram showing the consequences of a water deficit (ΔW) and the subsequent transitions in a bacterial glass.

Protein Modification and Synthesis

Evolution may have conserved structural features (of proteins) that might determine the abilities of proteins to associate with one another, i.e., through channeling (235). It is important to understand the environment of proteins in a dried cell. For example, it has been suggested that sulfhydryls may play a part in the stabilization of desiccated cyanobacteria (386), so what is known about the redox potential inside a dried cell? In exposed aerobic habitats, endolithic bacteria may be subject to redox potentials of around −700 mV (Eh_{Pt} [348]), presumably as a consequence of nitrogenase-catalyzed hydrogen evolution. Disulfide bridges stabilize the native state of proteins rather than determine the spatial arrangement of the polypeptide backbone (187). Approximately 70% of the coenzyme A in dormant spores of *Bacillus megaterium* is in disulfide linkage to protein (352). The rapid cleavage of coenzyme A protein disulfides upon germination is thought to be a simple mechanism for activation of metabolism and loss of heat resistance. It seems doubtful whether the use of disulfides can be viewed as a general mechanism to stabilize proteins in dried bacterial cells, because bacteria, as a group, do not synthesize disulfides under physiological redox conditions (89, 288, 320, 414). Premature disulfide formation in the cytoplasm may block secretion and is thought to occur only in the periplasm of gram-negative bacteria and in the extracellular media of gram-positive bacteria (258). One protein involved in disulfide formation is DsbA, a strong oxidant with an intrinsic redox potential (E_0') of −0.089 V (428).

The Hsp70/DnaK and Hsp60/GroEL chaperones function in the folding of polypeptides. It appears that what is generally a poorly understood process may involve the sequestration of an aqueous environment, shielded from most if not all nonnative hydrophobic interactions (258). The effects of chaperones would seem to be particularly relevant when desiccated cells are rehydrated, and even a role for chaperonins as compatible solutes has been suggested (163).

With respect to protein stability, extracellular proteins or cell surface-associated proteins must be faced with more drastic perturbations upon drying than their cytoplasmic counterparts. The cell surface proteins of halobacteria represent the only convincing examples of prokaryotic glycoproteins—there are no examples of intracellular prokaryotic glycoproteins (219). The significance of protein-associated sugars is discussed further below.

Proteins exhibit marginal stability, equivalent to only a few weak interactions (350). Expressed in ΔG_{stab} per residue, the free energy is below the level of thermal energy. Molecular adaptation to extreme conditions may be accomplished by the accumulation of minute local structural changes. In addition, "extrinsic factors" (not encoded in the amino acid sequence) may be of importance (191). However, no general strategies of stabilization of proteins have been established for any of the stress parameters in nature (189). Are there environmental conditions in a given biotope required to generate or maintain the functional state of proteins? What determines the limits of growth and reproduction in extreme biotopes? Is there a hierarchy of stress parameters regarding selective pressure (189, 362)? In recent years there has been a flurry of reports of so-called water stress proteins in a wide range of taxa, particularly in plants (356). Identifications of the roles of these proteins—including the Lea proteins, osmotins, and dehydrins—and particularly the uncovering of evidence for their interaction in the water relations of cells have not been forthcoming (21, 32, 43, 69, 356). Interestingly, a eukaryotic histone-like protein has been reported to respond to cell shrinkage or swelling through changes in its phosphorylation state (340), and salt-dependent protein phosphorylation in the cyanobacterium *Synechococcus* sp. strain PCC 6803 has recently been described (157). However, the prospects for finding proteins that are induced specifically in response to cell drying in order to provide a direct measure of protection, both to themselves and to other cell components by effecting measurable biophysical interactions, would seem to be remote. The idea is very attractive, of course, from the perspective of genetic manipulation of desiccation tolerance in sensitive cells. Proteins, such as those induced in response to oxidative stress, may exert indirect effects on cell stability (242). The water stress proteins (Wsp) of the cyanobacterium *N. commune* (Fig. 15) were initially thought to confer a stabilizing effect on desiccated cells in view of (i) their abundance (they accumulate to around 70% or greater of the total soluble protein) and (ii) their great stability (immunoblotting has revealed that Wsp proteins are stable in desiccated cells stored for decades) (168, 345). Our recent data tend to discount a structural role for Wsp, but they do point to a very subtle and critical role in desiccation tolerance (see below).

Membrane Modification

Alteration of the lipid content of membranes is of major importance in response to environmental stresses (265, 324). There is a comprehensive literature on the role of membrane fluidity and lipid composition on survival of bacteria at extremes of temperature and salinity, but equivalent data for changes associated with the drying of cells are few (208, 336, 337). Maintenance of membrane integrity in anhydrobiotic organisms represents a central mechanism of desiccation tolerance (54, 77). In addition to its ability to preserve the integrity of proteins and enzyme activity, trehalose can stabilize membranes (76, 77, 80, 83, 224). Membranes dried without trehalose undergo vesicle fusion, change in morphology, and loss of Ca transport activity upon subsequent rehydration (81). It appears that the dry vesicles, as well as liposomes, may be stored for at least 6 months without loss of stability, providing that they are protected from oxygen (82). A similar enhancement of stability can be achieved with dried cells if they are stored under an inert gas phase (64, 291).

What is the basis for the protection of membranes with

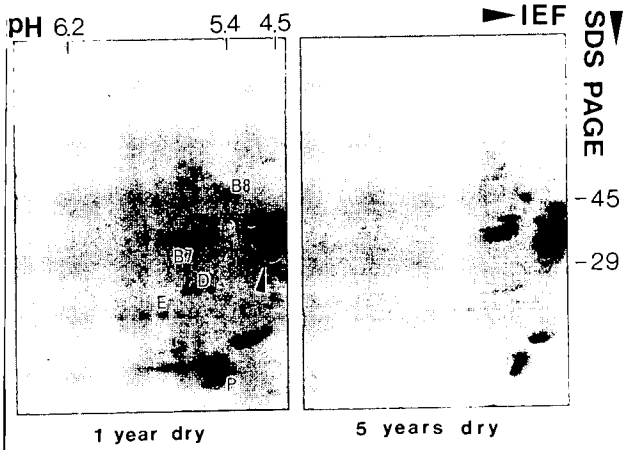


FIG. 15. Wsp proteins are the most abundant soluble proteins in desiccated *N. commune*. Shown are two-dimensional gels of protein extracts from cells desiccated for 1 and 5 years. P, phycobiliproteins. The arrowhead indicates the cluster of Wsp isoforms. Other letters refer to marker protein constellations (295). Markers to the right are molecular masses in kilodaltons. Note that Wsp is the most abundant soluble protein. Recent work has shown that Wsp is secreted (168); the true abundance of Wsp may be greater than that shown in the gels, since no attempt was made, in this experiment, to retain the secreted portion for gel analysis. IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Reproduced from reference 345 with permission of the publisher.

trehalose? The phospholipids in membranes are hydrated; in the case of phosphatidylcholine, some 10 to 12 water molecules are hydrogen bonded around each phosphate (around 20% of the water in the membrane). When that water is removed, the packing of the head groups increases, which leads to increased van der Waals interactions among the hydrocarbon chains (81). As a result, the phase transition temperature, T_m, increases enormously. For experimental systems the T_m may be elevated from around −10 to around 60°C. As a consequence, dry lipids would be in a gel phase at room temperature—a temperature that, under a normal state of hydration, would permit the liquid-crystal phase of the membrane to be achieved. As a result, when such dry lipids are placed in water, they would be expected to undergo a phase transition and become "leaky." Trehalose appears to depress the phase transition temperature of the dry lipids after water has been removed and maintains them in the liquid-crystal state (81, 224). Trehalose can depress the first-order phase transition of dry phospholipids to a temperature close to that of hydrated bilayers, leading to a transition that is referred to as the L_k-to-L_l transition (221). In dry mixtures of trehalose and 1,2-dipalmitoyl-*sn*-phosphatidylcholine (DPPC), nuclear magnetic resonance spectroscopy measurements indicate that the sugar is in close proximity to the hydrophilic region from the phosphate head group to the interfacial regions (221). The eight hydroxyls on each trehalose are all available for hydrogen bonding to the phosphate and carbonyl groups of the lipids. Furthermore, the sugar is thought to occupy some space between lipid molecules. The lowering of the transition temperature of DPPC in this model system may involve spacing of the acyl chains to permit their disordering (221).

An understanding of the role of sugars in the stabilizations of membranes in dried cells has been derived from studies with pollen. The T_m for dry pollen is very low, considerably lower than the T_m for dry phospholipids isolated from the same pollen (81). For *Typha* pollen, 97% of the total soluble sugars present is sucrose (25% of the dry weight). Hoekstra and colleagues have provided evidence that it is this endogenous sucrose in dry, intact pollen grains that is responsible for the depression of the T_m relative to that seen in isolated membranes (172–176). Sucrose was able to depress the T_m for purified and dried microsomal membranes from 58 to 31°C. The sucrose content at which minimal T_m was achieved was 3 g g of membranes^{-1}, and the calculated mass ratio of sucrose to phospholipid that achieved maximal reduction of the T_m was 6 or less.

A depression of T_m in pollen grains can also be achieved through an increase in the degree of unsaturation of membrane phospholipids (81, 175). In this case, there is an inverse correlation between the amount of sucrose present and the degree of unsaturation. Cells utilizing unsaturation, however, tend to have a shorter lifetime in the dry state, presumably because of increased susceptibility to oxidation of their lipid components.

An accumulation of a nonreducing disaccharide, trehalose or sucrose, and the synthesis of lipids with an elevated degree of unsaturation are two strategies that permit pollen phospholipids to exist in the liquid-crystal phase at room temperature when dry. As such, rehydration does not lead to a phase transition, and damage following rehydration, through leakage, is circumvented (224). Both of these strategies would seem to be within the capabilities of bacterial cells. In fact, almost 60% of the total phospholipids in the purified cytoplasmic membrane of *Nostoc* UTEX 584 was found to be 20:3ω3 fatty acid (265).

Trehalose is a substrate for trehalase, and the activity of the latter is likely to play an important role in the kinetics of stabilization of whole cells. Recently, it has been shown that the binding of yeast trehalase to membranes requires that the enzyme be in its active (phosphorylated) state (89). The significance and basis of this reversible binding are unknown; however, phosphorylation and dephosphorylation reactions are crucial signals that are known to induce diverse metabolic cascades in both prokaryotic and eukaryotic cells.

Trehalose—a Panacea for Water Stress?

Two features of the water relations of cells have taken on significance in recent years. The first is the realization that water molecules play a critical role in structure-function relationships of proteins, and the second is the documentation that certain sugars contribute to the stabilities of proteins, membranes, and whole cells when water is removed from them. Under conditions of moderate water deficit, compatible solutes appear to be important components of mechanisms that contribute to the maintenance of viability. Under the most extreme water deficit, however, only the disaccharides trehalose and sucrose seem to afford protection. Disaccharides appear to be the most effective stabilizers of dried enzymes in vitro, and the protective effects of trehalose, in particular, have attracted most attention (53, 224, 309). In assessing the effects of trehalose, it is necessary to consider the following: what are the properties of trehalose, what is the proposed mechanism of protection, and is trehalose an all-purpose protectant?

Many chemical properties of sugars, such as their reducing properties, depend on the free hydroxyl group of the hemiacetal (at C-1 of the aldoses or C-2 of the ketoses). Disaccharides in which both hemiacetal hydroxyls have reacted with one another possess entirely different properties. Both sugar components exist in their full acetal form. Such oligosaccharides are not reducing, show no mutarotation, and form no osazones. The simplest natural representative is trehalose [1-α-

glucosido-1-α-glucoside; *O*-α-glucosyl-(1,1)-α-D-glucoside]. Trehalose is the only nonreducing oligosaccharide of glucose. A second representative is sucrose (α-glucopyranosido-β-fructofuranoside). In sucrose, fructose is present in the furanose (less-stable) ring form. Fructose is closely related to D-glucose, and free fructose is most stable in its pyranose form (six-membered ring). Only in oligosaccharides, polysacacharides, and several phosphate esters is the furanose five-membered ring system realized—as in sucrose. Why anhydrobiotic plants should rely upon sucrose and not trehalose is not known.

Because trehalose and sucrose seem to be accumulated by many different cell types in response to water stress, attention initially focused on the reducing property of these sugars as the basis for their protective effect. The property of being nonreducing, however, does not, in itself, constitute a property for protecting proteins (81, 83). Glycerol, without any reducing groups, fails to show particularly enhanced protective effects, although it is used extensively as a cryoprotectant of bacterial cultures. Largely on the basis of these facts, it is generally believed that trehalose is physiologically more relevant than sucrose in terms of its efficiency and the stoichiometric amounts required for protection. Other properties of trehalose that may be of importance include the inability to participate in browning reactions that lead to insoluble, discolored protein products after long-term storage (76). Glycerol, for example, cannot prevent browning reactions. It has been speculated that the ability of sugars to hydrogen bond to water could reflect the spacing of OH groups on the hydrocarbon backbone of the sugar and that equatorial groups on pyranose and cyclitol rings could fit precisely into the water lattice of bulk water; the experimental data fail to support this proposition (76). What is important in the mechanisms of stabilization by trehalose if the reducing property is not?

The interaction of trehalose with membranes has received more critical attention that its interaction with proteins (see, e.g., reference 220). The mechanism for the latter may include exclusion phenomena, although trehalose also seems to interact directly with the dry protein probably by hydrogen bonding of -OH groups to polar residues in the protein, but it is not yet clear how this interaction leads to stabilization (53). Crowe and colleagues have provided evidence for a direct interaction between trehalose and lipids. The sugar is thought to replace water molecules around the polar head groups of the phospholipid in the dry state (129). This "water replacement hypothesis" has been discussed at length (64, 67, 74–84). Direct evidence for trehalose binding to phospholipid membranes has been obtained for dry phospholipid vesicles. Apparently, hydrated membranes do not undergo interactions with trehalose, sucrose, or glucose (18). Trehalose does not react with proteins at elevated temperatures, as reducing sugars do, and its solutions are more readily dehydrated. The glass transition of trehalose solutions is also atypical with respect to other sugars. Another important observation is that transition metals enhance the effects of protection—a feature that is thought to reflect the general inhibitory effect of zinc, for example, on enzymes in vitro (56). Curiously, if phosphofructokinase is partially dried it is inactivated; addition of proline, which stabilizes phosphofructokinase in solution, leads to stabilization during partial dehydration, but activity is lost at lower water contents. In contrast, trehalose maintains activity over the range of water deficit, including most extreme desiccation.

In desiccated *N. commune*, the amounts of trehalose and sucrose were comparatively small at around 0.1 mg of disaccharide g (dry weight) of colony^{-1}, but the extracellular glycan constitutes the bulk of the colony dry weight, and the sugar concentrations may, in fact, be sufficient for the protection of intracellular components (170). However, by using *E. coli* trehalase-specific antibodies, two cyanobacterial proteins were detected within minutes of rehydration, and their appearance matched the time at which trehalose levels became undetectable (169). These data are consistent with reports from Panek's group that trehalose in desiccated yeast cells is rapidly turned over upon cell rehydration.

It is clear that the synthesis of a disaccharide such as trehalose or sucrose is sufficient to afford protection from drying for liposomes, enzymes, membrane components, and dried cells. Not surprisingly, it has been suggested that the engineering of trehalose synthesis in cells will provide a means to manipulate desiccation tolerance (81). An important consideration for a protective effect on cell components is that the effect must be mediated through, and incorporated within, the stringently regulated physiological tension of the bacterial cell. For such cells, an accumulation of trehalose may protect intracellular components, but what of the outer leaflet of the cytoplasmic membrane and the different faces of the outer membrane of gram-negative bacteria? A significant recent finding of the group of Panek is the role of the trehalose carrier in the dehydration resistance of *Saccharomyces cerevisiae* (108). As expected, trehalose must be present on either side of the bilayer to stabilize dry membranes. A specific trehalose carrier translocates trehalose from the cytosol to the extracellular environment. Mutants that lacked the transporter accumulated trehalose but did not survive dehydration. Mixing those same mutants with trehalose increased their resistance.

A clear correlation between trehalose or sucrose accumulation and the maintenance of integrity in anhydrobiotic cells has been established (see above). In contrast, there are numerous reports in the literature on the accumulation and turnover of trehalose in bacterial cells, when the physiological status of the cells and their fate after drying are very hard to assess (374). Cells of *Rhizobium leguminosarum* bv. trifoli TA-1 cells can accumulate 0.13 g of trehalose g of protein^{-1}, yet the cells lose viability beyond 0.35 M NaCl (38). Surveys of the solutes accumulated in response to water stress in bacteria growing in situ indicate that there are clear preferences for which solute should be accumulated. For example, of 22 strains of cyanobacteria shown to accumulate low-molecular-mass carbohydrates in response to salt stress, only 4 accumulated trehalose (368). In rhizobia that do make trehalose, trehalose may accumulate until stationary phase is reached, and then it is rapidly metabolized. Trehalose accumulates in response to anaerobiosis (1% [vol/vol] oxygen) in rhizobia and decreases at ambient O_2 levels, when oxidative effects would appear to be at their most acute (177). Trehalose synthesis also appears to be correlated with a number of cell processes that do not involve water stress (177). In a filamentous cyanobacterium, *Phormidium autumnale*, trehalose accumulated up to 6.2 μg μg chlorophyll a^{-1} in response to matric water stress to −5 MPa. At lower activities (up to −25 MPa; a_w = 0.83), the concentration of trehalose dropped to approximately half of this level (167). A similar response was achieved for another cyanobacterium that accumulated sucrose in response to matric water stress.

Sheaths, Capsules, Slimes, and MDOs—a Mechanism beyond Trehalose?

Do bacterial cells that show resistance to air drying secrete trehalose? Perhaps they do. One thing that many certainly do is to secrete conspicuous amounts of EPS. Here, I first review what is known about these EPS layers and then attempt to

sketch the outlines of what could be a general mechanism of protection in anhydrobiotic bacterial cells.

Extracellular investments are a conspicuous feature of many bacterial cells. These investments may have the appearance of diffuse slimes or of rigid layers with a defined and complex ultrastructure (303). Many bacteria live within an EPS in environments that range from soil to the human lungs (325). These EPS layers are formed by the accumulation of various types of polymeric substances of high viscosity around bacterial cell walls; they tend to be hygroscopic, often contain more water than the bulk environment, and may decrease the rate of water loss from the cells. It is widely believed, and generally stated, that EPS provide bacterial cells with a means to survive drying, yet studies on specific responses of polysaccharide synthesis to drying are few and the mechanisms of sensing, induction, and regulation remain little studied (110). Protective roles for EPS have been confirmed in some studies (204), yet others failed to show any obvious correlation between the ability to produce EPS and a capacity for desiccation tolerance (269). Functions attributed to EPS, in addition to protection against desiccation, include anchorage to the substrate, protection against phagocytic predation, the masking of antibody recognition, and prevention of lysis by other bacteria and viruses (381). The EPS of *Beijerinckia* spp. may also protect the cells against oxygen damage (19). The presence of proteins, uronic acids, pyruvic acids, and *O*-methyl, *O*-acetyl, and sulfate groups in these layers emphasizes their complexity and also suggests that a number of enzymes would be required to degrade (and synthesize) the polysaccharide. The occurrence of carbonyl, carboxyl, hydroxyl, and sulfate groups provides a means to attach cations. In the latter respect, these sheaths may scavenge metals that may be used either in physiological processes, such as nitrogen fixation, or as toxins against predators.

EPS, such as capsules, tend to have a very low affinity for various dyes, and electron microscopy studies have established that in general they are less electron dense than the cell wall and the cytoplasm (331). Nontuberculosis mycobacterial species contain a variety of trehalose-containing lipopolysaccharides. Isolates of the tubercle bacillus are generally devoid of these but have simple acyltrehaloses (27, 134, 223). There are no indications that these structures provide any measure of membrane stabilization with respect to the drying tolerance of members of the family *Mycobacteriaceae*; however, it has been proposed that because of their structure and amphipathic nature, they may represent a "pseudo-outer membrane" (50). Eubacteria and archaebacteria have other surface layers that constitute an interface between the cell and its environment. Although the functions of these surface layers are not readily apparent, it has been suggested that they may serve a protective role by modulating environmental stress (363). The proteinaceous sheath of *Methanospirillum hungatei* GP1 is a resilient, proteinaceous, paracrystalline bilayer structure (364). The sheath is rigid, presumably because of covalent bonding in combination with weaker bonds (ionic, hydrophobic), and it contains disulfides. An important feature of these layers is that because they may be stress-bearing structures, new material must be inserted without loss of cell turgor. Surface structures of *Desulfurococcus mobilis*, another archaebacterium, have an unusual protein lattice at the surface, providing almost an exoskeleton, that has been speculated to afford protection from water stress (421). One component of the cell surface components of *Halobacterium* spp., bacteria that are subjected to salt stress in situ, provides the only convincing example of a prokaryotic glycoprotein (219). The cell surface glycoprotein has a mass of 120 kDa and is extremely acidic, with 20% Asp or Glu, 1 mol of cell surface glycoprotein per 40 to 50 mol of uronic acids, and 40 to 50 mol of sulfate per mol of glycoprotein in ester linkages.

The EPS of bacteria represent an additional cell compartment, which may contain the bulk of the water associated with a single cell (Fig. 2). Studies with a soil *Pseudomonas* strain—yellow pigmented and oxidase positive—showed that when dried at −1.5 MPa, the cells remained embedded within the EPS and were less distinct at the electron-microscopic level (325). At −1.5 MPa the EPS held 5 times its weight in water, and at −0.5 MPa it held 10 times its weight in water. The water content of the acidic heteropolysaccharide of *Gloeothece* sp. strain ATCC 27152 was 98.8% when cells were grown under N_2 and 99.5% when they were grown under $NaNO_3$ (381). These structures are engorged with water. Why does this water not simply flow out of the sheath, especially if many of the carbohydrate components appear to be water insoluble? It seems that these sheaths must represent well-mixed gels. Liquid water is restrained by the small amount of gel material because of the force of mixing. To separate water from a gel, work must be applied against this force (see reference 420 for a more extensive discussion). The sheaths of *N. commune* UTEX 584 have distinctive gel-sol transition zones, implying that the underlying structure of the sheath and its water-bearing properties can be modulated during the growth of the cells (23, 169). *N. commune* grows as spherical, resilient cartilaginous colonies under matric conditions at low humidity (76); however, the derived strain, *N. commune* DRH1, exudes polysaccharides that have much lower viscosity in liquid media (169). In view of the copious amounts of water trapped in these types of extracellular gels, their structural analysis has proved to be difficult (331). It is also widely stated, largely on the basis of what seems to be anectodal evidence, that certain structures observed in the electron microscope are artifacts and are the result of the "collapse" of cellular material during embedding for electron microscopy. As a consequence, while analytical data are available, structural analysis of extracellular structures and elucidation of structure-function relationships remain to be performed. Particular controversy has surrounded the interpretation of the capsular polysaccharide (M antigen; colanic acid) of *E. coli* (331). The data of Schmid (347), however, clearly document that in dehydrated and fixed cells the capsule is readily observable with the light microscope but with the same fixed material the capsule cannot be imaged by the electron microscope—clearly, electron-microscopic analyses cannot be used as the basis to describe these structures as dehydration artifacts.

The EPS of cyanobacteria provide some of the most complex examples of bacterial sheath structures, and they have been well documented in early and contemporary literature (136, 410). Many of these have an intricate ultrastructure (311), and, especially in communities growing in situ, they tend to be pigmented (see below). For the most part, these external layers of polysaccharide are reminiscent of the glycocalyxes, slimes, and capsules of other eubacteria (248, 381). For example, in gross morphology, the appearance of packets of cells of the cyanobacterium *Myxosarcina* spp. and those of the archaean *Methanosarcina* spp. are virtually indistinguishable. In one desiccation-tolerant cyanobacterium, *Crinalium epipsammum*, isolated from sand dunes of the Dutch coast, the cell wall was found to be hydrophilic and thickened and contained poly-β-(1,4)-glucan (cellulose, known to retain water efficiently [94]). The cells were also elliptical. The presence of this polymer and the shape of the cells are two properties that have been suggested to aid survival of this organism, although the significance of the latter property seems obscure.

Early studies on the biosynthesis of EPS in the cyanobacterium *Anabaena flos-aquae* indicated that they are derived from intracellular polysaccharides of the same composition (244). Synthesis appeared to be intracellular, and the products were thought to diffuse through the cell wall. At least for the sheath of *Gloeobacter violaceus* (349), no phosphodiester bonds appeared to be involved in the binding of the sheath to the peptidoglycan layer. Of the different substrates tested with *Anabaena flos-aquae*, only D-fructose was able to substitute efficiently for CO_2 in sheath synthesis (244). Similarly, fructose was the most effective carbohydrate tested that induced floc formation in the eubacterium *Azospirillum brasilense Sp7* (ATCC 29145) (338). It was concluded that overproduction of exocellular polymers induced the flocculent growths, which consisted of nonmotile, refractive, highly desiccation-resistant forms entangled within a fibrillar matrix.

EPS may be produced under both hypoosmotic and hyperosmotic conditions. What could be the nature of the regulation? In members of the family *Rhizobiaceae*, periplasmic cyclic β-(1,2)-glucans are involved in osmoregulation in media with low ionic strength (39). The glucans are synthesized to obtain an increased osmolarity in the periplasm in order to minimize differences in osmotic pressure across the inner membrane (240). In media of high osmotic strength (0.5 M NaCl), the production of cyclic glucans is strongly repressed in *Agrobacterium tumefaciens* and *R. meliloti*, glycogen synthesis is inhibited, and trehalose is accumulated. In contrast, other cells, e.g., *R. leguminosarum*, excrete up to 1,600 mg of glucan liter^{-1} in the presence of 200 mM NaCl. It has been postulated that the outer membrane of this strain becomes more permeable when exposed to salt, and constant loss of glucans prevents end product inhibition (39). Cyclic oligosaccharides and membrane-derived oligosaccharides have similar compositions and are regulated in a similar fashion. Membrane-derived oligosaccharides of *E. coli* are periplasmic glucans variously substituted with *sn*-1-phosphoglycerol, phosphoethanolamine, and *O*-succinyl ester residues (365, 382). The synthesis of membrane-derived oligosaccharides and of analagous periplasmic glucans in other gram-negative bacteria is subject to strict osmotic regulation. Adaptation to growth at low osmolarity appears to involve the signaling functions of the periplasmic glucans, which are themselves regulated osmotically (335). They may represent a focal point in the hierarchy of an osmotic signaling system and must be considered in the context of cell drying. Also, in this context it is important to note that osmotic regulation occurs principally at the level of modulation of enzyme activity rather than at the level of gene expression. A general role for periplasmic oligosaccharides in osmotic adaptation of gram-negative bacteria as ecologically diverse as enteric bacteria and soil bacteria has been suggested (240, 287). Studies with *mdoA* mutants indicate that a certain minimal ionic strength in the periplasm is crucial for normal porin regulation that is mediated through EnvZ, the proposed sensor of external osmolarity (135). Additional evidence that pressure or stretch is a signal for behavioral response to osmotic upshift is the identification of mechanosensitive ion channels which are stretch activated (225).

It is now becoming clear that the synthesis of EPS in bacteria not only is complex but also requires the coordinated expression of sets of genes that respond to changes in the water potential of the cell and its environment. The regulation of EPS synthesis is even more complex than it first appears, because it may also occur together with the coordinated expression of other systems such as those under oxygen control (148). Several different EPS systems are under active investigation. The M antigen (colanic acid; capsule) is distributed widely in enteric bacteria. The capsule is made only under high osmolarity, at low temperature, and at low humidity; in fact, it production is favored under matric as opposed to osmotic conditions (200, 257). Although the function of the capsule is unknown, it has been suggested that it may protect cells from desiccation when they are outside of the host. Synthesis of this capsule involves assembly from nucleotide sugars of a repeating polysaccharide containing glucose, galactose, glucuronic acid, and fucose (147). The genes necessary for this synthesis are scattered around the *E. coli* map and include *galE* (UDP galactose 4-epimerase) at 17 min, *galU* (glucose-1-phosphate uridylyltransferase) at 27 min, *manA* (mannose phosphate isomerase) at 36 min, and the *cpsA–E* cluster mapping near *rfb* (rough phenotype; TDP-glucose oxidoreductase) at 44 min. Synthesis of the capsule is complex. Transcription of the *cps* gene cluster is regulated by three positive regulators, RcsA (373), RcsB, and RcsF, and two negative regulators, RcsC and Lon. RcsC and RcsB may be sensor and effector, respectively, of a two-component regulatory system. RcsB interacts with *ftsZ*, a cell division gene, implying that the *rcs* system may be part of a global regulon. In view of the importance of cell volume changes in the responses of cells to water stress, it would be of interest to see how *rcsB* expression is influenced during the slow drying of *E. coli* cells.

Genes encoding the *E. coli* K4 capsular polysaccharide, a fructose-substituted chondroitin, have been cloned and expressed (103). Expression of the K4 capsular polysaccharide is also complex and requires coordinated expression of protein products for transport through the periplasm and outer membrane, translocation across the cytoplasmic membrane, and polymerization (42). Some analyses of the genes have been carried out. The *exoYFQ* operon is involved in the transport of the succinoglycan cell surface polysaccharide in *R. meliloti* (139).

Alginate is an EPS in *P. aeruginosa*, and its presence permits encapsulation of the cells and protects them from phagocytosis in the cystic fibrosis environment (127). Activation of synthesis occurs in response to growth in the lungs of patients with cystic fibrosis but also under high osmolarity, suggesting that alginate excretion could be the relict of an adaptation to drying. The expression of the genes involved in alginate production and secretion have been well characterized. The *alg* operons are activated at two promoters upstream of *algD* and *algC*. Signaling, like that of capsular polysaccharide synthesis, is mediated by a two-component system. An ATP/GTP-dependent protein kinase, AlgR2, undergoes autophosphorylation and transfers a PO_4^{3-} group to a DNA-binding response regulator, AlgR1. The phosphorylated form of AlgR1 has a high affinity for binding at upstream sequences in the *algC* and *algD* promoters. Evidence that mobile genetic elements are involved in EPS synthesis of a marine strain, *P. atlantica*, has also been obtained (22). The element that inserts and excises from the *eps* locus has the properties of an IS element.

Bacteria respond to desiccation by channeling energy and nutrients into polysaccharide production. For example, *Pseudomonas* sp. contains more EPS than protein when desiccated (325). It seems necessary that EPS production be coordinated within the overall carbon metabolism of the bacterial cell. In this regard, the regulation of glucan biosynthesis is likely to be important, and relatively facile methods are now available to clone the structural and regulatory genes involved in glucan biosynthesis from any bacterium of interest (328).

So, what of the role, if any, that these EPS components play in the stabilization of air-dried cells? Recent studies by Goodrich and colleagues have provided possible clues. Three synthetic carbohydrate derivatives (glycolipids), triethoxycholes-

terol, maltosyltriethoxycholesterol, and galactosyltriethoxycholesterol, were shown to have subtle effects on the physical properties of membranes (142, 143). The carbohydrate portion of each derivative appeared to mimic the effects of water. First, there was an expansion of the lipid lattice normally associated with lipids when they pass from the pretransition to a liquid-crystal state. Second, the carbohydrate at the membrane surface altered the organization of the acyl chains and carbonyl groups. The lipid linker functioned to position the carbohydrate group between the lipid molecules of the membrane, in the region of the hydrophilic portion of the lipid head group. These derivatives were shown to offer protection following freezing and thawing, and the effects were maximized at a carbohydrate-to-lipid molar ratio of 0.4:1. The data were consistent with a water replacement hypothesis, yet the amount of derivative required to induce cryoprotection was on the order of 100 times smaller than that recorded for the amount of trehalose and sucrose required to reduce probe intermixing to comparable levels. Even more important was the observation that there did not appear to be any significant differences in samples containing derivatives with galactose or maltose as the terminal sugar. This latter result suggests that the cryprotective effect is a characteristic of saccharides in general and is not specific to mono- or disaccharides. What these studies seem to be hinting at is that carbohydrates in general and EPS in particular may function as very efficient protective agents if they have an appropriate orientation with respect to the target. Application of drying in the presence of compatible solutes is being used to successfully store erythrocytes (140, 141, 144). It seems reasonable to question whether similar compounds and similar effects may play a role in anhydrobiotic cells. Is it possible that the nature of the mechanism described for synthetic glycolipids is applicable to dried bacterial cells that elaborate carbohydrate sheath layers? A feature of the latter is an intimate association between the EPS and the outer membrane layer—an association that is accentuated when the extracellular layers dry and shrink. Bacteria elaborate a wide range of carbohydrate derivatives, including many glycolipids, and there are plenty of unusual examples to consider in regard to the mechanism described above. Gliding bacteria produce carotenoids that include sugar-linked acyl chains, and their lipopolysaccharides also contain the unusual sugar 3-*O*-methyl-D-xylose (321). One bacterial compound appears to be an excellent candidate. Cord factor or (α-trehalose 6,6′-dimycolate) is a cell wall glycolipid of *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Corynebacterium* species and is thought to play a role in the pathogenesis of some of these bacteria through prevention of fusion of bacterium-containing phagosomes with primary or secondary lysosomes (366). Cord factor was found to be more effective than free trehalose in preventing membrane fusion. This effect was thought to reflect immobilization of the trehalose component at the membrane surface through its hydrophobic anchor. In this way, the glycolipid could increase hydration force; alternatively, it could act as steric block to fusion. In view of the results of Goodrich et al., it seems possible that cord factor could, in principle, provide a measure of protection to dry membranes.

The most obvious components of the cell walls of many types of bacteria are their EPS layers. Figure 16 shows a section through a portion of a desiccated colony of *N. commune*. The chemical and physical features of the EPS layer, a complex glycan, have recently been described in detail (169). The prominent features shown in Fig. 16 include the spherical, intact cell of a filament that permeates the dense, copious extracellular glycan and an unstained, electron-translucent layer that immediately surrounds the cell and separates it from the glycan. This translucent layer is not some artifact of drying; in fact, an electron-translucent layer of comparable dimensions and location is a diagnostic feature of the akinetes (spores) and heterocysts of many cyanobacteria (see Fig. 2a and 3b in reference 25 and Fig. 10 in reference 377). The structure is also reminiscent of the translucent spore cortex layer of *Bacillus* spores (see Fig. 1 in reference 261). The singular distinction here, however, is that every vegetative cell in a filament of *N. commune*, in the desiccated state, has such a translucent layer. To place this fact in some perspective, it should be noted that under appropriate culture conditions all of the vegetative cells in filaments of some cyanobacteria may differentiate synchronously into akinetes (see Fig. 3 in reference 35). These akinetes of cyanobacteria are generally regarded as being freeze tolerant and desiccation tolerant, although critical studies to support these assumptions, as well as explanations of the mechanisms that provide the basis for these properties, are lacking (252, 377). Curiously, akinetes of one strain when frozen in liquid nitrogen in the presence of dimethyl sulfoxide—a known chaotropic agent—were reported to show no loss in viability upon storage (60). Are desiccated filaments of *N. commune* simply chains of akinetes? This would be a simple explanation to account for the desiccation tolerance of this cyanobacterium, but there are a number of reasons why it is both unsatisfactory and untenable, not the least of which is the obvious one that we conveniently remove a consideration of desiccation tolerance from a vegetative cell of *N. commune* to the equally misunderstood akinete. The peptidoglycan layers of *N. commune* cells lack the thickness that is characteristic of akinetes (252; see Fig. 10 in reference 377). More importantly, rehydration of desiccated *N. commune* cells leads to marked changes in the appearance of the extracellular glycan (Fig. 17) (169) and to the resumption of metabolic activities, but it does not lead to any outgrowth or germination of the cells. In contrast, a developmental cue leads each akinete, upon revival, to divide into a short trichome that grows from, and breaks out of, the mother akinete (see Fig. 5 to 9 in reference 35). Perhaps extant akinetes, elaborated by several different genera of cyanobacteria, and the vegetative cells *N. commune* share certain features that provide them with an enhanced capacity to tolerate desiccation. Are the translucent layers of the different cell types comparable? Is the outer layer of an akinete the remnant of a more extensive carbohydrate layer such as the bulk glycan retained by colonies of forms such as *N. commune*? The translucent layer of *N. commune* cells is appressed to, and in intimate contact with, the cell membrane and the outer sheath layer. What could be in this layer? In *Anabaena cylindrica*, the homogeneous translucent layer of heterocysts and akinetes contains largely polysaccharide (426). The lack of post-staining suggests that lipids are absent, although at least for heterocysts this homogeneous layer may also contain phosphoglycolipid intermediates (426). Analysis of desiccated material of *N. commune* prepared with specific stains for analysis by light microscopy suggests that sugar-containing compounds are present in this layer and that they are antigenically distinct from the bulk glycan (169). Critical-point drying leads to removal, or possibly rearrangement, of the contents of this translucent layer, suggesting that they may be volatile (169). As cells are rehydrated, however, there are marked changes in the structure of the translucent layer. Could the translucent layer be a glass, which interacts with the outer membrane in the way that the glycolipids described by Goodrich et al. (142, 143) interact with membranes? The extracellular sheath layer is composed of a complex, high-molecular-mass polysaccharide. Biopolymers and synthetic polymers

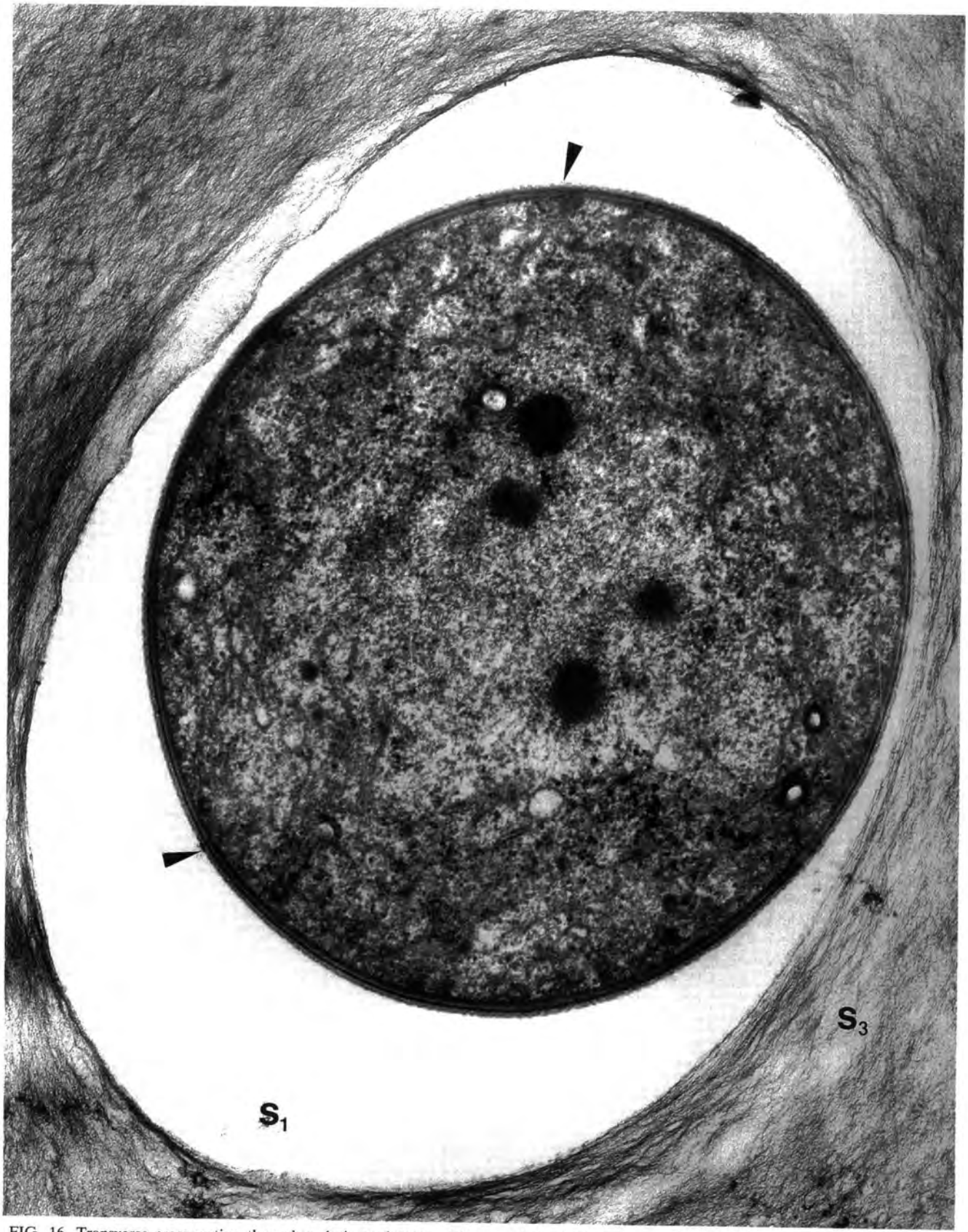


FIG. 16. Transverse cross-section through a desiccated colony (12 years dry) of *N. commune* CHEN (169). The section includes a single desiccated cell of a filament with an intact cell envelope (arrows), the electron-translucent layer S_1, and fibrous extracellular glycan (169). Magnification, ×37,500. Micrograph 10016 by Donna R. Hill.

FIG. 17. Influence of rehydration on the extracellular sheath of *N. commune* CHEN. Same material as Fig. 16, but rewetted for 15 min prior to fixation. Note the marked changes in structure and staining characteristics of the S_1 and S_3 layers and the intact cell membrane (arrowhead). Magnification ×60,000. Micrograph 10040 by Donna R. Hill.

form glasses, so that it seems not unreasonable to suggest that a considerable portion of a dried *Nostoc* colony may be in the glassy state. These dried colonies have the consistency of dried bacon rinds: they are friable and easily crushed to a powder. They can be heated and frozen without a loss of cell viability. Wetting leads to an instantaneous change in physical properties, including considerable swelling, and to the consistency of a stiff yet malleable gel. However, the colonies never form slimes or diffuse aqueous gels under matric conditions. It is expected that the structural analysis of the glycan will provide much information on the mechanisms for desiccation tolerance in this organism.

In summary, it seems likely that EPS synthesis represents a focal point of the ability of some bacteria to express desiccation tolerance. Table 4 summarizes those properties of EPS that may be important in this regard.

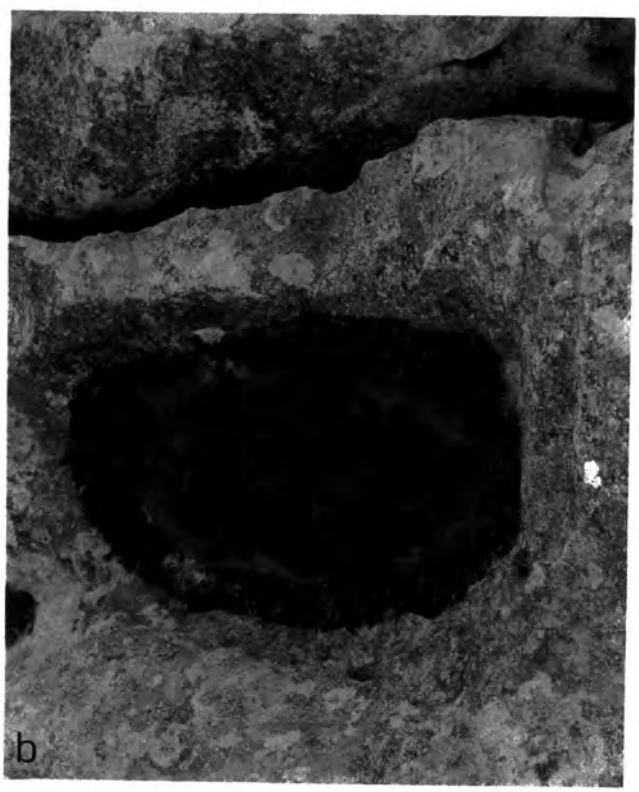
Photoprotective Pigments

General aspects of UV radiation and a discussion of the role of UV pigments in desiccation tolerance has been reviewed by Whitton (415, 416). Communities of terrestial cyanobacteria often appear black or brown (Fig. 18), and many other communities have highly pigmented sheaths upon close inspection. The colors of many of these pigments change readily with pH, a feature that is apparent when cells are removed from rocks or sediments by using mild acids. One pigment that is unique to, and common in, terrestial cyanobacteria is the sheath pigment scytonemin. Scytonemin is a yellow-brown lipid-soluble pigment that can undergo reduction to a red form, and it has a broad absorption spectrum centered around 400 nm depending on the conditions of pH and solvent (130). In *N. commune* communities, the pigment is easily extracted by mild abrasion of the colonies in the presence of 1% Nonidet P-40 and 6 M urea (169). Scytonemin appears to be present predominantly in nonplanktonic species, and its structure has recently been solved (312). The pigment is dimeric with a molecular mass of 554 Da, it is optically inactive, and it is composed of a skeleton of indolic and phenolic subunits (Fig. 19A). It has been suggested that the pigment is formed from

TABLE 4. Essential features of a protective extracellular biopolymer

Feature
(i) High water retention, probably with an ordered structure and intricate network of fibers
(ii) Complex repeating structure that requires several enzymes for dissolution and therefore presents a poor substrate for utilization by competitors
(iii) Ideally should be toxic to prevent grazing by eukaryotes, or should provide immunoglobulin G masking if the cell is a pathogen
(iv) Should have absorption properties that provide scavenging of cations, metals [see (iii)], etc.
(v) Attachment to cell surface should permit new growth without loss of turgor
(vi) Regulation—inducible/constitutive, CO_2 transport problem
(vii) Manipulative biophysical properties (gel-to-sol transition to permit motility of cells within sheath); viscosity/polymerization should be controlled by cell-encoded and secreted enzymes
(viii) The skew, twist, and shear properties upon drying should not be disadvantageous
(ix) Low matter transport
(x) Because of (vii), the cell should have a means to scavenge and take up short-chain-length sugars
(xi) Induction, synthesis, and secretion of the polymer should be stringently regulated, as should (x)
(xii) A CO_2-concentrating mechanism may be advantageous [see (vi)]
(xiii) Capacity to form a glass
(xiv) Intimate contact with the cell surface [leaflet of the outer membrane; see (v) and (viii)]


a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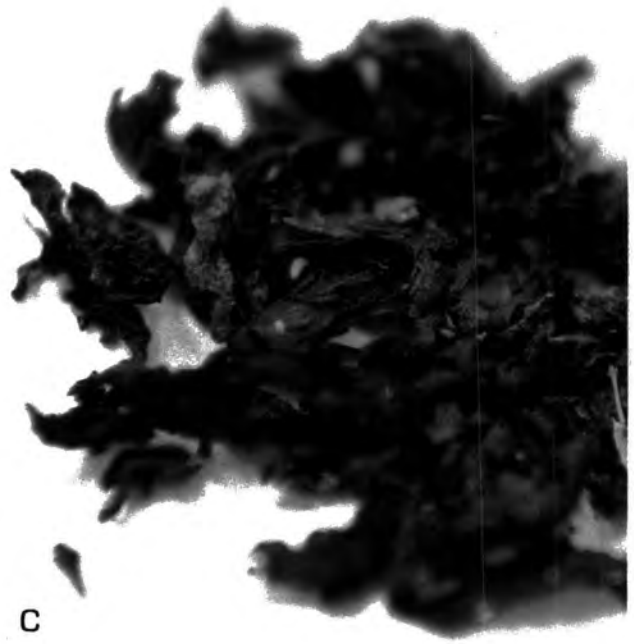
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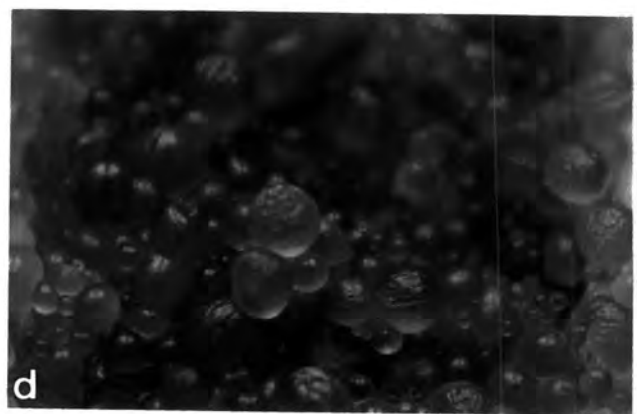
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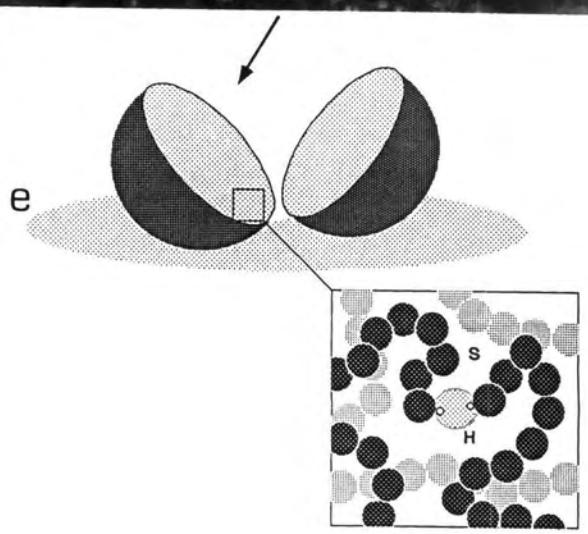
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S
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the condensation of tryptophan and phenylpropanoid-derived subunits (312).

Another class of UV-absorbing pigments present in many strains of cyanobacteria is the mycosporin-like amino acids (MAAs). MAA synthesis is not restricted to nonplanktonic species as seems to be the case for the sheath pigment scytonemin (131). Chemically, MAAs contain a substituted cyclohexenone linked to an amino acid (or its imino alcohol). These compounds have maximal absorbance from 310 to 360 nm and are water soluble. The UV-A/B-absorbing mycosporin of *N. commune* is colorless, water soluble, and secreted (168, 344). This pigment absorbs at 312 and 330 nm and contains a number of chromophores bound to a polysaccharide core (Fig. 19B). The structure of one of these chromophores, E_{335}, has recently been solved, and it has been shown to contain serine, threonine, and xylose (30). This MAA is unique because of its covalent attachment to carbohydrates. It is unknown whether the pigment is secured to the extracellular glycan of *N. commune* (Fig. 16 and 17). A potential role for Wsp polypeptides (Fig. 15) in the synthesis and/or modification of these pigments is described below (168). A survey of different laboratory-grown strains of cyanobacteria showed no significant absorption in the spectral region where these water-soluble pigments pigments absorb (344). In contrast, cultures of *N. commune* CHEN and *N. commune* DRH1 showed induction in response to UV irradiation.

The responses to UV radiation of liquid-grown cultures of cyanobacteria have been measured. The presence of MAAs provided only a modest increase in UV resistance when cells of *Gloeocapsa* were desiccated, and it was assumed that inoperative physiological and photorepair mechanisms were the cause (132). With other strains there appeared to be some overlap, as deduced from the two-dimensional protein indices, in the responses to UV irradiation and nalidixic acid. One protein produced under UV irradiation was a 33-kDa polypeptide with a pI of approximately 4.2 (253, 254); these properties are consistent with those of the Wsp polypeptides of *N. commune* (345) (Fig. 15). The conditions of desiccation are clearly important for consideration of the roles of pigments when studied in the laboratory situation; however, measurements suggest that MAAs probably contribute to protection of cells during active growth and desiccation in situ (132).

Other types of bacterial pigments have been implicated in protection of communities from radiation. However, the production of a gold-orange pigment in a methicillin-resistant strain of *Staphylococcus aureus* was not correlated with resistance of this strain to drying (114). Chromogenic bacteria from the Antarctic are frequently reported. All *Pseudomonas* cultures isolated from ice cores in Antarctic sediments produce an exogenous, water-soluble, dark-brown melanin-like pigment in tyrosine-containing media (2). This "pyomelanin" has features that suggest that it may be a MAA.

The thick and dense carbohydrate coats of *Deinococcus* cells have been implicated in the UV resistance of this bacterium, although the generalized comments as they pertain to both the desiccation resistance and radiation resistance of *Deinococcus* species are unclear (359).

Colony Structure

A conspicuous feature of many bacterial colonies that grow exposed to air is that they tend to be spherical. For a given volume, a sphere presents the minimal surface area to the vapor phase, thus retarding the net rate of evaporation. The water present in the colony—usually the interstitial component present in the extracellular wall layers—reduces the net diffusion of gases by 4 orders of magnitude relative to air (274). Bacterial colonies therefore undergo changes in water content, shape, and diffusion characteristics as they are dried. The shapes and forms of bacterial colonies may play an important role in determining the extent to which cells evade damage from drying, oxygen, and other perturbants. The intrinsic capacitance of soybean nodules containing *Bradyrhizobium japonicum* was measured as 0.29 MPa^{-1}, indicating that the nodules can release relatively large amounts of water from the symplast with only small changes in total nodule water potential. Estimates of the bulk modulus of elasticity of the nodules ranged from 0.91 to 2.60 MPa, indicating a high degree of elasticity (313), and the modulation of water volume of the nodule was thought to influence nodule permeability and thus the amount of oxygen entering the nodule. Oxygen exclusion is accomplished in *Frankia* vesicles by an envelope that contains two hopanoid lipids (26). The extracellular glycan of *N. commune* DRH1 imparts a spherical structure to the colonies, and the centers of these tend to be reducing (169, 170) (Fig. 17). Some strains of *N. commune* also synthesize an oxygen-binding hemoprotein (cyanoglobin) when they are starved of oxygen (297). Perhaps the need to scavenge oxygen is one consequence of abundant sheath biosynthesis.

The greater susceptibility to desiccation of the *R. leguminosarum* group of rhizobia compared with the slow-growing rhizobia is correlated with the different amounts of water retained by the bacteria at any relative vapor pressure, as opposed to the rates of water movement into or out of the bacteria (46). The higher retention of water by the former group appears to be related to a greater availability of adsorptive surface area and to higher surface energies rather than to differences in internal solute concentrations.

Genetic Mechanisms

E. coli has been subjected to all manner of insults, including extremes of heat and cold, osmotic shock, starvation, and high pressure, in order to understand the plasticity of its gene expression (255, 413). Comparable studies on the drying of *E. coli* are lacking. Even though this bacterium is not considered to be especially tolerant of drying, such studies could identify whether desiccation overlaps with other stresses and may help reveal some of the fundamental aspects of desiccation sensitivity.

Genes that are likely to be involved in the responses of cells to desiccation can be inferred from a study of the targets of desiccation damage. One gene that may be important is the putative sigma factor *rpoS* (*nur*, *katF*), which appears to be of major importance for regulating switches in responses to

FIG. 18. Life in the dry lane. (a) Karst scenery near Malham Tarn, Ingleborough, England. (b) Small depressions (ca. 0.3 m in diameter) in limestone support populations of *N. commune* whose colonies appear black and cartilaginous when rehydrated. (c) Desiccated colonies of *N. commune* collected in situ (approximately actual size); the dark-brown color of the colonies is due to the UV-absorbing pigment scytonemin (see the text and Fig. 19). (d) Spherical colonies of *N. commune* DRH1 (ca. 5 mm in diameter) grown under isopiestic water equilibration. Note the brown pigmentation due to scytonemin. Each colony contains numerous filaments of the cyanobacterium. (e) Schematic representation of the interior of a single sphere (shown in panel d). S, sheath; H, heterocyst.

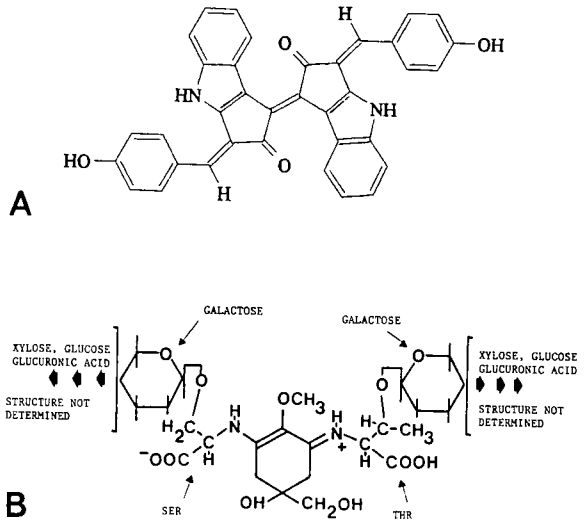

FIG. 19. Solved structures of cyanobacterial UV-absorbing pigments. (A) The reduced (red) form of lipid-soluble scytonemin (312). (B) The E_{335} chromophore of the water-soluble UV-A/B-absorbing pigment (UVP) from *N. commune* (30).

environmental fluxes such as stationary-phase growth, regulation of catalase activity, exonuclease III activity, acid phosphatase activities, and near-UV resistance (165). The *rpoS* (*katF*) gene product (σ^{38}) is a second principal σ factor of RNA polymerase in stationary-phase *E. coli* cells. This sigma factor is involved in the transcription of type III ("gearbox") promoters such as the one upstream of the *fic-pabA* operon (378). In contrast to the detailed studies of spore outgrowth, there are no data available for the promoter specificity of the RNA polymerases present inside drying or rehydrating bacterial cells. Of 20 proteins induced under osmotic shock, 5 were also starvation proteins (195). Interestingly, although starvation, growth rate modulation, and osmoregulation seem to involve similar proteins, neither σ^s nor trehalose is required for adaptive thermotolerance (164). A number of osmotically inducible genes (*osmA* to *osmK*) are also growth phase dependent (156, 164). Such dual control was found for *osmB*, a6.9-kDa polypeptide modified with a lipid moiety, that may be an outer membrane protein. The function of *osmB* was speculated to cross-link the outer membrane and peptidoglycan in a manner that restricts growth rate damage during water removal (199).

In view of the destructive effects of oxygen during desiccation, the genes involved in oxygen-scavenging mechanisms are likely to be important in the tolerance of bacterial cells to air drying. *E. coli* has three isozymic forms of SOD:MnSOD (*sodA*, 88 min), FeSOD (*sodB*, 38 min), and a hybrid enzyme that contains one subunit each of Fe and MnSOD (162). The regulation of *sodA* by Fur (ferric uptake regulation protein), Arc (aerobic respiratory control protein), and Fnr (fumarate nitrate reduction/regulation of anaerobic respiration) is independent of the superoxide response regulon *soxRS* (162). Products of *soxRS* and *soxQ* activate oxidative stress proteins such as SodA as part of the global response (71). SoxR is a regulatory protein that is part of a global response mechanism responding to the presence of $O_2^{\cdot -}$ (152, 387). In addition, it appears that SoxR can respond to aspects of the redox status of bacterial cells other than $O_2^{\cdot -}$ (230). The *soxRS* regulon is controlled by a two-stage system in which SoxR protein is a redox-sensing transcriptional activator of *soxS*, whose product subsequently activates the various *soxRS* regulon genes (262). A FeSOD has been found in heterocysts of *Anabaena cyclindrica* Lemm. (51), and a parallel increase in the levels of this enzyme and nitrogenase was noted in cells grown in the light. However, as mentioned elsewhere, despite the presence of these enzymes, heterocysts of laboratory-grown cells are prone to damage through air drying (278). The protective effect of *oxyR* expression is due to the induction of enzymes capable of scavenging active oxygen species e.g., catalase (*katG*), Mn-containing SOD (*sodA*), glutathione reductase, and alkyl hydroperoxide reductase (*ahp*) (372). The cascade of effects is induced when OxyR becomes oxidized (380). A survey of a range of cyanobacteria indicated that there are two distinct physiological groups with respect to oxygen-protective mechanisms—those that synthesize ascorbate peroxidase to scavenge H_2O_2 by using a photoreductant as electron donor, and those that scavenge H_2O_2 only with catalase (243).

UV-induced photoproducts can be recognized and repaired by several systems in *E. coli*, including photoreactivation (*phrB*), excision repair (*uvrABCD*), and postreplication repair (*recA*) (215). Apurinic and apyrimidinic sites left in DNA as by-products of DNA glycosylases are worked on by AP endonucleases that cleave at either the 5′ or 3′ side of the AP site.

Some sugars offer a degree of protection for enzymes, membranes, and cells against short-term drying. It is also clear that for effective desiccation tolerance by bacterial cells, constitutive synthesis must be necessary and synthesis of sugars can represent only one component of a multicomponent system directed at cell stabilization. Trehalose and sucrose appear to be the principal stabilizing solutes in anhydrobiotes; however, the role of other compounds known to be produced by bacteria in response to water deficit must be appraised (86). *E. coli*, which is not desiccation tolerant, can utilize trehalose as both a carbon source and an osmoprotectant (164). It is synthesized from UDP-glucose and glucose-6-phosphate via trehalose-6-phosphate. Accumulation is regulated on two levels: synthesis of trehalose-6-phosphate synthase is induced by osmotic stress (and apparently other conditions [see above]), and the synthase is activated by potassium glutamate (137, 376). Trehalose synthesis is dependent on *otsA* and *otsB*, encoding trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively, both of which are induced at high osmolarity. Stressed *E. coli* cells regulate the cytoplasmic level of trehalose by a futile cycle involving overproduction, excretion, and degradation to glucose, which is reutilized (376). Degradation is mediated by a periplasmic enzyme, trehalase, encoded by *treA* (33, 156). The latter permits cells to utilize trehalose at high osmolarity! The K_m for trehalose of this trehalase is high and may represent the evolution of a catabolic enzyme that uses a compatible solute as a carbon source. *rpoS* directs expression of *otsA*, *otsB*, *treA*, and *osmB* (164), which are not only stationary-phase induced but also osmotically induced. As discussed above, it has been suggested that the engineering of trehalose overproduction in cells would provide a measure of tolerance toward drying (77). Irrespective of whether the overproduction would provide tolerance, it is clear that it would cause a number of problems with respect to carbon source regulation, utilization, coordination with glycogen metabolism, etc.

ECOLOGICAL CONSIDERATIONS

Desiccation is likely to play a determinative role in the ecophysiology of bacterial communities that grow in aerophytic environments; on and inside of rocks; on and in soils and sediments; in the phyllosphere; in crusts, accretions, dusts, and

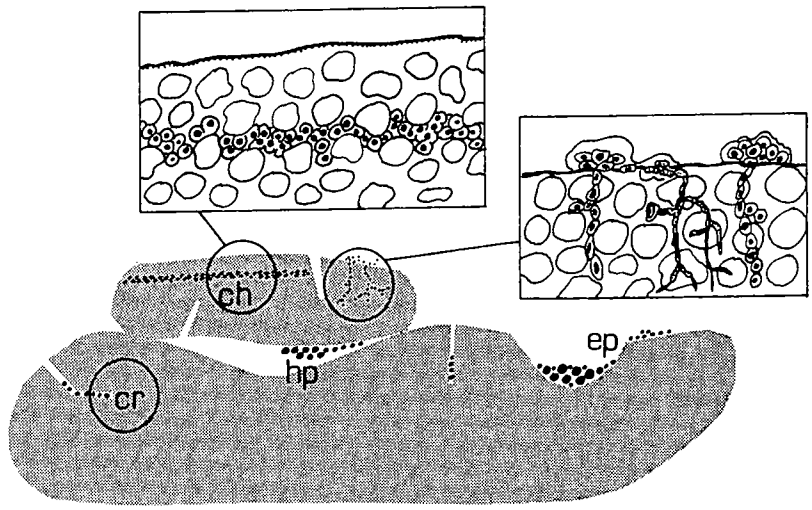

FIG. 20. Interactions of prokaryotic cells with rocks. ch, chasmoendolith; cr, cryptoendolith; ep, epilith. A rock-boring endolith is shown (right inset). The chasmoendolithic communities of cyanobacteria in Antarctic rocks (123–126, 197, 256) are discussed further in the text. A euendolith is shown in Fig. 22.

aerosols; and on human and animal skins (Fig. 20). An important factor to consider in desiccation-tolerant communities is their overall growth rate and longevity. Terrestial limestones in regions with extended dry spells require years for recolonization, despite a ready immediate source of inoculum. In contrast, marine rocks, as freshly exposed substrates, can be colonized within days (290). Here, wetting frequency is of importance, as is position in the tidal zone (218, 290).

Bacterium-Air Interface

In a consideration of the available data on the influences of interfaces on microbial activity, changes in water activity were thought to have an indirect effect (390). Water equilibrium across the bacterium/air interface or colony/air interface occurs when the water potential in bacterial cells equals that of the surrounding atmosphere (water vapor). At 20°C the value of $RT/\bar{V}_w$ is 135 MPa, which, in consideration of equation 5, indicates that extremely large and negative values of Ψ_{wv} ($\tilde{\Psi}_m$) are possible (Fig. 8). When the water potential of the population is in equilibrium with air, the temperatures of each may be quite different (290). The actual value of $\Pi_{\text{bacterium}}$ will depend on the its physiological status and the ambient conditions.

Hydrophobicity

Cell surface hydrophobicity (or its antonymn, hydrophilicity) is thought to play a major role in determining the distribution and activities of microbial populations (87, 102). An assessment of whether particular cells have a hydrophobic character relies on the results of a number of tests (97, 341). One test, for example, relies on whether the cells partition to the organic phase in an aqueous mixture that contains *n*-hexadecane (102). Details of these tests and some of their drawbacks are discussed in reference 102. Implicit in the consideration of cell surface hydrophobicity is that it occurs at an interface—one that separates a cell engorged with water (!) from its environment (Fig. 10). Ironically, a detailed study of the distribution of the hydrophobic character of cyanobacteria has focused solely on strains isolated from benthic (submerged) environments (115); all strains were shown to have a hydrophobic character. In contrast, cyanobacterial mats covering extensive areas of the sandy soil in arid southern Israel were described as being hydrophilic (20). Is this what one would expect, and is a predisposition to show cell surface hydrophobicity any indication of a tendency to be more or less tolerant of a water deficit? Consider the origin of the hydrophobic effect. It arises from the tendency of water molecules to maximize their hydrogen bonding around a nonpolar molecule (so decreasing enthalpy [105]). In doing so, these water molecules lose some of their rotational degrees of freedom—they become more ordered—and the decrease in entropy associated with this ordering is thermodynamically unfavorable. The hydrophobic interaction is spontaneous ($-\Delta G$) and endothermic ($+\Delta H$), and the reaction is favored entropically ($+T\Delta S$). As may be expected, the hydrophobic interaction is highly temperature dependent and leads to a structuring of water molecules at the hydrophobic interface. Overall, the surface of a bacterial cell will have regions of hydrophobic and hydrophilic character (182). Would a predominantly hydrophobic cell surface hinder or encourage water loss upon drying of the cell? Water loss may be accelerated from the surface of hydrophobic cells during their drying. Desiccated crusts of microbial populations from both marine and terrestial origins initially resist wetting, as is evidenced from the pronounced beading of water that takes place at their surfaces. Such beading could serve to prevent the cell from being committed to rehydration too early should the water availability be limited, or it could enhance the release of perennating structures (e.g., baeocytes and hormogonia) before their subsequent entrapment (and protection) by the swelling gel-like mass of the colony. The hormogonia of some cyanobacteria are hydrophobic; in fact, a "skin" of such hormogonia covers the surface of liquid cultures of some *Nostoc* spp. within 24 h of transfer of the cells to fresh media (291). However, there is no indication that hormogonia are any more tolerant of drying than are vegetative cells; the fact that they lack a sheath component suggests they may, in fact, be more sensitive to drying. Photoprotective pigments found in some bacterial colonies include those which are water soluble as well as those which are lipid soluble (Fig. 19). The former, but not the latter, are released upon rehydration of desiccated colonies (2, 169).

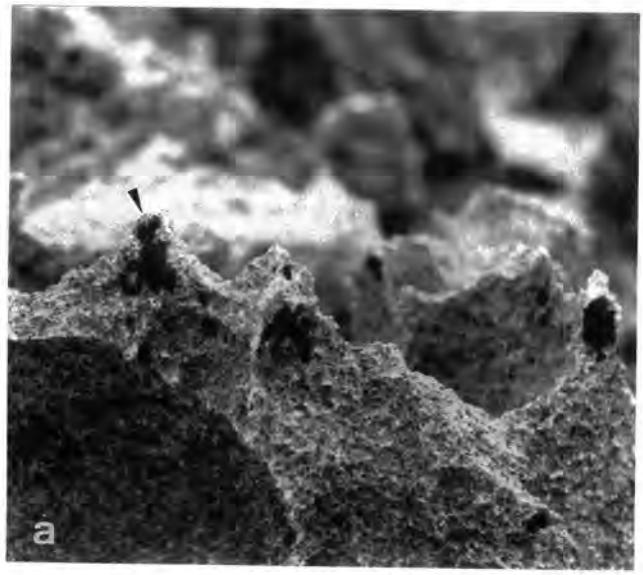

FIG. 21. Extreme xerophytic habitats colonized by the desiccation-tolerant cyanobacterium *Scytonema* sp. (a) Upper intertidal zone along south shore of Aldabra Atoll, Indian Ocean (290, 308). Actual size; arrow indicates tufts of filaments. (b) Individual filaments are calcified, and the laminated sheath (S) contains the dark-brown UV-absorbing pigment scytonemin. H, heterocyst; V, vegetative cell. Colonies colonizing pneumatophores of the mangrove *Avicennia* sp., upper intertidal along the coast of the Sinai desert, Gulf of Elat, Israel (292, 293) (inset); the filaments form collars around prop roots and are exposed at low tide. The collars may retain water for some time after exposure (99) but ultimately are completely desiccated for parts of the tidal cycle (292).

Model Systems—the Cyanobacteria

Cyanobacteria dominate the bacterial populations of extreme environments such as deserts (90, 271), thermal springs (400), hot brines (100), frigid lakes (268), soda lakes (63), and the nutrient-poor open ocean (118). Marine mats are dominated invariably by *Microcoleus* species (283, 306, 308). In terrestial localities, growths of *Tolypothrix*, *Scytonema* (Fig. 21), and *Nostoc* spp. form visually conspicuous mats and crusts in exposed habitats from the Tropics to the polar regions (290, 416, 417) (Fig. 18).

The deserts of hot and cold regions support a range of different bacterial communities. Where hot brines accumulate, and along the shores of coastal sabkha and lagoons, communities of *Halobacterium* spp., photosynthetic bacteria, and cyanobacteria accumulate under the surface of, and within, salt crusts (34, 99, 293). Here the surface crusts are often populated by intensely pigmented diatoms that offer protection to underlying prokaryotic populations (293). Where water is more scarce, the populations are restricted to rocks in which the bacteria accumulate below the surface. Bacteria may colonize the rock surface (epiliths); may actively bore into the rock substrate (euendoliths [218] [Fig. 20 and 22]); may seek out cracks, fissures (chasmoendoliths), and the microspaces of porous rocks (generally under a crust) (cryptoendoliths); or may remain at the stone-sediment interface (hypoliths) (Fig. 20). Endolithic organisms in hot deserts are subject to much more severe environmental stress than those in cold deserts because of the sudden changes between warm-humid and hot-dry conditions that can occur. This apparently is the reason for the exclusion of eukaryotic organisms in the endolithic microbial communities of hot desert rocks which are dominated by cyanobacteria and heterotrophic eubacteria (122, 256, 271).

The work of Imre Friedmann and his colleagues has provided comprehensive data on the physiological ecology of the bacterial communities that populate rocks in the Dry Valleys of the Ross Desert, Antarctica, which is considered, perhaps justifiably, the most extreme environment on Earth (122–126, 256, 429). Here the nanoclimate is the primary factor that influences growth of the bacteria. When rocks are saturated with water, the light flux is increased 40-fold, to almost 0.2% of the incident radiation. Rocks receive between 425 and 1,050 h of light per year depending on their orientation. Prolonged periods of evaporation can be measured in rocks following a fall of the very restricted amounts of snow, and this evaporation may continue for days or even weeks. Biological nitrogen fixation—a process that seems to be sensitive to short-term drying of cells grown under laboratory conditions—is rare here. Cyanobacteria dominate three of the five cryptoendolithic communities found in the sandstone rocks. A green zone of lichen-dominated community is formed by an association of *Hemichloris antarctica* (a green alga) and cyanobacteria. In the

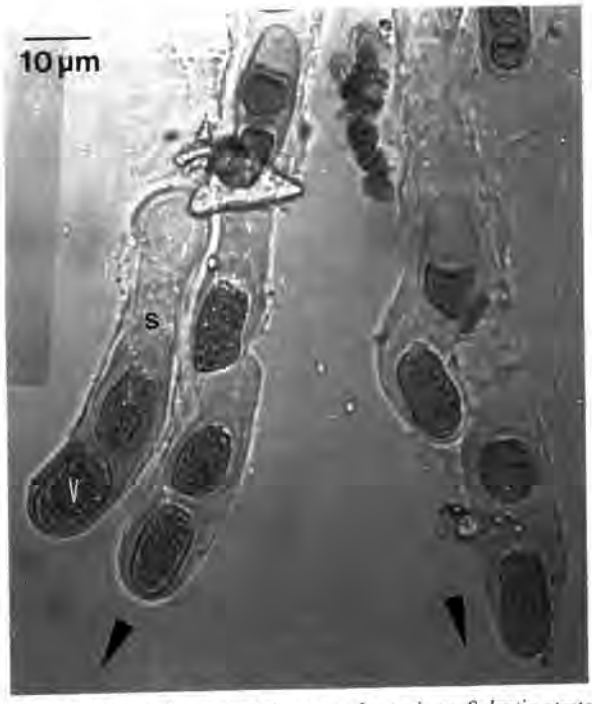

FIG. 22. A rock-boring marine cyanobacterium, *Solentia stratosa*. Pseudofilaments formed by single cells in a communal mucilage bore vertically into limestone rock substrata (arrows).

lower zone of the communities, *Micrococcus roseus*, *Deinococcus radiopugnans*, *Brevibacterium* sp., and *Arthrobacter* sp. are present. It is noteworthy that the hot-desert and cold-desert *Chroococcidiopsis* (cyanobacteria) strains appear to belong to a single species—the most xerotolerant cyanobacterium (see below)? Growth of a community dominated by a red-pigmented *Gloeocapsa* sp. is favored in continuously wetted boulders where liquid water is available; with decreasing moisture, this cyanobacterium is replaced by a lichen-dominated community. The different cyanobacterial communities colonize rocks according to strict preferences and requirements of pH and relative humidity. A *Gloeocapsa-Hormathonema* community colonizes rocks, between pH 7.3 and 8.2, that contain one-third the amount of Fe_2O_3 of rocks where lichens predominate (125). Moisture gradients are responsible for the distribution of microorganisms and occur largely in response to snow melts.

These cyanobacterium-dominated communities appear to be the oldest on Earth. Preliminary radiocarbon dating of cryptoendolithic microbial communities of the Ross Desert (McMurdo Dry Valleys) indicated a ^{14}C deficiency corresponding to approximately 1,000 years (31). Productivity measurements of cyanobacterium-dominated cryptoendolithic communities at Battleship Promontory, Ross Desert, were 4 µg of C m^{-2} $year^{-1}$, with a net turnover of lipid carbon of 19,000 years (197). These latter measurements require some adjustment (reduction), because temperatures below 5°C were not considered in the calculations. Even so, the adjusted numbers still are of the order of 10^4 years. The disintegration of rock-inhabiting communities in Antarctica is induced by salt weathering; grain-by-grain abrasion through frost, salt, or wind action; and polishing by eolian weathering (126). It would seem that successful colonization of rocks by these organisms, over some distance, can be achieved only if they have a tendency to withstand the most extreme cold and air-drying.

Blackened and desiccated colonies of *N. commune* are a characteristic feature of karst regions, where the brittle growths appear scattered over the exposed limestone (343, 346) (Fig. 18). Here, the colonies are subjected to repeated cycles of wetting and drying interspersed with short or extended periods of desiccation (Fig. 18). The mechanisms involved in the desiccation tolerance of this form appear to be varied. Compound 20:3ω3 constitutes 58% of the total fatty acids present in the purified cytoplasmic membrane of *Nostoc* strain UTEX 584 (265)—a feature that would be expected to contribute to membrane fluidity. Cells contain sucrose and trehalose (169). Field materials of *N. commune* elaborate a complex extracellular glycan (Fig. 16 to 18), which is secreted in copious amounts by liquid cultures of *N. commune* DRH1 and which lends a spherical appearance to colonies grown on solid media (Fig. 18) (168). When they are rehydrated, colonies secrete water-soluble UV-A/B-absorbing pigments that constitute up to 10% of the dry weight of the desiccated cell mass. The dark-brown appearance of the colonies is due to scytonemin (Fig. 18 and 19). Analyses of the two-dimensional protein index of cells of laboratory-grown cultures of *Nostoc* strain UTEX 584, following their exposure to different water stresses, failed to identify any novel classes of protein synthesized in response to drying (294, 295). In fact, rehydrated cells underwent a rapid turnover of protein within a short period of rehydration (295). Similar studies with colonies of *N. commune* collected in situ provided quite different results. A group of acidic proteins with molecular masses of 32 to 39 kDa constitute the bulk of the soluble protein (345). The proteins are very stable, and their synthesis was induced in laboratory-grown cultures in response to drying (Fig. 15). These "water stress proteins" (Wsp) were initially thought to have a structural role in cell stability in view of their abundance and their high content of hydroxylated amino acids (serine, threonine, and tyrosine). Recent studies suggest a related but more subtle role. The three Wsp polypeptides (of 32, 37, and 39 kDa) appear to be isoforms; they are secreted, accumulate in the extracellular glycan, and show homologies with carbohydrate-modifying enzymes (168). The polypeptides copurify with an associated 1,4-β-D-xylanxylanohydrolase (EC 3.2.1.8) activity that is inhibited by Wsp-specific antibodies—a property that suggests some role for Wsp in the modification of the extracellular glycan. This appeared puzzling at first, because the purified extracellular glycan of *N. commune* contains no, or very little, xylose (169). Xylose is found, however, in one of the chromophores of the secreted UV-A/B-absorbing pigment (Fig. 19B) (30). Wsp polypeptides and UV-absorbing pigments form complexes with each other in the absence of salt but appear to exist in a monomeric state in the presence of salt (168). These ionic interactions are expected to be attenuated in situ through drying and wetting of colonies and the resultant changes in salt concentration. The secretion of Wsp polypeptides, water-soluble UV-absorbing pigments, and extracellular glycan represents considerable metabolic investments by the cells—it remains to be determined if and how these processes are related and coordinated. One additional feature of the water relations of *N. commune* deserves mention. Upon rehydration, cells accumulate massive amounts of a singular polypeptide, cyanophycin, which quickly disappear (Fig. 23). Cyanophycin, first characterized by Simon (see reference 6 and references therein) is found only in cyanobacteria, it is nonribosomally synthesized, and it contains only arginine and aspartic acid, in a 1:1 molar ratio (multi-L-arginyl-poly-L-aspartate) (5). The amounts of cyanophycin that accumulate in rehydrated cells are dramatic. Inspection of Fig. 23 suggests that these inclusions exclude at least 50% of the volume of the

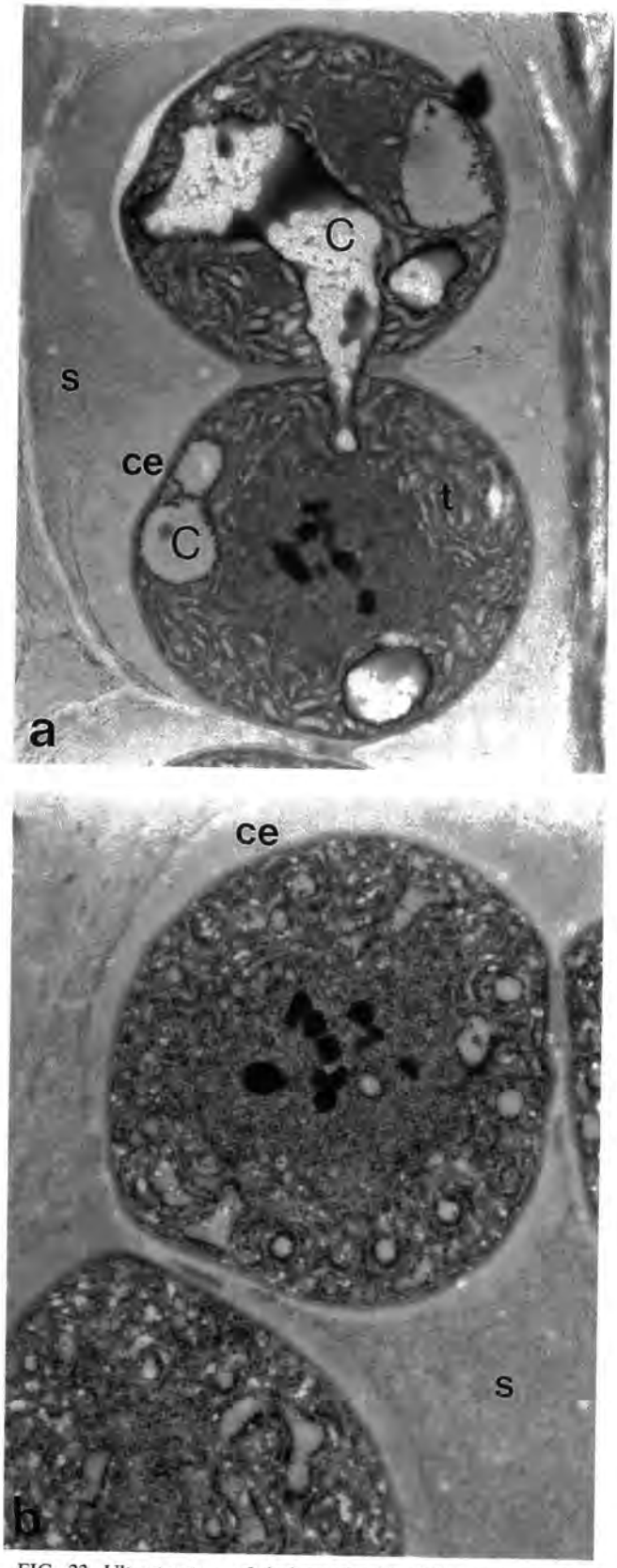

FIG. 23. Ultrastructure of desiccated cells of *N. commune* HUN following 24 and 72 h of rehydration. C, cyanophycin granules; s, extracellular glycan (note the fibrous ultrastructure); ce = capsule-like envelope; t, thylakoid membranes. Note that the cells are structurally intact despite long-term desiccation (years). Reproduced from reference 279 with permission of the publisher.

cell compartment during rehydration. The arginine and aspartic acid used for cyanophycin synthesis presumably derive from rapid protein turnover upon rewetting of the cells, because the onset of nitrogen fixation requires longer periods of rehydration (298, 326, 343). It is interesting that arginine · HCl is a noncompatible solute and, while it is preferentially excluded from globular proteins in the native state, it may interact with the denatured state through its guanidinium group and thus induce structure destabilization (384). Although lysine and to some extent valine are also noncompatible in this sense, it is possible that the scavenging of argine by multi-L-arginyl-poly-L-aspartate synthase contributes to some stabilization of proteins. Can or does cyanophycin function as a compatible protein solute?

Many bacteria have the capacity to withstand a certain degree of air drying under laboratory conditions, and this is also true of many cyanobacteria. The indications from our studies with strains of *N. commune* are that desiccation tolerance represents a complex array of interactions at many different levels in the cell, and there appears to be a battery of mechanisms to diminish the effects of air drying. It is difficult, therefore, to gauge how the tolerance of the garden variety ("weeds") of cyanobacteria has been modified by long-term culture (decades) in liquid media. One feature readily lost from some cyanobacterial strains following repeated subculture is the production of a conspicuous extracellular sheath (291)—often convenient, as this makes manipulation under laboratory conditions more facile. Not surprisingly, immobilization of such strains in alginate beads (43), or simply allowing them to dry in solid media (agar) in a petri dish, can improve their long-term stability.

Here is a story that returns to the question of bacterial glasses, alluded to above, and it includes an account of perhaps the most desiccation tolerant type of cyanobacterium. The normally attractive appearance of asphalt shingle roofs, on buildings from Canada to the southeastern region of the United States, frequently becomes an eyesore as a result of the development of dark stains commonly referred to as "black fungus" and "algae" (8–10). The growth is most pronounced on light-colored shingles and, not surprisingly, has generated considerable investments of time and money from roofing manufacturers in view of consumer dissatisfaction. The growth is caused by a form of the genus *Chroococcidiopsis* (291), a coccoid cyanobacterium that dominates the rock-dwelling communities of hot and cold deserts (122, 123). On clear summer days the dried, dark-pigmented microscopic spherical colonies of this organism are baked on the roof at temperatures that exceed 85°C (291). In winter, freezing and sublimation exact equivalent water stresses. In response to wetting, which could be a long-term downpour or, worse, a transitory shower, the gelatinous colonies become visible within seconds. Here, as for terrestial communities and intertidal communities that become desiccated, the cells are subject to an array of changes in temperature, water deficits, convection, etc. Observations that the growth was absent or markedly reduced in areas immediately below and around metal structures such as galvanized roof vents and copper or lead flashing led to the development of shingles in which heavy metals, particularly zinc, had been incorporated (260). While it became clear that the incorporation of calcium carbonate in the fillings of the shingle promoted the "algal" growth, the use of shingles free of these fillers and containing zinc granules has been less than successful. There are three especially ironical facets to this story. The first is the very existence of this unusual, virtually monospecific, anhydrobiotic "life on the roof"—a prime candidate in the search for bacterial glasses. The second is that

trial experiments (see exhibit 9 in reference 9) by 3M Corporation probably represent the largest single outdoor experiments ever conducted on the stress responses of bacteria! The third is that the incorporation of zinc in shingles should have been attempted as a control measure. Cyanobacteria are tolerant of heavy metals in general and of zinc in particular, a metal that appears to enhance the stability of dried proteins (17, 80, 155, 353)!

TECHNOLOGIES FOR DRIED CELLS AND ENZYMES

Immobilized Cells, Enzymes, and Biopolymers

Desiccated cells are immobilized cells and their enzymes are immobilized enzymes. It has been recognized that the carbohydrate investments of cells may contribute substantially to their resistance to air drying, and the entrapment of cells and enzymes in polysaccharide gels is an immobilization technique that has been applied extensively in different systems (203, 427). Compared with free-living cyanobacterial cells, matrix-immobilized cells exhibit superior temperature tolerance and storage longevity of photoinduced electron transport, and desiccation-sensitive proteins such as phycobiliproteins are more stable (273). Owing to the desiccation resistance and radiation resistance of *Deinococcus* (*Micrococcus*) *radiodurans*, it has been suggested that packets of dried cells could be used as a dosimeter for sterilizing doses of radiation (359). Studies of the skew and uniaxial deformation of immobilized photosynthetic bacterial cells in polymer matrices can provide important information on the positioning of natural chromophores or artificially introduced dyes in their membranes (119). The introduction of a drying event to such immobilized cells and their components—effectively mimicking the lifestyle of desiccation-tolerant cells—has been used to achieve further stabilization (247). These studies suggest that not all polymers may afford the same degree of protection. The study of the characteristics of desiccation-tolerant cells, such as their extracellular biopolymers and the genes involved in their mode of synthesis, can provide important information for use in methods to stabilize cells, inoculants (including seeds), and proteins such as liposome-encapsulated hemoglobin for extended periods (141, 194, 198, 205, 206, 247, 332, 333, 427). A knowledge of desiccation tolerance is providing the means to develop desiccated-synthetic-seed technologies, encapsulation technologies that rely heavily upon polymer mass transfer characteristics, etc. (146, 319). Desiccation-tolerant cells can be used effectively in biosensors (427), and they may have utility in long-term space travel. Novel techniques have been developed to characterize the nucleic acids of dried cells for use in forensics (52). The use of compatible solutes such as trehalose has already made it possible to more efficiently retain enzymatic activity and to prevent desiccation damage when cells and cell components are dried (53; see below).

The sol-gel process is a technique that can be used to prepare transparent oxide glasses by hydrolysis and polycondensation of alkoxides (109). These gels can be supplemented with macromolecules that become entrapped in the growing covalent gel network. Xerogels (glasses) can be formed from the aged gels by slow evaporation of the solvents (methanol and water). The fine-pore networks in dried gels (<100 Å [<10 nm]) do not scatter visible radiation, and they allow the diffusion of small molecules. In many respects, including their formation (solution, gelation, ageing, and drying), these xerogels are comparable to the extracellular gels of bacteria (also the cytoplasm of a desiccated cell?), and as such they may offer considerable potential for the analysis of aspects of the biochemistry of the desiccation of bacteria. The characterization of new biopolymers from desiccation-tolerant bacteria will provide data of use to the modeling of liquid transport processes (391).

It has been suggested that dried films of bacteriorhodopsin from *Halobacterium halobium* can be repeatedly used as a real-time holographic medium with characteristics that outperform other existing films of similar type. Bacteriorhodopsin from *Halobacterium halobium* 96N was successfully entrapped in a sol-gel glass and was found to retain its light-sensitive and spectroscopic properties (411). Such a product may have implications in molecular computing, holography, and the general area of molecular electronics.

Damp Enzymes

Enzymes catalyze their reactions in either direction depending on the equilibrium position, which can be attenuated by water availability. Enzyme catalysis in water-restricted environments has attracted considerable attention in recent years (250, 251, 396). Two experimental approaches have been used to study "damp" enzymes. The first makes use of the fact that certain surfactants aggregate in organic solvents and form thermodynamically stable micelles in the presence of limiting amounts of water (396). The "reverse" micelles have their polar head groups in contact with the encapsulated water and their hydrophobic tails in contact with the organic solvent, and they can solubilize enzymes and proteins. Because the micelles are optically transparent, it is possible to make direct measurements of enzyme kinetics. The second approach uses mixtures of enzyme catalysts and organic solvents (133, 322). By introducing organic solvents to enzyme-catalyzed reactions, hydrophobic substrates may be used, and it is possible to reverse hydrolytic reactions. Since enzymes are insoluble in most organic solvents, only simple immobilization techniques are needed (322). The cell surface enzymes of desiccation-tolerant cells which must function, or at least remain functional, under "damp" conditions may be particularly useful in industrial processes that rely on catalysis in organic solvents. Such enzymes may also be useful in enzyme assays that require

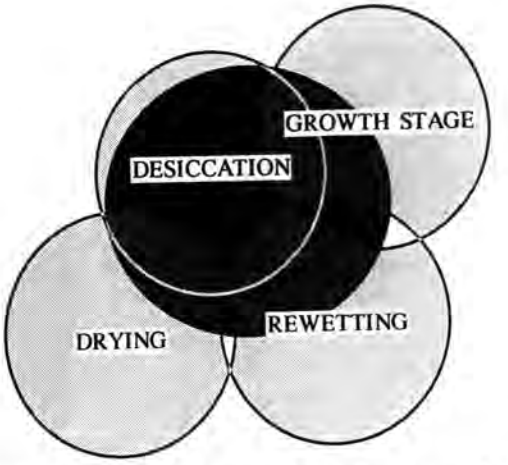

FIG. 24. The interactions between the different events that occur during the removal and addition of water to a prokaryotic cell. The darkened oval is a subjective indication of the relative extents to which these different events influence, or contribute to, the overall tolerance or sensitivity of the cell. Note that the interactions between certain events (unshaded) indicate that they play no direct part in desiccation tolerance.

immobilization to membranes. A secreted phosphomonoesterase of *Nostoc* strain UTEX 584 was found to be stable during desiccation and rapidly activated upon rehydration, and the recombinant protein has since been shown to be amenable to immobilization (237, 305, 418, 431). A test kit for methanol based upon lyophilized methanol dehydrogenase (stabilized with trehalose) from *Hyphomicrobium* X has been devised (14).

Water Replacement

Roser has suggested that the drying of proteins, cells, viruses, pharmaceuticals, etc., in the presence of trehalose can replace freeze-drying as the method of choice for their preservation (329). The ability to dispense with dry ice and bulky packaging is one example of a method that would clearly be of interest to companies that supply DNA modification enzymes, for example, to the scientific research community. Techniques that involve trehalose drying are now described in several patents filed with the International Patent Corporation Treaty (329). The University of California has a patent on liposome stabilization (81), and methods to dry erythrocytes have also been placed under patent law (140, 141, 144).

CONCLUDING COMMENTS

Figure 24 illustrates a subjective impression of the degree to which the different phases of water deficit, and their associated physiological consequences, contribute to desiccation tolerance, compare with Fig. 11. What is remarkable about desiccation tolerance is not what is known but what is not known. The real barrier to our understanding seems to have been, and continues to be, an inability to fathom the complexity of the state of dried (and wet!) cytoplasm—desiccation tolerance is a manifestation of the unique properties of water, and the basis for many of those properties remains obscure. Only very recently has there been a revival of the realization that water plays the pivotal role in biological integrity and cellular function. While the important role of trehalose in the stabilization of air-dried cells appears to be beyond question, there are many questions left unanswered with regard to life without water. More so than many other problems in contemporary cell biology, the study of desiccation tolerance requires, and will benefit from, the application of a judicious mix of biophysics, structural biochemistry, and molecular ecology to the study of whole cells and their purified components. Whether trehalose drying can indeed encompass all of the problems in anhydrobiotechnology remains to be seen. And what of the future for desiccation-tolerant bacteria? To satirize a hypothesis of water biophysics, now out of favor, this is the tip of the "iceberg."

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Ancient Prokaryotes - Water, Water, Everywhere?

MALCOLM POTTS

The emerging phylogeny of the Bacteria, Archaea and Eucarya has provoked, and continues to sustain, an intense fascination with the nature of ancient cytoplasm and its paleophysiology. The deepest branches within the Prokaryota - the present roots of the Archaea and Bacteria - are defined by the nucleotide sequences of the small-subunit rRNA's of thermophilic microbes and it is thought, and argued compellingly, that "life arose in a very warm environment." The seeking out of novel - hopefully old - prokaryotes, from the most inhospitable of our planet's environments, has taken on an impetus that can be rationalized in simple terms: there may exist representatives of old, unbroken lineages, that stretch from the earliest segment of the Archean.

Extreme - in the eye of the beholder?

During a military excursion through the limestone karst region of the Burren, County Clare, in Eire, Oliver Cromwell noted, with words to the effect, that there were "Not enough trees to hang a man, not enough soil to bury a man, and not enough water to drown a man." Today, the term extreme, or inhospitable, tends to be equated with ancient. Investigations of hot brines and salterns, caldera, sulfurous sea vents, boiling mud pots, and the like, have provided a source of organisms whose physiologies have come to shape our ideas as to the likely forms of primitive life. It is generally stated, and it is perhaps even widely assumed, that the new era of genome sequencing can answer all the nagging questions to do with the origins of cellular functions.

Such optimism, however, is at odds with the fact that even understanding the form and structure of cytoplasm in extant living cells remains one of the most intractable, and controversial, problems in cell biology.

The environments colonized by the Archaea constitute a broad range of ecological niches. These niches, however, have one very striking, if not transparent, feature in common - they contain water, and plenty of it. Where life originated remains unknown but it is thought that it occurred in an environment maintained far from equilibrium - such as would be found in the fumaroles and thermal vents that occupy the current attention of many microbial ecologists and physiologists. In the debate over the form of ancient cytoplasm, and the nature of the environment in which arose, a peculiar property of certain prokaryotic cells - the capacity to tolerate acute water-deficit (desiccation) - has escaped attention. The prospect that some old bacterial communitites struggled in dry (and hot) environments deserves consideration. The dehydration, and subsequent rehydration, of a bacterial cell generates a continuum of states in a system that is poised far from equilibrium. The consequences of the superimposition of thermal stress and water deficit would certainly fulfill the paleoenvironmental criteria that have been discussed and developed by J. William Schopf and colleagues.

The fact that no known Archaea form spores may be one explanation why investigators have not been persuaded to consider (hot) air-drying as a stress vector, or aerosols as potential refuges for these organisms. Evenso it should be noted that eubacterial spores contain significantly more water than is found in desiccated cells where the water content may be as low as 0.02 g H_20 per gram of cell solids. In the context of extreme it can be noted that the water deficit in the archaean *Halobacterium* spp. - caused by an intracellular salt accumulation of around 5M - pales in comparison to the magnitude of the water stress that is imposed upon airborne eubacteria as they navigate aircurrents on a summer's afternoon.

Desiccation and the Paleobiological Record

Those bacteria that can withstand the removal of virtually all of their cellular water, the anhydrobiotes, present a unique opportunity to understand a stress that may have constituted a major barrier to the distribution and activities of primitive cells. One group of prokaryotes in particular did make desiccation tolerance its business and that same group, the Cyanobacteria, constitutes a point of reference for the interpretation of micro fossils and stromatolitic structures that form part of the paleobiological record. Some cyanobacteria have been shown to retain viability following more than a century of storage in the air-dried state. E. Imre Friedmann has pioneered studies on the anhydrobiotic communities of desert regions. The unicells of one desiccation-tolerant cyanobacterium, *Chroococcidiopsis*, exist within the inner confines of the rocks of both hot and cold deserts, including the Dry Valley's of Antarctica - arguably the most extreme environment on Earth. Morphologically similar sheath-enclosed colonial unicells, as well as ensheathed trichomes, that may be of cyanobacterial origin, occur in the ~3465-million-year-old sedimentary rocks of the Towers Formation, northwestern Western Australia. These trace fossils have the remnants of clearly distinguishable extracellular polysaccharides. Production of such investments in extant forms may represent the primary strategy for tolerating desiccation. Irrespective of the species composition of ancient stromatolites it seems that at least some of these structures must have been built under conditions where the cells were periodically exposed and then subjected to air-drying. Paradoxically, a discussion of desiccation does appear in studies of bacterial-like trace fossils but only in the context of the degree to which air-drying may have introduced structural artifacts that require cautious interpretation.

Slow Evolutionary Change - The Eoanhydrobiote

Active studies of the Precambrian fossil record have proceeded only for some quarter of a century, a time that has witnessed the formal recognition of prokaryotes and eukaryotes and the

emergence of the Archaea. Speciation, generalization, and exceptionally long-term survival may have been pthe key elements involved in primitive prokaryotic evolution. As a group, the Cyanobacteria is considered to reflect the pressure of these elements and Schopf has explained how modern-day representatives may have undergone an inordinately slow (hypobradytelic) rate of evolutionary change. The paleoenvironmetal, stromatolitic, microfossil and carbon-isotopic data from units in the 3.5 to 3.3 Ga in age are consistent with the early archean existence of oxgen-producing cyanobacteria - with their presence possibly extending into the early archaean. The property of desiccation tolerance may have arisen very early in the rise of ancient cyanobacteria, and in other primitive prokaryotes - the *eoanhydrobiotes* ? - that may have led a "life in the dry lane." That fact provides a new and a different perspective with which to consider the constraints enjoyed by early life, the places to seek them out, and the means to study them.

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Nitrogen fixation by *Nostoc* colonies in terrestrial environments of Aldabra Atoll, Indian Ocean

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The rates of acetylene reduction were compared *in situ* for six different types of terrestrial and semi-aquatic *Nostoc* colonies on Aldabra as an indication of their rates of nitrogen fixation. The rates per unit chlorophyll *a* were all rather similar during standard assays in mid- to late morning, with a mean rate of C_2H_4 production for all experiments of 0·0388 nM C_2H_4 μg chl *a* $^{-1}$ min^{-1}. The rates for *Nostoc* colonies were at least ten times those for mature cushions of *Tolypothrix byssoidea*. Marked variation in rates of C_2H_4 production occurred throughout the day, with the peak rates occurring in early afternoon. Three of the types of *Nostoc* were re-wetted in the laboratory one year after they had been dried. All showed high rates of C_2H_4 production after a lag of up to one day. The lag was much shorter in a population of *N. commune* freshly collected in England. It is suggested that colonies of *Nostoc* are especially suitable for comparative studies of nitrogen fixation in different parts of the world, in view of the ease with which viable colonies can be stored for later extraction of chlorophyll or laboratory experiments.

Introduction

Species of the genus *Nostoc* are among the most widely distributed of any blue-green alga, and many, including obvious macroscopic forms such as *N. commune* Vaucher (Whitton & Sinclair, 1975) and *N. pruniforme* Ag. (Mollenhauer, 1970), are probably cosmopolitan. Many are known to fix atmospheric nitrogen (Stewart, 1973), and in view of the presence of heterocysts, presumably they can all do so. Baas Becking (1951) emphasized the importance of *N. commune* on coral atolls in the Pacific, as it plays a pioneer role on every atoll, and is a dominant in a certain phase of vegetational succession. Rates of nitrogen fixation for terrestrial growths on atolls have not been reported, but rates 'comparable with those found in managed agriculture' were reported for a marine *Nostoc* on Eniwetok (Enewetak) Atoll by Mague & Holm-Hansen (1975).

At Aldabra Atoll, conspicuous growths of *N. commune* occur in small depressions in the limestone platin (Whitton, 1971), and four other species forming macroscopic colonies, *N. carneum* Ag., *N. piscinale* Kütz., *N. pruniforme* and *N. sphaericum* Vaucher, occur in other types of habitat (Donaldson & Whitton, 1977b). The present study reports *in situ* and laboratory studies on several forms of *Nostoc* from Aldabra, together with a brief comparison with *Tolypothrix byssoidea* (Berk.) Kirchner, the most widespread terrestrial alga on the atoll.

A range of papers dealing with many aspects of Aldabra (9°24′S, 46°20′E) are included in Westoll & Stoddart (1971). More recent data are also available about the climate (Stoddart & Mole, 1977), chemistry of freshwater pools (Donaldson & Whitton, 1977a; Whitton & Potts, 1977) and on the terrestrial and freshwater algae (Donaldson & Whitton, 1977b). The present studies were all carried out in the wet season (see Table 2). The length of the dry season varies from year to year, but colonies of *Nostoc* probably sometimes remain for about 3 months with insufficient water to become fully re-wetted.

Materials and methods

Nitrogen fixation was estimated using the acetylene reduction assay technique. The rates of acetylene reduction were expressed in relation to chlorophyll *a*, and also in some cases, to unit area of community. An initial attempt to relate rates to the total N content of colonies was later discarded. There was large variation within one population in total N, when related to volume, dry weight or chlorophyll *a* content of colonies. (It seems probable that this was due to the presence of non-algal N, such as animal

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excreta.) Studies were also included on materials which were dried, returned to the laboratory in Durham, and then re-wetted.

Algal colonies

Representative samples were taken from situations where a particular algal community was well developed. The various types of *Nostoc* used for experiments are described below. They are listed in an order which reflects the extent to which their habitats hold water after rain. All except *N. sphaericum* were taken from Île Picard (West Island); *N. sphaericum* was taken from Grande Terre (South Island), in pool CC 11 (Donaldson & Whitton, 1975) near Bassin Flamant.

A. *N. commune* Vaucher var. *flagelliforme* (Berk. *et* Curtis) Born. *et* Flah.: early in the wet season this form grows directly over shaded sand, but later also occurs connecting leaves of sedges and grasses.

B. Material intermediate between **A** and **C**: grows over coarse sand later in the wet season.

C. *N. commune*, resembling original description of species (Fig. 1A).

D. Small, firm, verrucose colonies, always including much *Phormidium jenkelianum* G. Schmid. These colonies grow in shallow depressions receiving about the same amount of wetting as **C**, but in an area with especially high phosphate levels. The association between the two species appears to be close, since the relative proportions are quite

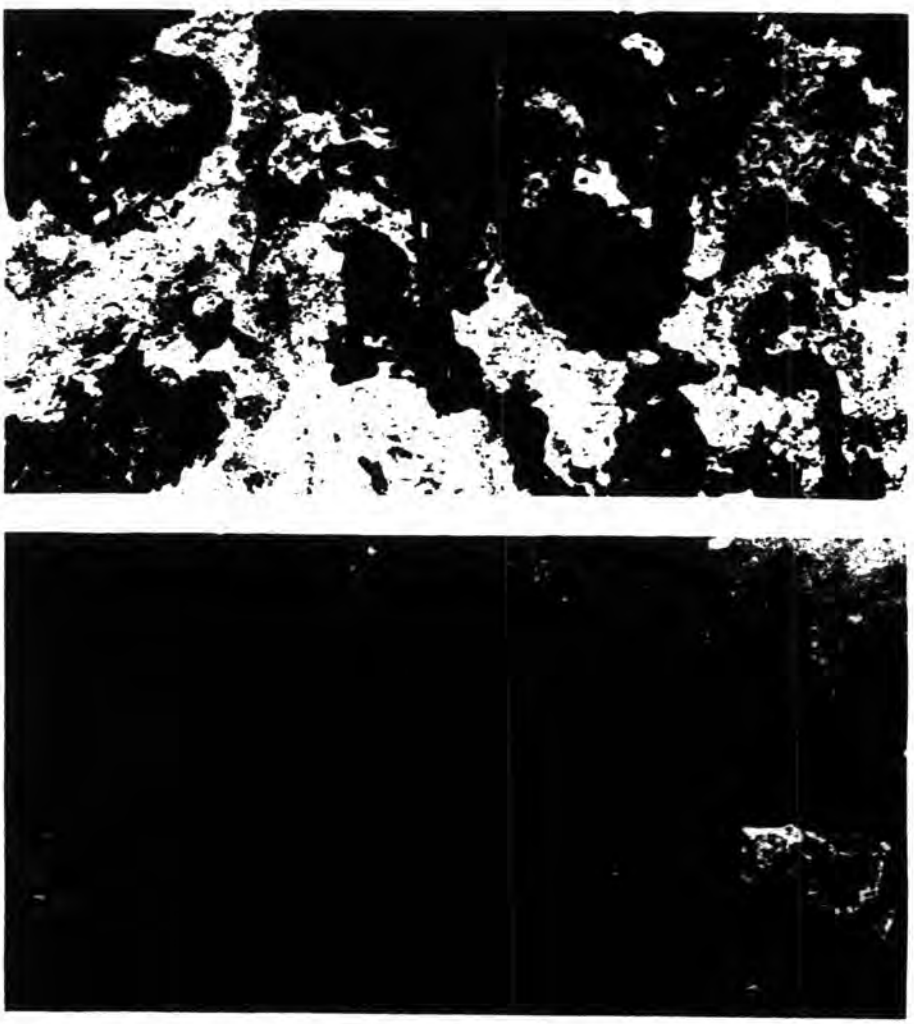

FIG. 1. (a) Type *Nostoc commune* in partially dried state overlying limestone platin with thin crust of *Tolypothrix byssoidea*; (b) *N. sphaericum* colonies in shallow pool.

similar in various colonies inspected. An estimate of the contribution of the *Nostoc* to the total biomass was made by comparing the relative volumes of the *Nostoc* and the *Phormidium*. This was done by measuring filament lengths and average cell width (and hence cross-sectional area) on homogenized material viewed in a haemocytometer cell. *Nostoc* was found to contribute about 70% of the total cell volume. (In order to compare acetylene reduction rates of the *Nostoc* in these colonies with that of the other *Nostoc* colonies, the assumptions are made that the chlorophyll content per unit cell volume is similar for both *Nostoc* and *Phormidium*, and that this *Phormidium* is not reducing acetylene.)

E. Round colonies intermediate between **C** and **F**, associated with pools that are more permanent than those where **C** occurs.

F. *N. sphaericum* Vaucher (Fig. 1B), grows in pools that sometimes hold water for many days.

Cores of *Tolypothrix byssoidea* (Berk.) Kirchner were taken from old cushions of this species overlying limestone pavé. Colonies of *Nostoc commune* from England were used as a comparison in some laboratory experiments. These were taken from the edge of a small, intermittent, calcareous flush at Tarn Moor (Sunbiggin), Cumbria, England. The vicinity of this flush has been described in detail by Holdgate (1955).

Field experiments

The following general assay procedure was used. Colonies were taken which had already been moistened naturally for at least some hours. An amount of alga was taken which gave about the same biomass per unit area inside the bottle as outside it. One ml of rain water was included in each 7 ml serum bottle. The bottles were then sealed and incubated *in situ* for 15 min prior to the addition of acetylene. One ml gas (East African Oxygen Ltd) was injected ($pC_2H_2 = 0{\cdot}17$ kN m^{-2}), 1 ml gas removed to equalize the pressure, and the bottles then incubated for a further 1 or 2 h. 'Dark' controls were obtained by wrapping bottles in foil. Four replicates were used for most assays, but where no standard deviation is shown in Table 2, only 1–2 bottles were used. All the standard experiments except one (Table 2) were carried out on sunny days, with cloud cover never exceeding 50% other than for transitory periods. Three or four temperature measurements were taken at intervals during the experiment in replicate serum bottles including alga; the mean values are given in Table 2. An indication of the ambient environment (air or water) was obtained with a thermometer in an open serum bottle filled with water. Dissolved oxygen measurements were made using a Lakeland Instruments Co. meter with a Mackereth electrode.

At the end of the experimental period, gas samples were removed with multiple-sample vacutainer needles and stored in non-sterile, non-silicone coated, 5 ml draw vacutainers (Potts & Whitton, 1977). The algal colonies were dried carefully under low light and sealed in polythene bags for subsequent extraction of chlorophyll *a*.

Laboratory experiments

Experiments were carried out after return to Durham with various dried materials similar to those used for field assays. Details of the materials are included with the results. Assays in the light were carried out with continuous illumination (warm white fluorescent source, 3000 lx). Experiments on the influence of temperature were carried out on an aluminium block with a temperature gradient from 56° to 20°C, and with a light intensity of 5000 lx. Laboratory acetylene reduction assays were carried out in 250 ml conical flasks with 25 ml medium, $pC_2H_2 = 0{\cdot}17$ kN m^{-2}, and usually three colonies; the flasks were sealed with Suba-seal closures (manufacturer: Freeman, Spaincross, Barnsley). Gas samples were collected by a method similar to that used in the field.

The measurement of water uptake by a dry colony was carried out by immersing the colony in medium and then taking it out again at various intervals for weighing. Excess water was removed by absorbent paper. At the end of the experiment the colony was dried at 105°C to obtain the dry weight. The percentage water content at a particular time is given by:

$$\frac{W_t - W_d}{W_s - W_d} \times 100$$

where W_t = wet weight at time t
W_d = dry weight
W_s = final wet weight

Culture medium

The culture medium was one modified from that of Allen & Arnon (1955), which has proved successful in growing many algae from Aldabra. This medium

contains (in mg l^{-1}): Na, 91; K, 56; Mg, 19·4; Ca, 18·0; Mn, 0·5; Fe (as EDTA chelate), 4; Co, 0·01; Cu, 0·02; Zn, 0·05; PO_4-P, 55·9; Cl, 153; SO_4-S, 25·9.

Analysis of gases

The samples stored in vacutainers were analysed for acetylene and ethylene, using a Varian aerograph series 1200 gas chromatograph.

Chlorophyll a

Chlorophyll *a* was extracted from the colonies used in field assays after return to Durham. The colonies were first incubated overnight in medium (see below) at 32 °C in the light (3000 lx), and then incubated with 95% methanol in 30 ml McCartney bottles for 15 min at 70 °C in the dark. Both pre-treatment and the use of methanol (as opposed to acetone) proved essential for complete extraction of the chlorophyll. Extracts were cleared using pressure filtration through glass fibre discs, and the absorbance then measured at 665 nm before and after acidification with HCl. Chlorophyll *a* was calculated using the formula given by Marker (1972), but with a constant derived from a different 'acid' factor; a mean value of 1·85 for the 'acid' factor has been found by us for a wide range of field and laboratory blue-green algae, and this has been used in all the present calculations. (For *Nostoc commune*, the actual value obtained was $1·86 \pm 0·054$, $n = 200$.) Critical comparison of data from various sources must await a thorough study of the methods used for estimating absolute amounts of chlorophyll *a*. For any given chlorophyll extract, the lower the 'acid' factor used in making calculations, the greater the amount indicated for the chlorophyll *a*, and hence the smaller the rate indicated for C_2H_4 produced per unit chlorophyll *a*.

Laboratory experiments on wetting, drying and re-wetting colonies of type *N. commune* indicated that this procedure had no detectable effect on the levels of chlorophyll *a* extracted (Table 1) or on the ratio of chlorophyll *a* to phaeophytin *a*. The pigment extracts of *Tolypothrix byssoidea* included the brown sheath pigment. As absorbance of this at 665 nm is low, it has been ignored in making estimates of chlorophyll *a*. (Detailed study of variation within this species would, however, probably necessitate the separation of brown pigment from chlorophyll *a*.)

TABLE 1. Effect of drying and re-wetting on amount of chlorophyll *a* extracted from *Nostoc commune*. The twenty colonies chosen all appeared similar visually (range of wet weights among all colonies, 0·344–0·526 g).

	Colonies wetted	Colonies wetted, dried and re-wetted
$\frac{\text{Chl } a}{\text{Wet wt}}$	$1·89 \times 10^{-4} \pm 0·48 \times 10^{-4}$	$2·04 \times 10^{-4} \pm 0·57 \times 10^{-4}$

Results

Three main types of field experiment on rates of acetylene reduction were carried out: time course in individual serum bottles in the light; incubation of various communities *in situ* for 1 or 2 h, usually commencing at 1000 h; changes in one particular population during the day. The results of the time course studies indicated that the periods of 1 or 2 h used for the standard assay were suitable. Marked changes in rate during this period of incubation are apparently due mostly to changes in light intensity and/or temperature, rather than anything that might have been caused by incubation in limited volume, such as CO_2 deficiency. It is however not possible to assess the effects of raised temperatures in those cases where the temperature inside the bottle was slightly higher than the ambient temperature (Table 2). The results of the incubations for 1 or 2 h periods are shown in Table 2, and those of the changes taking place during the day in Fig. 2. Estimates of rates of acetylene reduction per unit area are also included in Table 2 for those instances where it was considered that the biomass per unit area in the serum bottles corresponded closely to that in the adjacent natural community.

Although both species and environment varied, the results of the assays summarized in Table 2 show that all the rates for *Nostoc* in the light were quite similar. If the results for all the experiments starting between 0930 and 1230 are pooled, then the rates range only from 0·0239 to 0·0567 nM C_2H_4 μg chl a^{-1} ($\bar{x} = 0·0388$, s.d. = ± 0·0123). All the rates for *Nostoc* found during the standard morning assays are much higher than those found during the three assays on *Tolypothrix byssoidea* ($\bar{x} = 0·00180$ nM C_2H_4 μg chl *a* min^{-1}).

The two studies on changes in rates during the day (Figs 2a, b) showed that much higher rates occurred in the early afternoon than in the morning, with a maximum rate for *Nostoc commune* of 0·170 nM C_2H_4 μg chl a^{-1} min^{-1}. The rates of fixation in the dark were relatively uniform throughout the day,

TABLE 2. Rates of C_2H_4 production by *Nostoc* and *Tolypothrix byssoidea* colonies. For explanation of colony types, numbers of replicates and other experimental details, see Materials and methods. L = light, d = dark

Species	Morphology of colonies	Date	Period of Time incubation (h)		Environmental	Mean temperature (°C)				μg chl *a* cm^{-2}		nM C_2H_4 min^{-1} cm^{-2}		nM C_2H_4		μg chl *a*$^{-1}$ min^{-1}	
						Air or water	Ambient					L	D	L		D	
										$\bar{x}$	*s.d.*	$\bar{x}$	$\bar{x}$	$\bar{x}$	*s.d.*	$\bar{x}$	*s.d.*
Nostoc commune	C	14.12.72	1000	1	open platin: full light	A				24·7	8·21	0·7830		0·0317	0·0170		
Nostoc commune	E	28.3.73	1000	2	shallow pool (W10)	W	33·8	33·8	33·8					0·0239	0·0097	0·0012	
Nostoc commune	E	29.3.73	1000	1	shallow pool (W10)	W	31·7	31·7	31·7	20·4	3·61	1·133	0·3879	0·0555	0·0102	0·0190	
Nostoc commune	E	1.6.73	1000	2	removed from W10 to open platin	A	34·8	34·8	33·8	84·2	15·80	1·980	1·517	0·0235	0·0089	0·0180	0·0064
Nostoc commune	C: old expanded	1.6.73	1000	2	open platin: full light	A	36·0	37·0	36·0	106	6·07	5·618	0·5507	0·0530	0·0154	0·0052	0·0031
Nostoc commune	C: quite dry before wetting	14.5.75	0900	1	rewetted for 1 h	A								0·0084	0·00074	0·00296	0·00049
Nostoc commune	C	15.5.75	0900	1	same community as 14.5.75	A								0·016	0·00185	0·0006	0·00049
N. commune var. *flagelliforme*	A	12.12.72	1000	1	coconut grove, ground	A	28·0					0·3651		0·0567	0·0067		
flagelliforme	A	28.3.73	0930	2	coconut grove, ground	A	28·3							0·0435	0·0092	0·0001	
flagelliforme	A	28.3.73	1130	2	coconut over *Fimbristylis*	A	33·8							0·0239	0·0092		
flagelliforme	B	1.6.73	1020	2	coconut over ground	A	30·5	30·5	30·5	61·3	21·45	2·879	0·4900	0·0470	0·0115	0·0080	0·0023
N. commune/ *Phormidium*	D	2.6.73	1100	2	open platin: full light	A	35·8	35·4	33·6	281	67·52	9·993	1·011	0·0356	0·0047	0·0036	0·0016
N. sphaericum	F	29.4.73	1000	2	from pool, incubated by Aldabra lab.	W	29·6	30·0	30·0	50·4	6·38	1·638	0·0605	0·0325	0·0132	0·0012	0·0010
Tolypothrix byssoidea		28.3.73	1030	2	pavé	A	34·8							0·00130	0·00160	0·00010	
byssoidea		1.6.73	1000	2	open platin	A	36·0	36·0	36·0	383	55·13	1·409	0·0972	0·00368	0·00133	0·00237	0·00220
byssoidea		7.1.75	1000	1	pavé: 100% cloud	A				77·4	29·2	0·0379	0·0185	0·00049	0·00049	0·00024	0·00012

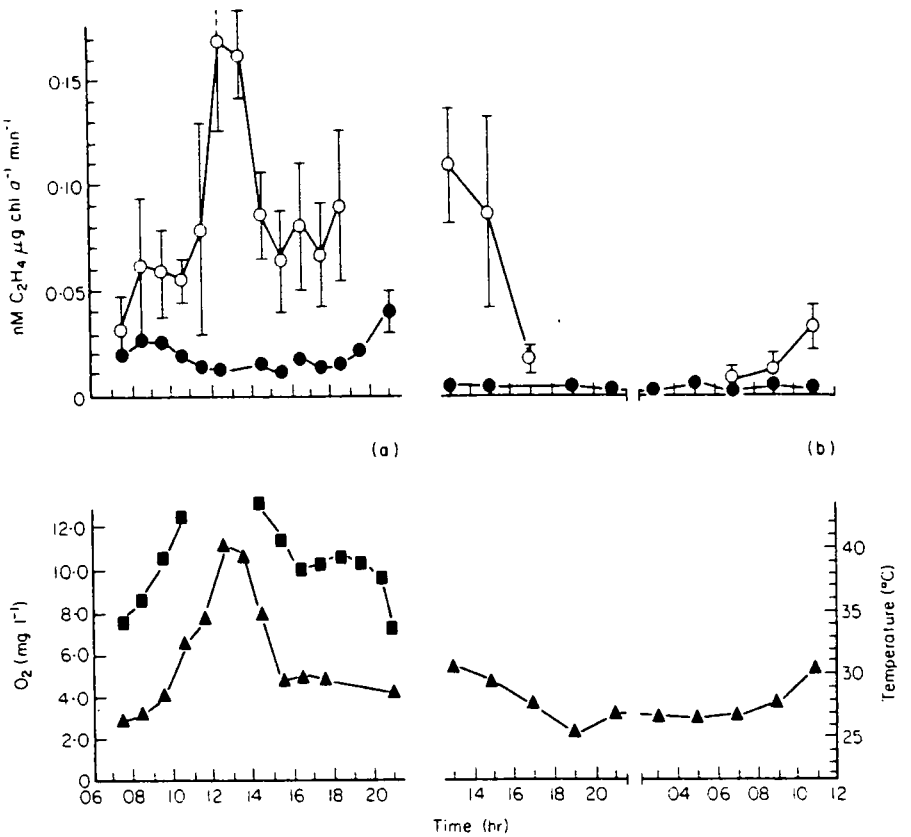

FIG. 2. Changes in rates of acetylene reduction during the day by (a) *Nostoc commune* and (b) *N. sphaericum*, together with changes in ambient temperature and (for a) dissolved oxygen. Rates are plotted for the mid-point of the period during which the assay was made. ○, light; ● dark; ■ dissolved oxygen; ▲ temperature. (Part of the dissolved oxygen curve omitted because the levels of supersaturation were above those for which instrument was calibrated; part of the night-time curve for B not shown because all rates were very low and similar to those at 2300 and 0300 h.)

but there was an increase in the rate of (dark) fixation by *N. commune* in the evening.

Laboratory experiments were carried out on the effects of re-wetting dried *Nostoc* colonies about one year after they had originally been collected on Aldabra. Recently collected *N. commune* from Tarn Moor, England (see Materials and methods) was included as a control. Three types of colonies from Aldabra were used, type *N. commune* (**C**), *Nostoc–Phormidium* (**D**) and *N. sphaericum* (**F**). When dry, colonies of all three were hard and brittle, but within a few minutes of medium being added, they became soft and pliable and had clearly increased in size. It was visually obvious that this response was more rapid in (Aldabra) *N. commune* than in *Nostoc–Phormidium* or *N. sphaericum*. This effect was shown clearly in time course studies (Fig. 3), in which *N. commune* reached 80% saturation in 5 min, whereas the other two types took longer than 1 h to reach this level. Uptake of water by Tarn Moor *N. commune* was also slower than that of Aldabra *N. commune* even when experiments on the former were carried out at 32°C.

All four populations of *Nostoc* showed more or less linear rates of C_2H_4 production during the second day of 2–3 day assays (Fig. 4). However, the behaviour with respect to C_2H_4 production did not correspond to that shown by moisture uptake. There was a much shorter lag in C_2H_4 production by the Tarn Moor *N. commune* (at 20°C) than for any of the Aldabra types (at 32°C), and in contrast with the water uptake results there was no indication that the lag in C_2H_4 production was shorter for Aldabra *N. commune* than for the other Aldabra types. The rates during the linear phases of C_2H_4 production by the four types are summarized in Table 3.

As colonies of *Nostoc* on Aldabra may be subject to marked changes in temperature during the day

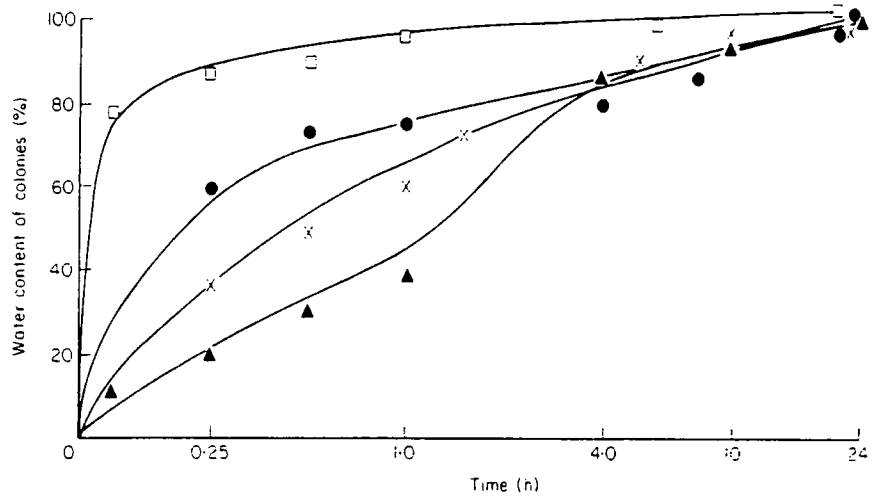

FIG. 3. Time course of water uptake by dry *Nostoc* colonies. □ *N. commune* (Aldabra); ▲ *Nostoc-Phormidium*; × *N. sphaericum*; ● *N. commune* (Tarn Moor).

TABLE 3. C_2H_4 production by *Nostoc* 1–2 days after re-wetting (see Fig. 4).

Type	Rate (nM C_2H_4 μg chl a^{-1} min^{-1})
Nostoc commune (Aldabra)	0·0531
Nostoc – Phormidium	0·0143
N. sphaericum	0·0744
N. commune (Tarn Moor)	0·0661

(Fig. 2a; Donaldson & Whitton, 1977a), a series of laboratory experiments was carried out to establish the influence of temperature on rates of C_2H_4 production by the various materials. As the variability in rates of C_2H_4 production by the colonies was quite high, only results which were demonstrated with a range of experiments are summarized here.

(i) When Aldabra *N. commune* was first incubated for prolonged periods at 32°C, and then incubated at a range of temperatures, the optimum rate of C_2H_4 production lay in the range 31–38°C, and fell to about 20% maximum rates at 22° and 44°C. The temperature of pre-incubation had a detectable effect on the rates of C_2H_4 production, and prior incubation at lower temperatures (20°C, 25°C), in particular, led to significant increases ($P < 0{\cdot}01$) in rates of C_2H_4 production at these temperatures. The maximum temperature at which detectable rates of C_2H_4 production were found was 46 C, whatever the previous incubation temperature.

(ii) The maximum temperature at which detectable

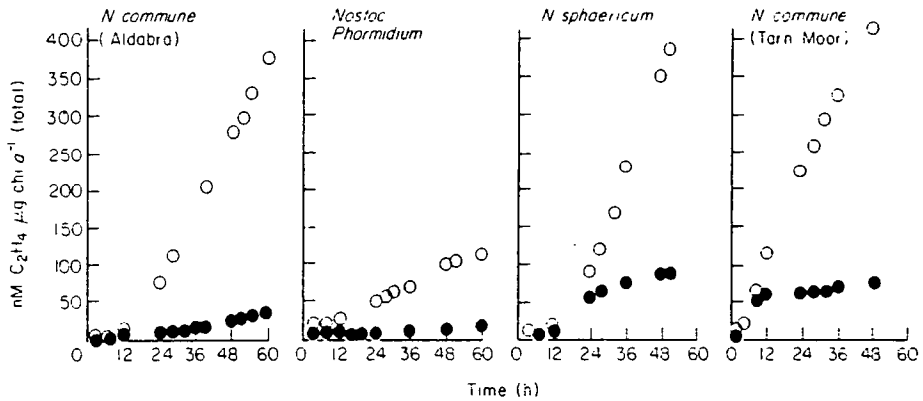

FIG. 4. Time course of C_2H_4 production when dry *Nostoc* colonies are rewetted. Aldabra colonies dried for one year then incubated at 32°C; Tarn Moor colonies dried for one week, then incubated at 20°C. ○ light; ● dark.

rates were found was also 46 C for both the *Nostoc–Phormidium* and the *N. sphaericum* colonies.

(iii) The maximum temperature at which detectable rates were found for Tarn Moor *N. commune* was 38 C. This alga showed quite similar rates of C_2H_4 production over a broad temperature range (22–34 C), and showed no obvious optimum under the conditions of culture used, which included prior incubation at temperatures from 20–32 C.

Discussion

All the *Nostoc* communities studied on Aldabra which had been fully wetted showed high rates of acetylene reduction. There was no obvious indication of differences in rates of reduction in the light between the various types of colony when incubated during the morning. In view of the range of forms and habitats which were sampled, the rates are all remarkably similar. The rates for *Nostoc* are all much higher than those for mature cushions of *Tolypothrix byssoidea*, although the difference is less marked when expressed per unit area rather than per unit chlorophyll. A comparison similar to that between *Nostoc* and mature *Tolypothrix byssoidea* can also be made for the intertidal zone of the lagoon on Aldabra where populations of several species such as *Rivularia* sp. have much higher rates than mature mats of *Scytonema* sp. when expressed per unit chlorophyll, but where the difference is less marked when expressed per unit area (Potts & Whitton, 1977).

There was considerable variation during the morning assays in the rates of reduction in the dark as compared with those in the light, but in general the rates of dark reduction, as a percentage of light reduction, were lower for *Nostoc* than found in the survey of intertidal communities. However, it is evident from Fig. 2 that rates of reduction in the light vary so markedly with time of day that it is difficult to make meaningful comparisons without taking measurements throughout the day. The rates of dark reduction found for *N. sphaericum* (Fig. 2b) were very low; it seems likely that this was due to the experiment being carried out several days after the colonies were first collected, when the cellular levels of photosynthate were low. The rise in rate found after dusk (Fig. 2a) is similar to the rise found by Horne (1975) in a stream *Nostoc* from sunny sites just prior to the onset of darkness. As time course experiments were not carried out in the dark within single serum bottles, it is uncertain whether there would be a similar rise in rates over several hours if colonies were transferred to the dark earlier in the day, or whether this effect is apparent only at the end of the daylight period when stored products are at their highest level.

The rates per unit chlorophyll recorded for some of the terrestrial *Nostoc* populations from Aldabra are among the highest recorded in the literature for *in situ* studies of blue-green algae. Comparisons are made difficult by the variety of methods used by different authors and sometimes also lack of information about the time of day, but a few observations on *Nostoc* are summarized in Table 4. In general the rates get less the further away from the tropics, but the data of Alexander (1975) for Devon Is. (Canadian Arctic) indicate a maximum rate almost as high as the maximum recorded for Aldabra. The mean rate found for the Aldabra terrestrial *Nostoc* populations during the morning assays is similar to the maximum recorded for any intertidal communities of the lagoon on Aldabra (Potts & Whitton, 1977), and also to the maximum found for a marine *Nostoc* on Eniwetok Atoll (Mague & Holm-Hansen, 1975). It is not possible to make a direct comparison of rates per unit chlorophyll with that for *Nostoc* in sub-tropical grassland reported by Jones (1977). Rates per unit area can however be compared (Table 2 against Table 4). The afternoon peak rate reported by Jones is about 40% that recorded during the morning for Aldabra *Nostoc* type B, the form probably most similar to that studied by Jones.

It is evident that, at least from terrestrial or semi-aquatic environments, colonies of *Nostoc* may be dried, and re-wetted for use in laboratory experiments long after they were originally collected. The observation that Aldabra *N. commune* initially took up water more rapidly than the other forms corresponds to general observations made on the atoll: the more transitory is the period of wetting for a particular form of *Nostoc*, the more rapidly does it take up water. The lag of up to one day in acetylene reduction shown by the Aldabra colonies assayed in Durham was almost certainly a consequence of long storage. Colonies of *N. commune*, in particular, are subject in the field to frequent cycles of wetting and drying, and the one field experiment showed that about half the probable maximum rate of acetylene reduction occurred after re-wetting for 1 h. In the laboratory assays, the rates after 1 day were of the same order as those found in the field during the morning assays. The results are however not

TABLE 4. Comparison of rates found in present study for *in situ* nitrogen fixation in the light with those of other authors. For purposes of conversion of data to standard format, it is assumed that the rate of C_2H_4 reduction is 3 × rate of N_2 fixation (Hardy, Burns & Holsten, 1973; Mague & Holm-Hansen, 1975), chl *a* = 1 % d.wt, N = 6 % d.wt and protein = 37·5 % d.wt. Data of Jones (1977) are included for comparison with data in Table 2.

Alga	Reference	Location		Experiment	Quoted rate	Rate, converted to standard format (μg N μg chl *a* $^{-1}$ min $^{-1}$)
Nostoc commune	this paper	Aldabra	9°S 46°E	terrestrial: afternoon peak	0·171 nM C_2H_4 μg chl *a* $^{-1}$ min $^{-1}$	0·016
Nostoc (various)	this paper	Aldabra	9°S 46°E	terrestrial: morning assays (mean)	0·0388 nM C_2H_4 μg chl *a* $^{-1}$ min $^{-1}$	0·0036
Nostoc	Mague & Holm-Hansen, 1975	Eniwetok	10°N 162°E	marine: range	2·83–5·00 nM C_2H_4 mg N $^{-1}$ min $^{-1}$	0·0016–0·0028
Nostoc	Jones, 1977	Pretoria	26°S 28°E	terrestrial: afternoon peak	19·2 mg C_2H_4 m $^{-2}$ h $^{-1}$ (= 1·14 nM C_2H_4 cm $^{-1}$ min $^{-1}$)	
Nostoc	Bunt *et al.*, 1970	off Florida	*c.*26°N 80°W	laboratory culture assayed in sea	11·5 nM C_2H_4 mg protein $^{-1}$ h $^{-1}$	0·00067
Nostoc	Horne, 1975	California	39°N 123°W	stream (site 3), clear-day: mid morning	*c.*1·6 nM C_2H_4 mg d.wt $^{-1}$ h $^{-1}$	0·00025
N. commune	Fogg & Stewart, 1968	Signy Is.	60°S 45°W		2·3 μg N mg d.wt $^{-1}$ d $^{-1}$	0·000159
N. commune	Horne, 1972	Signy Is.	60°S 45°W	phytoplankton	0·05 μg N 3·6 μg chl *a* $^{-1}$ day $^{-1}$	0·00000965
N. commune	Alexander, 1975	Devon Is.	approx. 75°N 90°W	no details	6200–53000 nM C_2H_4 g d.wt h^{-1}	0·00096–0·0082

strictly comparable due to the differing environmental conditions, there being a much lower light intensity and much higher nutrient levels in the laboratory assays.

The facts that at least some forms of *Nostoc* are cosmopolitan, that colonies of *Nostoc* are often free of any other algae, and that these colonies may be dried down for subsequent extraction of chlorophyll and laboratory experiments, all make this alga especially useful for comparative observations on rates of nitrogen fixation in different environments and in different parts of the world. It is suggested that when field studies of nitrogen fixation are carried out in different regions, local populations of *N. commune* should as far as possible always be included as a control, and that samples of this alga should be returned to the laboratory for subsequent comparisons under standard conditions. At the same time it is important to establish just how much variation in rates exists between apparently healthy populations at one site. It seems possible that *Nostoc* colonies are usually associated with rates of fixation that are high for a particular climatic region, and that communities with lower rates of fixation are usually dominated by other species of blue-green algae.

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Nitrogen Fixation (Acetylene Reduction) Associated with Communities of Heterocystous and Non-Heterocystous Blue-Green Algae in Mangrove Forests of Sinai

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Summary. High rates of nitrogen fixation (acetylene reduction) are associated with communities of heterocystous and non-heterocystous blue-green algae, which are widespread and abundant in the coastal mangrove forests of the Sinai Peninsula.

Heterocystous forms, particularly representatives of the Rivulariaceae, grow in aerobic environments, where nitrogenase activity may be limited by the availability of nutrients such as Fe and PO_4-P. Desiccated communities of *Scytonema* sp. reduce acetylene within ten minutes of wetting by tidal sea water. Communities dominated by the non-heterocystous *Hydrocoleus* sp., *Hyella balani*, *Lyngbya aestuarii*, *Phormidium* sp. and *Schizothrix* sp., occur in close contact with anaerobic sediments and reduce acetylene in the dark as well as in the light.

Nitrogen fixation in all these communities is light dependant and may be supplemented by an alternative source of reductant in the dark. The indications are that nitrogen fixation by these communities of blue-green algae, makes a significant contribution to the overall nitrogen input of the mangrove ecosystem.

Introduction

In the marine environment, nitrogen is considered to be the major nutrient limiting production (Mague et al., 1974; Ryther and Dunstan, 1971). Of the few processes through which nitrogen may become available, biological nitrogen fixation is thought to be the most important, although the data are still very inadequate for any generalizations about its quantitative significance (Potts and Whitton, 1977a). In coastal waters, nitrogen fixation is usually associated

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with communities of blue-green algae, which often form abundant and conspicuous growths in the intertidal and supralittoral zones. Stewart (1967) demonstrated in situ nitrogen fixation by epilithic populations of the heterocystous *Calothrix scopulorum*, growing along a Scottish shoreline. Seasonal variations in the fixation rates were attributed to temperature changes and desiccation of the communities during summer months. The mean fixation rate was 2.5 g N m^{-2} a^{-1}, representing about 41% of the mean total nitrogen present. In the nearshore lee of islands at Eniwetok Atoll, Pacific, Webb et al. (1975) measured appreciable rates of fixation by *Hormothamnion enteromorphoides* and *Rivularia* sp. The highest rate in the light was 390 nM N fixed m^{-2} s^{-1}. Also at Eniwetok, Mague et al. (1975) reported acetylene reduction by intertidal communities of *Nostoc*. They suggested that the rates found parallel the ambient light intensity, and could have supplied up to 11 µg N cm^{-2} day^{-1} to the coral reef ecosystem. The presence of epiphytic blue-green algae on marine angiosperms has prompted workers to examine these plants for nitrogenase activity (Stewart, 1971). Recent measurements by Capone and Taylor (1977) in shallow water environments of the Florida Keys, suggest that nitrogen fixation in the phyllosphere of the sea-grass *Thalassia testudinum*, could provide between 8 and 38% of the daily nitrogen requirement for leaf production. Colonies of the blue-green alga *Calothrix* sp. were apparent on the leaves whenever acetylene reduction was readily detected.

In contrast to the many reports of nitrogen fixation by heterocystous blue-green algae in marine environments, in situ studies with non-heterocystous communities are rare. Renaut et al. (1975) detected high but variable rates of acetylene reduction associated with a phycoerythrin-rich species of *Lyngbya*, growing along the rocky coastline of El Haroura, Morocco. Rates of up to 60 nM C_2H_4 produced g fresh wt^{-1} min^{-1} were measured under aerobic, as well as microaerobic conditions. In situ acetylene reduction was demonstrated in four non-heterocystous communities of blue-green algae, in the lagoon of Aldabra Atoll, Indian Ocean (Potts and Whitton, 1977a). The rates observed for communities of *Hyella balani* and *Microcoleus chthonoplastes* (rage 0.0051 to 0.034 nM C_2H_4 produced µg chl a^{-1} min^{-1}) were of the same order as those found in parallel experiments with heterocystous communities. However, at present, the only non-heterocystous marine species for which there are 'positive' field data i.e. using $^{15}N_2$, are the planktonic form *Trichodesmium erythraeum* (for refs see Carpenter, 1973), and *Microcoleus chthonoplastes* (Potts et al., 1978).

The present account investigates the nitrogenase activity of the dominant communities of blue-green algae growing in two mangrove areas of the Sinai Peninsula, and attempts to assess the potential significance of nitrogen fixation in this important, high productivity marine environment.

Geographical Setting

The mangroves of Sinai, the northernmost of the Indo-Pacific region, are formed of trees of a single species, *Avicennia marina* (Forst.) Vierh., which grow over

parts of a fossil-reef platform, extending between the Sinai desert, and the oligotrophic waters of the Gulf of Elat. A general description of these mangroves, and a brief account of the distribution of some of the communities of blue-green algae are given by Por and Dor (1975), and Dor (1975), respectively. A more detailed study of one area of the northern mangroves has recently appeared (Por et al., 1977).

The communities of blue-green algae found in these mangroves will be fully described in a separate account dealing with the distribution, abundance and taxonomy of blue-green algae in the intertidal zone of the Gulf of Elat; only simplified descriptions are included in this study.

Material and Methods

In situ

Two areas of mangrove were visited regularly between November 1977 and April 1978, the northernmost extremity at the Shura el Manqata (34°25′E, 28°13′N), and the southern promintory of the Sinai Peninsula at the Ras Muhammad (34° 15′E, 27° 43′N).

The communities selected for field assays were those estimated subjectively to be relatively abundant, widespread and as representative as possible of well-defined populations. The assay technique used has been described in detail (Potts and Whitton, 1977a). Cores were taken from uniform areas of populations, transferred to 7 ml glass serum bottles fitted with perforated aluminium caps each containing a 2 mm thick rubber liner, and preincubated for 30 min. After this time 1 ml acetylene was injected (Matheson and Co., USA) and the pressure equalized ($pC_2H_2 = 0.22$ atm.). Incubations were carried out in sea water, under ambient aerobic conditions. Assays lasted 60 min and were terminated by removing the gas phase to 5 ml-draw vacutainers (3206 U, formula 134, Becton and Dickinson, USA). Unless otherwise stated, all experiments took place between 0930 and 1100 h. The following controls were used: light, dark, light with glucose (final concentration 0.1 g l^{-1}), light with DCMU (final concentration 10^{-5} M, 3-3,4-dichlorophenyl-1,1-dimethylurea, an inhibitor of photosystem II). Gas samples were taken from interstitial sediments, as well as from assay bottles containing sections of pneumatophore free of epiphytic algae. These precautions were necessary as certain trees in swamps and under waterlogged conditions are known to generate ethylene (Clemens and Pearson, 1977). Also a recent study has shown high rates of ethylene production, from the bottom sediments of the Solar Lake, a coastal pool adjacent to the Gulf of Elat (Potts, in press).

Dissolved oxygen was measured with a Yellow Springs model 57 (Yellow Springs Instrument Co., USA). Readings of pH and Eh were taken using separate, portable Knick meters (Jürgens GmbH, FRG), fitted with glass pH electrodes (sinta membrane, 1 M KCl) and Pt-Ag/AgCl/3 M KCl Eh electrodes (see Potts and Whitton, 1977b).

Laboratory

Laboratory assays were carried out with moist samples of communities collected at the same time as in situ experiments. Sea water collected at the same times was used as the incubation medium. Assays were essentially the same as those carried out in situ, lasting for 60 min, under constant illumination of 500 μE m^{-2} s^{-1} and at 36° C. The following additions were made to the incubation medium in assays in the light, at the final concentrations indicated: DCMU (10^{-5} M), glucose (0.1 g l^{-1}), KNO_3 (0.5 g l^{-1}), KNO_2 (0.1 g l^{-1}), NH_4Cl (0.3 g l^{-1}), Fe (0.1 mg l^{-1} as $FeCl_3$-EDTA), $NaHCO_3$ (3×10^{-3} M), chloramphenicol (10^{-5} M, 10^{-3} M). An argon/carbon dioxide gas phase was used in one experiment (Ar/Co_2: 0.96/0.04 atm).

Gas samples were analyzed for acetylene and ethylene, using a Packard series 427 model gas chromatograph, fitted with a flame ionization detector (operated at 150° C) and a glass column packed with Poropak R (1.5 m). Calibration standards were prepared with high purity, spectroscopy-grade ethylene (Messr Griesheim GmbH, FRG).

Pigments were extracted from the algal cores in 95% methanol, at 70° C in the dark. Chlorophyll *a* was estimated using the equations of Potts and Whitton (1977a).

Macrosamples of each community were examined using a dissecting microscope, and mounted specimens studied under bright-field illumination and phase contrast (Zeiss TL microscope). Photographs were taken with a Zeiss attachment through Plan 25/0.45 and Neofluor 40.0/0.75 objectives, using Ilford FP4 black and white emulsion. Binomials were allocated using standard taxonomic texts (e.g. Geitler, 1932).

Filtered sea water collected from field sites, was analyzed for NO_3-N, NO_2-N and PO_4-P, using the methods detailed by Strickland and Parsons (1968).

Results

Non-Heterocystous Blue-Green Algae

Significant rates of acetylene reduction were associated with four widespread communities of non-heterocystous blue-green algae (Table 1). It was possible to demonstrate high nitrogenase activity in the same communities on separate occasions, although the data for one species, *Lyngbya aestuarii*, were rather variable, and in one population, no activity was detected. For the other community of *L. aestuarii* where acetylene reduction was measured, the rates were comparatively low. It was possible to isolate this species (uni-algal) on a medium lacking combined nitrogen (unpublished data).

With the exception of *L. aestuarii*, the rates of acetylene reduction measured for the other communities of *Hydrocoleus* sp. – *Schizothrix* sp., *Hyella balani* and *Lyngbya aestuarii* – *Schizothrix* sp., are all significantly higher than those measured for the heterocystous community of *Gardnerula corymbosa* ($P < 0.05$).

Acetylene reduction was always detected in the dark, although light rates were generally higher, some significantly so. In one experiment with a community of *Hydrocoleus* – *Schizothrix*, there are no significant differences between the light and dark rates ($P < 0.05$); on a second visit to this community, no dark acetylene reduction was detected.

Without exception, light rates in the presence of DCMU are all greater than those in the dark, and most, such as those for the *Hydrocoleus* sp. – *Schizothrix* sp. community, are significantly higher (125% difference). With or without the presence of DCMU the distinctions are not so clear. In several experiments the differences are insignificant, or the rates when DCMU is added are less than the normal light rates.

Glucose tended to stimulate acetylene reduction in the light, the greatest stimulation being 59% for the community of *Hyella balani*.

Figures 1 and 2 show details of two of the non-heterocystous communities studied.

Table 1. Rates of acetylene reduction associated with communities of non-heterocystous blue-green algae. Rates are expressed as nM C_2H_4 produced mg chl a^{-1} min^{-1} and are the mean of at least four readings. L = light rate, D = dark rate, G = light rate in the presence of glucose, DCMU = light rate in the presence of DCMU, $\bar{x}$ = mean, s = standard deviation, v = visit.

* Included for comparative purposes as this community grew in close proximity to the community of *Hyella balani*

Community	Environment	v	L		D		G		DCMU	
			$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
Hydrocoleus sp. – *Schizothrix* sp.	thick crusts, upper intertidal	1	29.92	9.737	–	–	32.69	8.135	31.66	0.1556
		2	24.86	1.217	26.49	17.91	30.39	5.211	31.21	6.364
Hyella balani	endolithic in coarse carbonate sand	1	12.45	5.233	6.739	5.421	19.91	0.6894	15.30	5.890
Lyngbya aestuarii – *Schizothrix* sp.	smooth crusts, upper intertidal	1	21.11	7.017	17.55	5.346	21.76	0.7780	21.16	1.626
		2	20.99	12.99	14.76	0.1000	23.29	4.179	18.87	4.405
L. aestuarii	'crinkle' mat, mid intertidal	1	3.263	0.2546	2.826	0.3600	3.443	4.800	2.991	1.102
		2	3.175	0.3076	2.364	3.000	3.339	6.340	2.496	0.3860
L. aestuarii	smooth film over rock absent 2 weeks later		–	–	–	–	–	–	–	–
* *Gardnerula corymbosa*	stromatolites, mid intertidal	1	7.070	0.4879	2.472	0.1948	5.080	2.302	5.800	0.6600
		2	10.78	11.36	3.433	1.359	5.030	3.600	4.668	0.4531

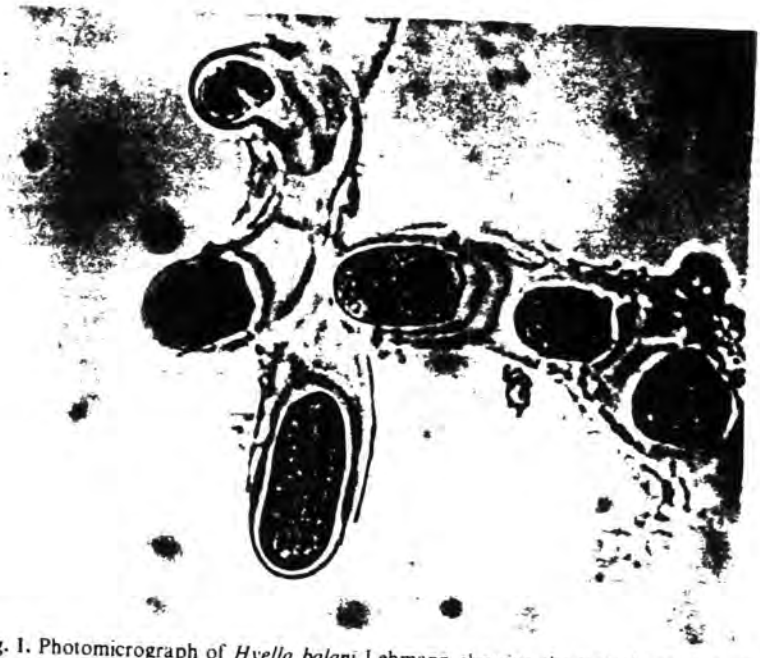

Fig. 1. Photomicrograph of *Hyella balani* Lehmann showing the typical granular, clavate end-cells of endolithic filaments. The slimy sheath is striated and shows pseudo-branching. Endolithic filaments were extracted from coarse-grained sediment, by treatment with dilute HCl. *Hyella* lent a green colour to the otherwise pale-grey, anaerobic sediment. The standing crop was measured as 20.69 μg chl *a* cm^{-3}: 0.644 μg phaeophytin *a* cm^{-3} of sediment. The scale bar is 5 μm

Fig. 2. Blistered, irregular crusts of a *Hydrocoleus* sp. – *Schizothrix* sp. community, binding fine-grained carbonate sediment. When wet these crusts become pliable and cartilaginous. The two species occur in two discrete layers; the *Hydrocoleus* forming a bright green layer at the surface, with a bright pink laminar of *Schizothrix* below this. The globules are hollow, as is evident from the bottom right-hand corner of the photograph. The telephone token is 18 mm diameter

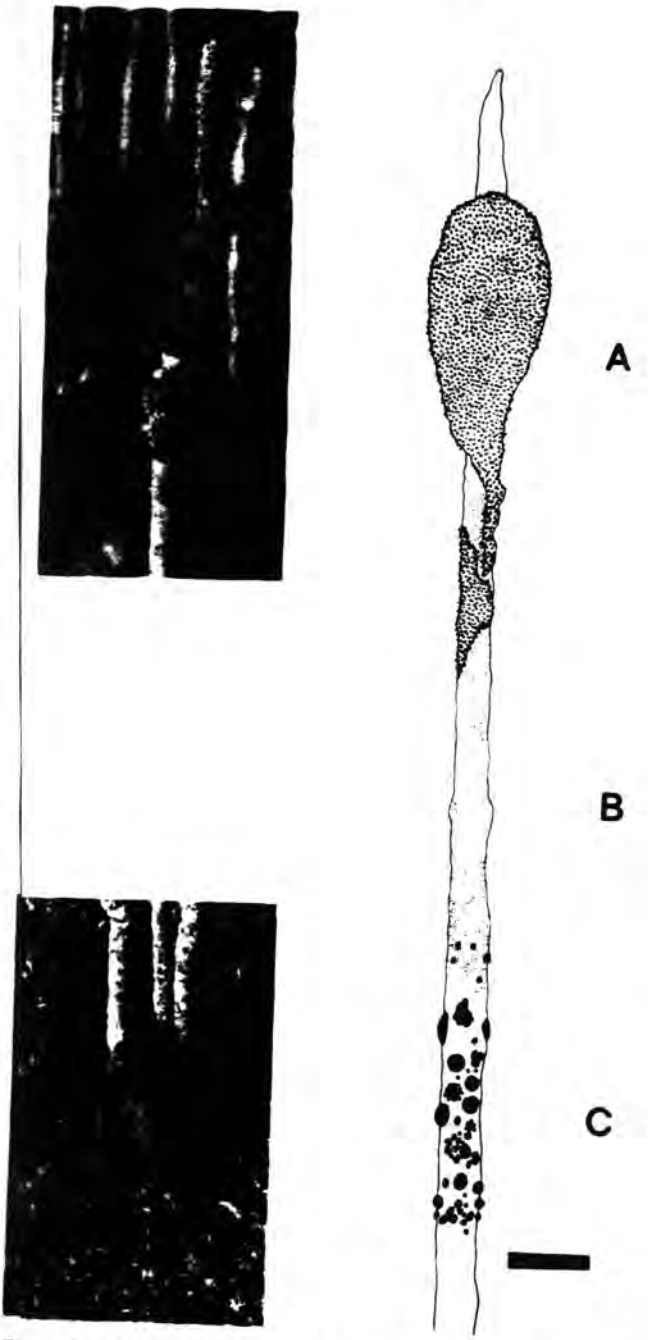

Fig. 3. Aerial pneumatophore of the mangrove *A. marina* showing the zonation of epiphytic heterocystous blue-green algae. A zone of *Scytonema* sp., B zone of *Calothrix* sp. C zone of *Kyrtuthrix maculans*. This zonation is typical of pneumatophores at Shura el Manqata, but at Ras Muhammad, the *Scytonema* zone is absent. The growth form of *Scytonema* is shown in the upper inset: friable crusts, binding sediment with the active photosynthetic layer *c* 4 mm below the surface. The lower inset shows the sediment surface, with the non-heterocystous *Phormidium* sp. forming oncoidal structures at the base of the pneumatophores. The scale bar relating to the line drawing is 20 mm

Table 2. Vertical distribution of nitrogenase activity on mangrove pneumatophores and in the sediment of the immediate vicinity. All rates are expressed as nM C_2H_4 produced cm^{-3} min^{-1}. Above the sediment surface, rates for the dominant communities of blue-green algae are expressed also as nM C_2H_4 produced mg chl a^{-1} min^{-1}. All values are the mean of at least four readings. L = light rate, h = presence or absence of heterocysts, $\bar{x}$ = mean, s = standard deviation

Area	h	species	L cm^{-3}		L mg chl a^{-1}	
			$\bar{x}$	s	$\bar{x}$	s
25 centimetres above the sediment surface	+	*Scytonema* sp.	1.044	0.6039	15.27	0.1414
15 centimetres above the sediment surface	+	*Calothrix* sp.	1.017	0.3619	16.23	5.777
5 centimetres above the sediment surface	+	*Rivularia* sp.	0.9969	0.2168	18.17	3.951
the sediment surface	−	*Phormidium* sp.	*0.07084	0.04853	11.94	6.650
0.5 centimetres below the sediment surface	−	*Microcoleus* spp.	0.01937	0.02294		
2 centimetres below the sediment surface			0.01042	0.01805		
4 centimetres below the sediment surface			0.01984	0.03436		

* dark rate measured here as 3.986 nM C_2H_4 mg chl a^{-1} (0.02622 nM C_2H_4 cm^{-3} min^{-1})

Heterocystous Blue-Green Algae

A marked vertical zonation of communities was always apparent on aerial pneumatophores of *Avicennia marina* (Fig. 3). Nitrogenase activity decreased towards the base of the shoots, with a sharp decrease at the sediment interface (Table 2). Although much lower, rates of acetylene reduction were still detected at a depth of four centimetres in the sediment. The variability in readings was generally greatest here.

The *Scytonema* community occurs in the upper intertidal zone at the highest part of the tidal range and it appeared likely that this species would be exposed to the most extreme environmental fluctuations. Nitrogenase activity measured over a 24 h period is shown in Fig. 4. The dotted line is used to give some indication of the state of wetting of the community. Prior to high tide at 1130 h, the community was completely desiccated and appeared as friable crusts. After wetting, the community remained moist and sponge-like for approximately 4 to 5 h. Dark rates are lower and much less variable than the light rates, with maximum activity at 1200 h. Rates in the light increased rapidly after first light (0500 h), and reached a maximum at 1100 h. A second peak was reached during the late afternoon (1600 h).

Ethylene was first detected with this community between 5 and 10 min after wetting. The rate of acetylene reduction then increased rapidly and became more or less constant after 20 min (Fig. 5). When chloramphenicol was added to the incubation medium, a similar pattern in the time-course of water uptake versus acetylene reduction was observed, with rates of the order of 30% less. Pre-incubating the *Scytonema* material in an anaerobic gas phase in the presence of a higher concentration of chloramphenicol, virtually suppressed acetylene reduction.

After 3 h pre-incubation, material of *Scytonema* showed an increase in nitrogenase activity (Fig. 6). After 120 min the specific rate began to decrease.

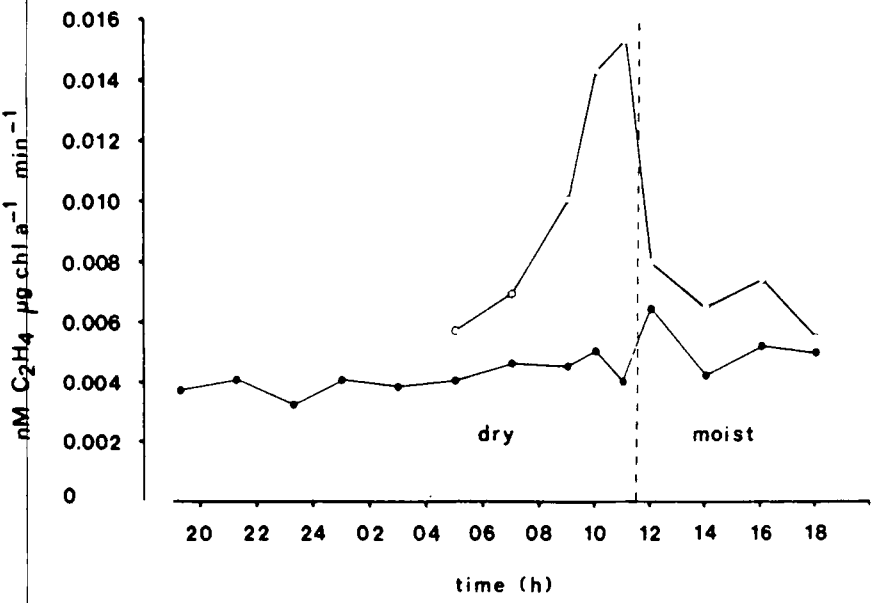

Fig. 4. The nitrogenase activity (nM C_2H_4 produced µg chl a^{-1} min^{-1}) of *Scytonema* sp., measured over a 24 h period ○—○ light rate, ●—● dark rate. The times given are the mid-points of 60 min assays. When the community was completely dried out, sea water was added before the assay for acetylene reduction. Each point represents the mean of at least three readings

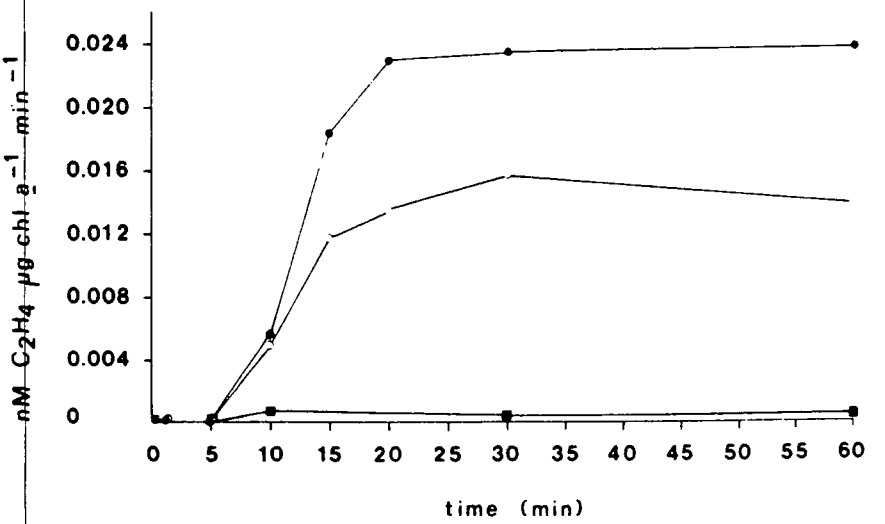

Fig. 5. Nitrogenase activity of *Scytonema* sp., in relation to water uptake by dried out communities: ●—● nitrogenase activity in the light, incubated with sea water under a gas phase of air; ○—○ nitrogenase activity in the light, incubated with sea water in the presence of 10^{-5} M chloramphenicol under a gas phase of air; ■—■ nitrogenase activity in the light, incubated with sea water and 10^{-3} M chloramphenicol, under a gas phase of Ar/CO_2 (0.96:0.04 atm)

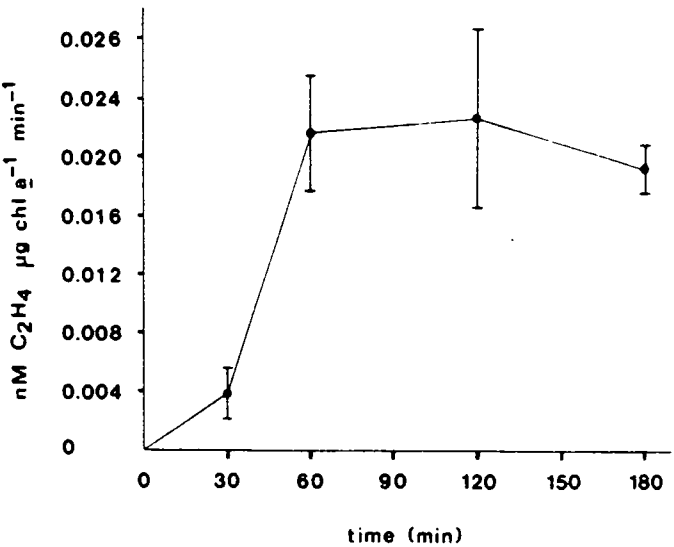

Fig. 6. Time course in acetylene reduction with *Scytonema* sp. Material was incubated for three hours in sea water at 30° C and 500 µE m^{-2} s^{-1}, and replicates were assayed for ethylene production after 30 min, 60 min, 120 min and 180 min. Each point is the mean of at least four replicates

Laboratory Experiments

Table 3 presents data from a range of experiments with *Hyella balani* and *Kyrtuthrix maculans*. Using the 'standard' controls of field assays (see Table 1), quite different results were obtained with the different species. The rate of acetylene reduction in the dark for *Hyella* is 10% higher than the light rate; no dark acetylene reduction was detected with *Kyrtuthrix*. DCMU significantly decreased the rate of reduction by *Hyella* (in the light), yet appeared to stimulate the rate in *Kyrtuthrix*. NH_4-N reduced the rates of reduction in both species, although rates still continued at *c* 11% of the control light rate. NO_2-N also decreased nitrogenase activity in these species, but the effect was not as pronounced as with NH_4-N. NO_3-N had a similar effect as NO_2-N in *Hyella*, yet stimulated the light rate by 22% in *Kyrtuthrix*.

HCO_3^- stimulated nitrogenase activity in both species, the effect being most pronounced in *Kyrtuthrix*. The most marked differences in the observed rates of acetylene reduction were produced with Fe and PO_4-P. Both stimulated rates by more than 100% in *Kyrtuthrix*, but suppressed nitrogenase activity in *Hyella*.

Environmental

Readings of pH vary little between the different environments (Table 4). The ranges in Eh and Eh_7 are large, with the lowest, most negative values being associated with sediments often smelling of sulphide. Particularly low values

Table 3. Comparative rates of acetylene reduction by *Hyella balani* and *Kyrtuthrix maculans* under a range of experimental conditions. Rates are expressed as nM C_2H_4 produced mg chl a^{-1} min^{-1}. $\bar{x}$ = mean, s = standard deviation. % = percentage difference between a particular rate and the control light rate

	Hyella			*Kyrtuthrix*		
	$\bar{x}$	s	%	$\bar{x}$	s	%
light	10.06	2.266		8.262	1.125	
dark	11.08	0.4137	+10	–	–	
light – DCMU	8.306	1.028	−17	9.435	1.222	− 14
light – glucose	9.715	2.338	− 3	12.99	0.7000	+ 57
light – KNO_2	8.572	3.051	−15	7.221	5.330	− 13
light – KNO_3	8.454	2.884	−16	10.05	6.668	+ 22
light – NH_4Cl	1.167	2.355	−88	1.111	2.976	− 87
light – $NaHCO_3$	12.53	2.884	+25	15.80	4.332	+ 91
*light – K_2HPO_4	8.888	0.2051	−12	18.56	2.117	+125
light – $FeCl_3$	4.081	4.947	−59	17.16	4.186	+108

* A decrease in the light rate was also observed in situ when phosphate was added to *Hyella* sediment

Table 4. Environmental data collected at the time of in situ assays. Values given are the mean of at least 12 readings. Values of Eh and Eh_7 are in mV, values of dissolved oxygen in mg l^{-1}. ND = insufficient water to cover the oxygen probe

Environment	Area	pH	Eh	Eh_7	°C	O_2
(i) tidal sea water entering lagoon, Shura el Manqata	surface	8.16	+155	+222	24.0	8.4
(ii) small pools in anaerobic sediment, *Hyella balani* endolithic in grains	pool water	8.30	+175	+251	27.5	9.0
	above sediment	8.50	+140	+227	27.8	
	−1 cm sediment	8.50	−110	− 27	27.0	
(iii) small pools in *Hydrocoleus – Schizothrix* mats	pool water	8.35	+166	+244	22.0	ND
(iv) surface of *L. aestuarii* mats	moist sediment	8.00	+155	+213	27.5	6.5
(v) centre of mangrove area	pool water	8.03	+140	+200	26.0	6.2
	surface of sediment	8.03	−190	−130	27.5	
(vi) sediment at the base of pneumatophores; oncolites of *Phormidium*	pool water	7.80	+ 10	+ 56	29.0	3.0
		7.80	+ 20	+ 66	25.0	

are associated with sediments in which *Hyella balani* grows as an endolith. Where it was possible to measure dissolved oxygen, highest values were obtained from tidal sea water entering the mangroves and standing water over *Hyella* sediment. Lowest values were measured in waters overlying dark, sulphide-smelling silts in the central areas of the mangrove forests.

Table 5. Nutrient chemistry of sea water from the lagoon at the Shura el Manqata, compared with the data of Sournia (1977). Values are expressed as $\mu g\ l^{-1}$, and are the mean of five readings. ND = not determined

Study	NO_2-N	NO_3-N	PO_4-P	pH	Eh	
present study	0.07	1.645	5.05	8.05	+240	December 1977 to February 1978
*Sournia (1977)	0.07	1.540	7.75	ND	ND	May 2 to May 28, 1976

* Measurements from a mangrove pool near Nabq

Nutrient data collected during the present study are very similar to those collected by Sournia (1977) during studies in 1976 (Table 5).

Discussion

The mangrove forests of Sinai are highly productive ecosystems which grow in extremely oligotrophic waters. Any input of nutrients such as nitrogen, is therefore likely to have a significant effect on the overall balance of the system. The present data show that nitrogenase activity is associated with a wide range of heterocystous and non-heterocystous blue-green algae which are abundant and widespread in these forests. Nitrogen fixation by these communities, especially by the more extensive non-heterocystous ones, would be one of the major factors influencing the productivity of this coastal ecosystem.

The measurement of high rates of acetylene reduction in the dark is not surprising, as many workers have suggested that dark nitrogen fixation may be supported by a source of dark-generated reductant e.g. pyruvate, malate etc. (Stewart, 1973; Gallon et al., 1975). Such high rates in the dark in the present study may be explained by the diminishing competition between acetylene reduction and photorespiration (Lex et al., 1972; Stewart and Pearson, 1970), and the development of microaerobic conditions in the assay bottles due to the cessation of oxygen evolution. Dark nitrogen fixation would be expected to have most marked effects on the overall nitrogen input, at those parts of the tidal cycle when the algal communities are desiccated for long periods during daylight hours, and wetted for long periods during the night. The build up of endogenous reductant will however be controlled in a similar manner, and will depend on photosynthetic activity during daylight, when suitably wetted. Although it was not possible to follow nitrogen fixation rates over a complete tidal cycle, it is likely that light and dark fixation rates vary markedly with the tidal cycle, causing a fluctuation in total nitrogen input.

Overall, the rates of acetylene reduction measured were light dependant. The fact that DCMU has a more pronounced inhibitory effect on acetylene reduction under conditions of high photorespiration, than under low oxygen tensions (see Wolk, 1973), may be of importance here, when one considers that all the communities described in Table 1 were associated with sediments

often smelling of sulphide. This leads to the consideration that certain of these communities are able to utilize sulphide as an electron donor in the photoassimilation of CO_2 (energy from photosystem I), when photosystem II is blocked by DCMU, and thus support acetylene reduction. This has already been suggested for communities of *Microcoleus chthonoplastes* in Farbstreifen-Sandwatt of the north German coastline (Potts et al., 1978).

Although glucose generally stimulated acetylene reduction in the light, the stimulation was often less than 10%. One should also note that many of the non-heterocystous communities occur at one to two centimetres depth in sediment e.g. *Hyella balani*. As light is certainly a limiting factor to growth at this depth, potential heterotrophy by these forms cannot be dismissed, and stimulation of nitrogenase activity by a carbon source cannot be attributed solely to heterotrophic bacteria.

It is possible to speculate on a spatial partition between two distinct environments in the mangroves. The first, a predominantly undisturbed, aerobic system, subject to high light intensities, high oxygen levels and extreme desiccation, and the second, a disturbed sediment system, with low oxygen tensions, lower light intensities and much less desiccation due to contact with sediment porewater and the tidal water table. *Kyrtuthrix maculans* and *Scytonema* sp. may be considered as representative of the former system, and *Hyella balani* of the latter. Under the low oxygen tension of the *Hyella* sediment, HCO_3^- levels which would inhibit photorespiration have little effect (see Table 3), but in the case of *Kyrtuthrix* where the rate of photorespiration is probably high, HCO_3^- stimulates nitrogenase activity.

Both Fe and PO_4-P are assumed to be limiting in marine waters, and at least for PO_4-P, this is probably the case in the waters of the Gulf of Elat, adjacent to the mangrove forests. It is interesting that both Fe and PO_4-P stimulate nitrogenase activity in *Kyrtuthrix*, yet not in *Hyella*. *Kyrtuthrix* belongs to the family Rivulariaceae, members of which are characterized by morphologically distinct hairs (Sinclair and Whitton, 1977). The formation of such hairs in many species of Rivulariaceae can occur through Fe and PO_4-P deficiency (Whitton et al., 1973). It is therefore likely that *Kyrtuthrix* and other species of Rivulariaceae found on pneumatophores, are defficient in Fe and PO_4-P, and that this has some limiting effect on nitrogenase activity. The depression of nitrogenase activity in *Hyella* is rather difficult to explain, although it is probable that neither Fe or PO_4-P are limiting in the mangrove sediments.

Desiccation appears to be one of the most important factors limiting nitrogen fixation by communities growing in exposed areas of the mangrove. Although forms such as *Scytonema* appear to be able to synthesize nitrogenase rapidly when wetted, the observed rates of acetylene reduction will depend on the nitrogenase activity of the material at the time it became dry. It is clear that in the Sinai mangroves, where desiccation and evaporation may be extreme and rapid, the tidal cycle and subsequent wetting of communities is possibly the most important factor controlling the frequency and extent of the nitrogen input through nitrogen fixation.

Compared with other marine environments where nitrogen fixation by blue-green algae is thought to be important, the rates observed in the present study

are comparatively high, although one should proceed with caution in any extrapolation towards nitrogen fixed, as opposed to acetylene reduced.

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Blue-green algae (Cyanophyta) in marine coastal environments of the Sinai Peninsula; distribution, zonation, stratification and taxonomic diversity*

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Eighty-four species of blue-green algae were recorded from the intertidal zone of the Gulf of Elat (Aqaba) and four hypersaline coastal pools. Heterocystous forms (sixteen) were restricted to the intertidal, although significant rates of nitrogen fixation (acetylene reduction) were measured in non-heterocystous communities in coastal pools. Species distribution, the colour and surface morphology of thick mats are determined by salinity, frequency of wetting and desiccation.

Distinct horizontal zonation patterns occur in coastal pools. Coccoid and non-filamentous forms such as *Entophysalis* and *Pleurocapsa* grow associated with gypsum crusts around the periphery of the pools, while filamentous forms (*Lyngbya*, *Microcoleus*) become abundant towards the central regions.

Complex stratification is apparent in many communities. In different coastal pools mat communities have a similar structure; *Lyngbya aestuarii* and pennate diatoms at the surface, then coccoid blue-green algae (*Aphanothece*, *Synechococcus*), a bright green layer of *Microcoleus chthonoplastes* and finally deeper layers of phototrophic bacteria. In mangrove forests, *Scytonema* and a number of species of Rivulariaceae form conspicuous growths on aerial pneumatophores while non-heterocystous forms are restricted to the sediment surface.

Introduction

Marine blue-green algae were the subject of far fewer studies by classical botanists than freshwater forms, and as a result they are poorly described and only a small number appear in determinative manuals. The ecological significance of Recent marine stromatolites and algal mats, and the lack of experimental data on their euryhalinity are considered to be valid reasons for treating these marine forms as separate taxa from freshwater ones (Golubić, 1973a). Experimental evidence to support the idea of a genetic distinction between marine and freshwater blue-green algae is given by Batterton & Van Baalen (1971) and more recently by Stam & Holleman (1975). Unfortunately, the obvious problems in allocating suitable binomials to marine forms have persuaded many workers to adopt the taxonomic conventions of Drouet (Drouet, 1968, 1973, 1978; and Drouet & Daily, 1956). Drouet's basic premise is that each species of blue-green alga has one genotype and many phenotypes or ecophenes (ecological growth forms); all ecophenes of the same species revert to the same phenotype when grown under the same conditions. Following this concept, Drouet has reduced some 3000 'described' species and subspecific taxa to thirty! However, this system does not withstand critical scrutiny, much of the classification is determinatively worthless and consequently it has received strong criticism (Bourrelly, 1969; Golubić, 1969; Nielsen, 1973; Stam, 1978; Stanier *et al.*, 1971).

Within recent years, the development of improved isolation and purification techniques has made possible a sounder appraisal of conventional taxonomy (Waterbury & Stanier, 1978), but modern taxonomic methods, for example the use of DNA-DNA hybridization and DNA base composition have been applied to relatively few blue-green algae and at present appear to be of little use to the determination of species in natural populations. Proposals to include the nomenclature of blue-green algae within the Bacteriological Code (Stanier *et al.*, 1978; Gibbons & Murray, 1978), offer little encouragement to the field investigator and it is clear

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that the development of a satisfactory and reliable determinative system will take some time (Komárek, 1973). Until such a system appears, it seems essential that studies of natural populations of blue-green algae should include precise and detailed morphological descriptions of the communities and dominant forms, in a format such that the data are readily available for comparison and, where necessary, revision. This is particularly important in the case of marine blue-green algae which as mentioned earlier are poorly described, yet have become the subject of an increasing number of studies by geologists and geochemists (Walter, 1976).

The present account describes communities of blue-green algae found in a range of marine environments in one relatively small area, the Sinai coast of the Gulf of Elat (Aqaba).

Location of study areas

The Gulf of Elat is a northern extension of the Red Sea (Fig. 1), separated from it near the Island of Tiran by a sill at a depth of 250 m. Waters in the Gulf are deep (maximum 1830 m), saline (41‰), oxygenated throughout their depth (4·8 to 5·0 mg l^{-1} O_2), extremely oligotrophic and have an average temperature at the surface of 21·5°C during winter and 24·5°C during summer (Klinker *et al.*, 1976; Sournia, 1977). Tides are semidiurnal, cotidal and have a mean range at springs of between 50 to 70 cm. The climate of the area is arid, with an average rainfall of around 20 mm yr^{-1} and evaporation rates in excess of 4 m yr^{-1} (Por *et al.*, 1977).

The intertidal zone is very narrow for most of the Gulf, in some areas less than 6 m in width. Beachrock is a conspicuous feature of the upper littoral for long stretches of the coastline. The intertidal widens in the alluvial fan of the Wadi Kid where mangroves line the coast for some 20 km (Por *et al.*, 1977; Potts, 1979a).

Several near-shore pools occur along the coast of the Gulf, all separated from the sea and receiving their supply of water through subterranean seepage. Three are discussed in the present account: The Solar Lake (Fig. 4) is hypersaline and heliothermal.

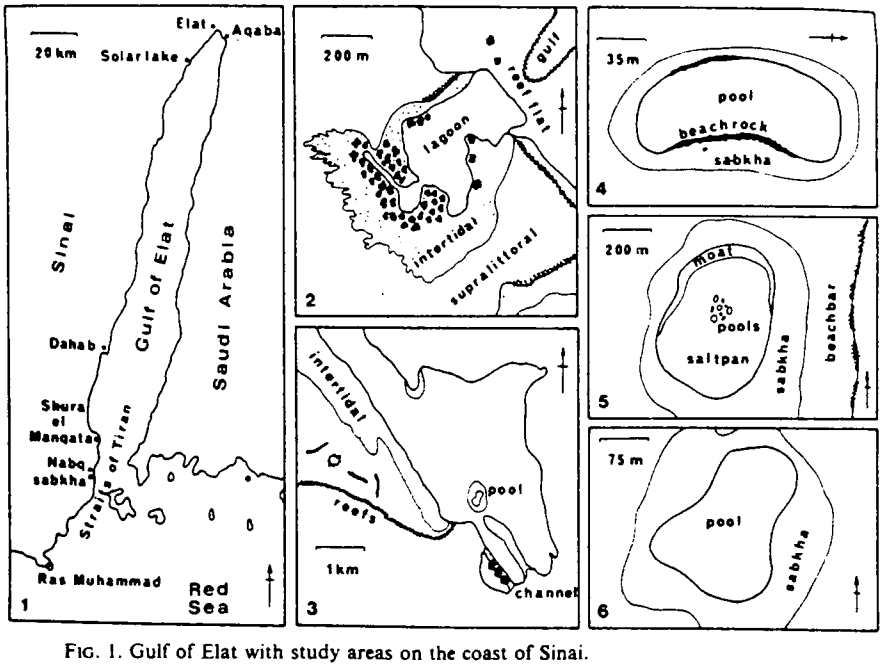


FIG. 1. Gulf of Elat with study areas on the coast of Sinai.

FIG. 2. Shura el Manqata mangrove forest (mangroves indicated by darkened areas).

FIG. 3. Ras Muhammad showing mangrove channel and Ras Muhammad Pool.

FIG. 4. Solar Lake near Elat.

FIG. 5. Sabkha area near Nabq.

FIG. 6. Ras Muhammad Pool.

During stratification (*c.* October to May) a chemocline separates an anaerobic hypolimnion from an oxidized epilimnion. Studies on physicochemical properties and photosynthetic microbial communities of the lake include those of Cohen *et al.* (1977a, b and c), Krumbein *et al.* (1977), Hirsch (1978) and Potts (1979b). An area of sabkha at Nabq (Fig. 5) is separated from the sea by a 2 m high bar of terrigenous sediments. The central area is a flat saltpan, mainly dry, except for a number of small pools. A narrow moat filled with water (50 cm deep) surrounds the saltpan. Salinity of the springs which appear in the moat is 40‰, rising towards the centre of the saltpan (350‰ in summer, 150‰ in winter). Ras Muhammad Pool (Figs 3 and 6) has features of both the Solar Lake and the coastal sabkha area (Friedman *et al.*, 1973).

Materials and methods

Studies were carried out from October 1977 to August 1978. Straight line transects were made in the intertidal zone at many points along the Gulf. More detailed transects were made in an area of the northernmost mangroves, the Shura el Manqata (Figs 1 and 2), and in the stunted atypical mangrove thickets of the Ras Muhammad (Figs 1 and 3). Transects were made at 90° to the coast, from the level reached by the highest spring tides, to the level of the Gulf waters at low tide. In coastal pools, transects were made from the peripheral region of sabkha sediments, towards the centre. Studies of coastal pools were supplemented by observations on saltwater fish ponds, constructed at the Heinz Steinitz Marine Biology Laboratory by the Israel Oceanographic and Limnological Research Co. These ponds contain high levels of phosphate, nitrate and nitrite; one pool (without fish) was selected for comparative studies.

A number of problems arise when one attempts to describe the various kinds of zonation and stratification found in coastal microbial communities. In the present study, *zonation* refers to a clear separation of visually different zones (in most cases corresponding to individual communities) along the surface of an intertidal gradient. 'Horizontal' zonation is not truly horizontal but is along a mild slope. Vertical zonation describes the situation where the zones are arranged along a slope with an angle of around 90°. *Stratification* is the separation of clearly defined strata (in most cases parallel to the surface) in a vertical section through a community. The prefixes micro and macro refer to scale alone in either instance of zonation or stratification.

Samples (10 cm^2) were collected along transect lines from visually conspicuous zones and also at 10 m intervals. A random set of twenty samples (each 10 cm^2) were removed from a particular community and 1 cm^3 subsamples used in pigment analyses.

Pigments were extracted from mat cores and rock samples in 95% methanol, in the dark at 70°C. Chlorophyll *a* and phaeophytin *a* were estimated spectrophotometrically (Potts & Whitton, 1977).

Nitrogenase activity was studied in representative communities using the acetylene reduction assay technique. Procedures used *in situ* are described in Potts (1979a).

Macrosamples of mats and rocks were examined first under a dissecting microscope. Subsamples were removed from visually distinct microstrata for more detailed microscopic study.

I have followed recent taxonomic studies (Golubić, 1973a, 1976; Le Campion-Alsumard, 1970; Schneider, 1976; Umezaki, 1961) and referred to classical taxonomic literature when identifying blue-green algae (Desikachary, 1959; Frémy, 1933; Geitler, 1932). Where it proved difficult to allocate a suitable binomial, several were placed in taxonomic categories not corresponding strictly to a description in a flora. This procedure has been developed for a computerised data recording system (Whitton *et al.*, 1978). This system delimits taxonomic units in certain genera on their range in cell width. Where size categories are used, a classical name has sometimes been given (one which corresponds to a classical description with a similar size range). In this case, the earliest classical description is given, even though this may be described originally as a freshwater form.

Observations and results

Distribution in the intertidal

The type of beachrock found in the upper littoral along much of the Gulf of Elat is shown in Fig. 7. When wet the surface appears dark olive-green to brown due to a more or less complete cover of *Rivularia* sp. and *Isactis plana* Thuret. Other Rivulariaceae become important locally; *Calothrix pilosa* Harvey grows as thick black felts in a narrow zone parallel to the shore and conspicuous olive-green

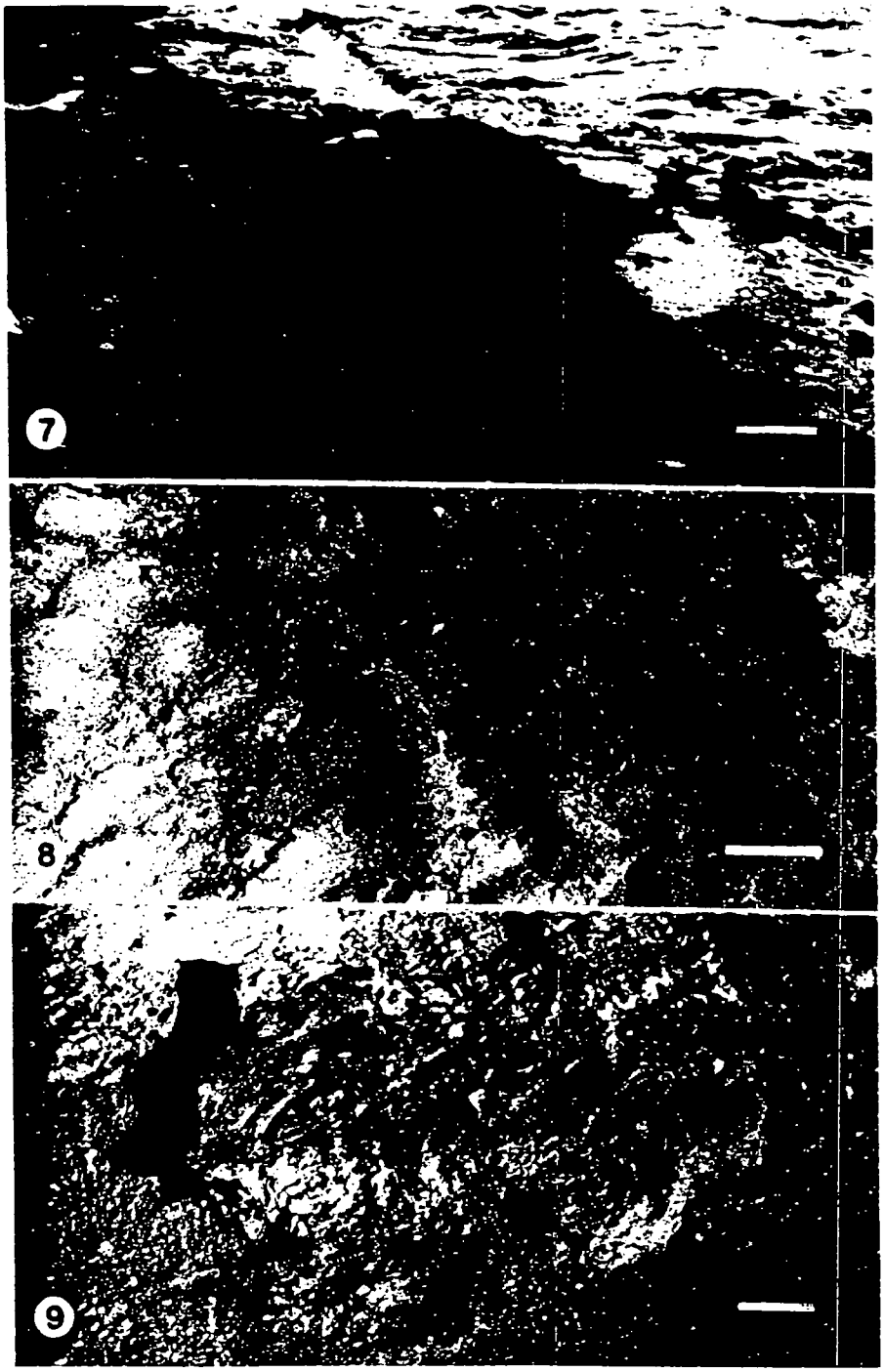


FIG. 7. Beachrock near Taba (scale bar = 50 cm).

FIG. 8. Crusts of *Lyngbya aestuarii* in supralittoral of Shura el Manqata (scale bar = 10 cm).

FIG. 9. Lithified stratiform stromatolites of *Microcoleus chthonoplastes* (scale bar = 10 cm).

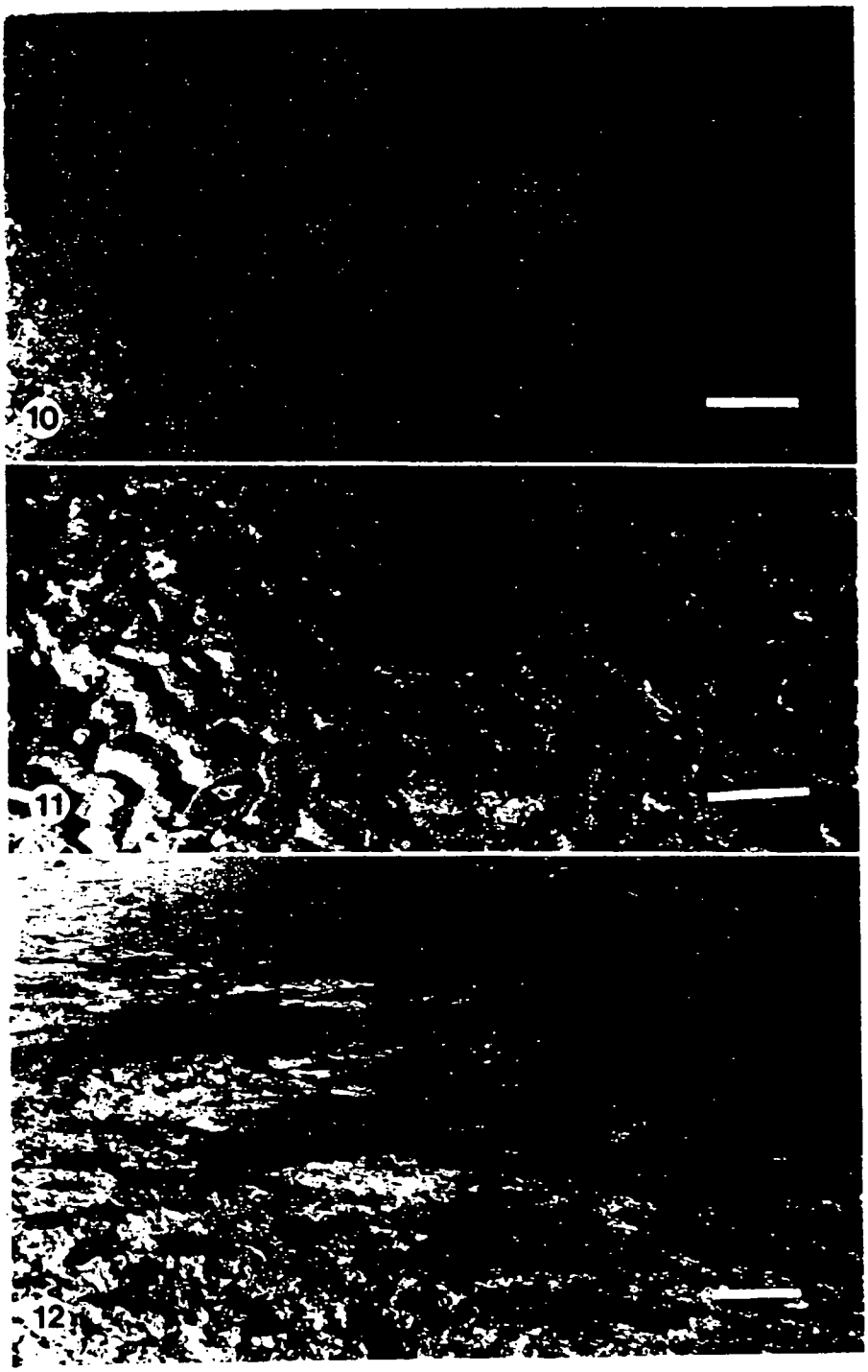


FIG. 10. Lithified sediment with *Gardnerula corymbosa* (scale bar = 5 cm).

FIG. 11. 'Brain' mat of *Aphanothece* sp., sabkha (scale bar = 10 cm).

FIG. 12. Periphery of sabkha area at Nabq showing: *Pleurocapsa* nodules (bottom left), polygons of *Microcoleus* and 'moat' (scale bar = 50 cm).

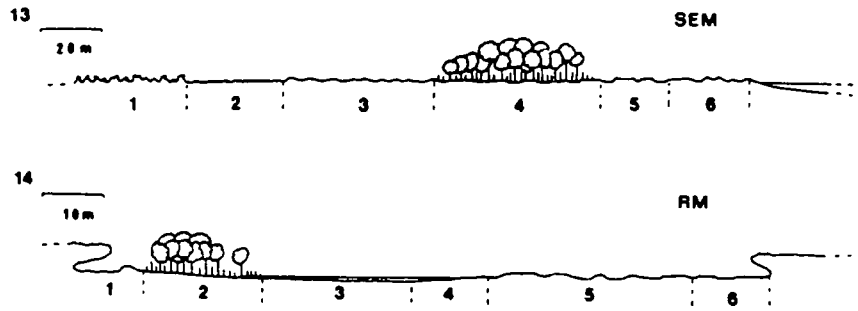


FIG. 13. Transect through Shura el Manqata (zone 1 is furthest from the mangrove lagoon).
FIG. 14. Transect through Ras Muhammad channel (zone 1 is to the West).

films of *Kyrtuthrix maculans* (Gomont) Umezaki form an irregular (1 to 2 mm thick) cover over beachrock near Dahab. Towards Elat, the beachrock zone narrows and species of Rivulariaceae, common elsewhere, are absent. The epilithic flora here is dominated by a phycoerythrin-rich species of *Oscillatoria* that forms patchy brown films up to 2 mm thick. The basal and central portions of these films contain vast numbers of the purple phototrophic bacterium *Thiosarcina* sp. (*Chromatiaceae*). Beachrock in this area is often obscurred totally by thick felts of Chlorophyta (*Cladophora* sp., *Enteromorpha* sp.) which appear at irregular intervals throughout the year.

The horizontal zonation of blue-green algae in the mangrove forests of the Shura el Manqata (Fig. 2) may be considered as representative of most of the northerly mangrove areas (Fig. 13). Buckled and irregular crusts of desert sediments mark the boundary of the upper supralittoral and the flat coastal plain (Fig. 13, zone 1; Fig. 8). *Lyngbya aestuarii* Liebmann (Fig. 20) grows in these crusts, in a 2 to 3 mm thick layer at a depth of 5 mm. This layer appears bright blue-green when the crusts are wetted. *Lyngbya aestuarii* is the only photosynthetic form here and surface growths are absent. This same species forms a thicker 'crinkle'-type mat community covering extensive areas in the upper littoral (Fig. 13, zone 2). These mats are brown and leathery, often with a thin white salt layer over the surface. Closer to the mangrove lagoon (Fig. 2) increased wetting frequency modifies the surface morphology of the *L. aestuarii* community, here it appears as a 2 cm thick, blue-black laminated mat (Fig. 13, zone 3). Heterocystous forms such as *Scytonema* sp., *Kyrtuthrix maculans* and *Rivularia* spp. become abundant only within shaded mangrove thickets where their communities display a marked vertical zonation on aerial pneumatophores of *Avicennia* (Fig. 13, zone 4; Table 1). *Scytonema* sp. forms a distinct structure, a thick collar in the shape of a 'wasp-nest' around the extreme upper portion of the mangrove pneumatophores. The collar is usually 3 to 5 cm thick and is restricted to the top 5 to 8 cm of the mangrove roots although sometimes it may cover them completely. Over the sediment surface between the dense mangrove root system, stromatolites of a *Lyngbya* sp. appear as discrete pale green domes, up to 5 cm in diameter and 2 to 3 cm high. This *Lyngbya* sp. forms also thin films over the sediment. In localized areas, the sediment surface is sometimes covered completely by free-rolling balls (aegagropiles) of the eukaryotic species *Valonia* sp. and *Chaetomorpha* sp. (Chlorophyta). In the most sheltered parts of the mangrove thickets, another type of aegagropile is found; pale brown, cartilaginous structures, 2 to 10 cm in diameter composed entirely of dead and empty sheaths of the species of *Lyngbya* described above and *Schizothrix* sp. The portion of the ball in contact with the sediment may become black, this colour changes to

TABLE 1. Vertical zonation of blue-green algae in mangrove forests of the Shura el Manqata.

	Approximate height above the sediment surface (cm)
**Scytonema* sp.	20–30
**Calothrix crustacea*	15–20
**Kyrtuthrix maculans*	5–15
**Rivularia* spp.	5–10
Lyngbya sp.	0–3
Gloeothece sp.	0–0·25
Lyngbya aestuarii	at surface
Microcoleus chthonoplastes	at surface
Hydrocoleum sp.	at surface
Schizothrix sp.	at surface

* Epiphytic on *Avicennia* pneumatophores.

pale brown when the ball is rolling freely. Out of the mangrove thickets the sediment surface appears as a bright blue-green mosaic of films of *Gloeothece* sp. (Fig. 15) that stabilize the sediment to a depth of 3 mm (Fig. 13, zone 4). The cells of this species are pale yellow in colour. Smooth mats of *Hydrocoleum* sp. – *Schizothrix* sp. and 'blistered' crusts of *Lyngbya aestuarii* – *Hydrocoleum* sp. form a thick cover over lithifying beachrock. Red cartilaginous crusts of *Schizothrix* sp. grow around small pools in the beachrock, extending sometimes down into the standing water. *Isactis plana* is embedded often in these crusts, visible macroscopically as a mass of tightly packed 'pin-heads'. A range of lithified beachrock structures is found in this area, the simplest being those formed by the stratiform stromatolites of *Microcoleus chthonoplastes* (Fig. 9).

The mangrove thickets at the Ras Muhammad are an impoverished variant of those further north and there are significant differences in the type of blue-green algal communities found there. A unique feature is the deeply undercut fossil limestone reef which in places reaches 2 m in height (Fig. 14). Epilithic communities of *Entophysalis granulosa* Kützing and endolithic *Hormathonema luteo-brunneum* Ercegović colour the exposed cliff yellow-brown in the upper 0·5 to 1·0 m and black below (Fig. 14, zone 1). There is a pronounced boundary between those pneumatophores close to the cliff devoid of epiphytes and those nearer the channel which have a cover of *Rivularia* spp. (Fig. 14, zone 2). Characteristic 'collars' of *Scytonema* sp. are absent. Sublittoral oncolites of *Microcoleus chthonoplastes* are present in the channel (Fig. 14, zone 3). At low tide an extensive carbonate mudflat is exposed along the eastern side of the channel (Fig. 14, zones 4 and 5). Most of the sediment appears grey-green to a depth of 8 cm due to endolithic communities of *Hyella balani* Lehmann, below this depth the sediment is black (a smell of H_2S was usually apparent). In places, lithification of the mud has produced large plates and polygons. The surface of these plates appears covered in masses of gelatinous black spots (Fig. 10); these are the subaerial apices of calcifying filament branches of *Gardnerula corymbosa* (Harvey) J. De Toni. Zone 6 is characterized by stromatolites of *Microcoleus chthonoplastes*.

Distribution in coastal pools

Each of the three coastal pools exhibits a marked horizontal zonation, each zone differs from those adjacent in colour, surface morphology and the dominant algal community (Table 2). The zonation is remarkably similar in each of the pools with several exceptions. The conspicuous diatom films typical of the perimeter of the Solar Lake are absent in the sabkha area at Nabq and are found only intermittantly in the Ras Muhammad Pool. Similarly, *Lyngbya aestuarii* which forms a broad zone of thick black 'tufted' mats in 30 cm deep water around Ras Muhammad Pool appears as discontinuous patchy films over the surface of mats in Solar Lake and was not recorded from the sabkha area. An additional zone was recognized in the sabkha area (not shown in Table 2), where salinity reaches its maximum values in the centre of the saltpan. Here, small pools and depressions appear pink due to communities of *Halobacterium* sp.

Varying rates of gypsum and halite precipitation

FIGS. 15–20. Scale bars = 10 μm.

FIG. 15. *Gloeothece* sp.

FIG. 16. *Synechococcus* sp.

FIG. 17. *Aphanothece* sp.

FIG. 18. *Johannesbaptistia* sp.

FIG. 19. *Dactylococcopsis* sp.

FIG. 20. *Lyngbya aestuarii*.

FIG. 21. Mat from zone 6 of Shura el Manqata (not to scale)—dashed lines indicate coloured strata, a = sediment, b = *Isactis plana*, c = *Lyngbya aestuarii*, d = *Hydrocoleum* sp., e = dead filaments and sheaths, h = sulphide zone and *Hormathonema violaceo-nigrum*, i = beachrock with endoliths.

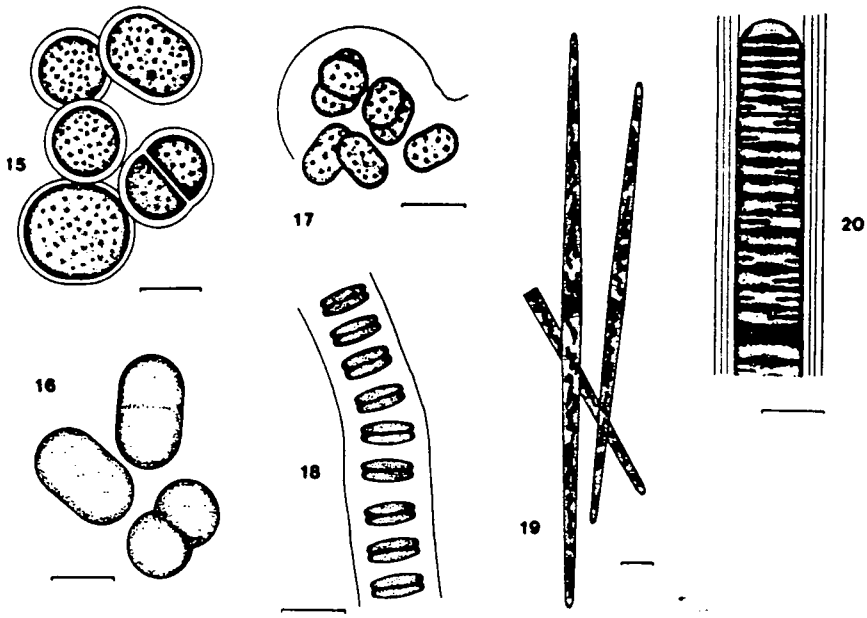
FIG. 22. Mat from zone F of Sabkha area (not to scale)—a = salt crust, b = *Aphanothece* sp. (orange layer), c = *Microcoleus chthonoplastes*, d = *Chromatium* sp., e = *Thiocystis* sp., f = black sulphide strata.

FIG. 23. Shallow-water mat of Solar Lake (not to scale)—a = *Lyngbya aestuarii*, b = pennate diatoms and *Aphanothece* spp., c = *Microcoleus chthonoplastes*, d = *Chromatium* sp., e = *Chromatium* sp., f = layers of degrading and semi-preserved material, g = stromatolitic sequences.

lead to a range of morphologically distinct structures in the pools. In the Solar Lake, crusts have formed 'beachrock'-type plates around the shore. In the sabkha area, *Aphanothece* sp. occurs associated with 'brain'-type salt crusts (Figs 11 and 17). Pronounced colour differences occur across short distances and separate zones. For example, the boundary between zones E and F coincides with standing water. Where the mats are exposed they appear orange, where wet they are red and have a smooth surface.

General comments on stratification

Stratification was observed in many communities



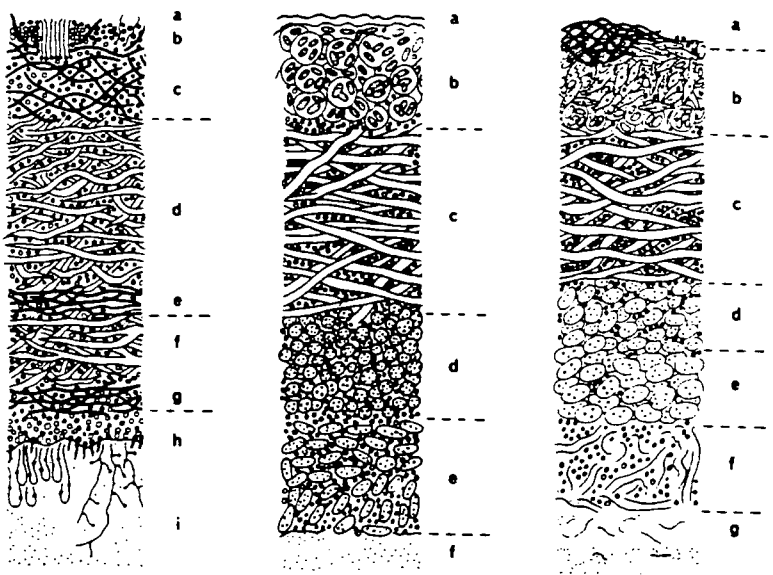


TABLE 2. Horizontal zones and dominant algal communities at the surface sediments of the Solar Lake (SL), the sabkha area at Nabq (S) and the Ras Muhammad Pool (RM)

Pool	Peripheral crusts around pool A	Seepage springs and macroalgae B	Gypsum crust zone close to water C	Diatom zone yellow D	Red zone E	Orange zone F	Thick mat zone: Black G	Thick mat zone: Green/brown H
SL	*Pseudanabaena*		*Entophysalis*	pennate diatoms		*Aphanothece*	*Lyngbya*	*Microcoleus*
S	*Pleurocapsa*	*Rhizoclonium*	*Pleurocapsa*		*Aphanothece*	*Aphanothece*		*Microcoleus*
RM	*Pleurocapsa*	*Enteromorpha*	*Pleurocapsa*	pennate diatoms	*Phormidium*/ *Synechococcus*	*Synechococcus*	*Lyngbya*	*Microcoleus*

usually apparent superficially as differences in colour. The most simple example is the occurrence of an active photosynthetic layer at a certain depth in sediments (Table 2, zone A). Sometimes this active green layer exists below a surface layer of deeply pigmented sheaths (yellow, orange, brown, blue-black), for example in communities of *Scytonema* sp., *Lyngbya aestuarii* and *Microcoleus chthonoplastes*. This feature of a yellow to orange-red coloured layer over a green layer was also observed on a larger scale in the Solar Lake. Waters from the epilimnion when filtered coloured the filter bright orange, this was due to gas vacuolate populations of *Dactylococcopsis* sp. (Fig. 19). Waters from the anaerobic hypolimnion appeared vivid green due to dense growths of Oscillatoriaceae.

Microstratification

Different coloured strata were particularly pronounced in colonies of *Rivularia* and *Isactis*. Moving from the basal to distal regions of a single colony there occurred a dark green layer of healthy cells, a yellow to yellow-brown layer *c.* 200 μm wide, a more or less colourless layer which included the hairs (150 μm) and a light pink zone at the extreme outer edge of the colony.

The most complex patterns of microstratification were found in thick mature mats from mangrove forests and coastal pools (Figs 21–23). The microstrata may be repeated once in a single sequence (Fig. 22), or many times with alternating layers of sediment, dead filaments and algal growth (Fig. 23). The different microstrata usually contain a single dominant species which is, with the exception of the surface layer, a motile form. Thick mats of *Lyngbya aestuarii–Hydrocoleum* sp.–*Schizothrix* sp. grow over lithified sand and beachrock usually with its own separate microstratification of shallow and deep-boring blue-green algae and deeper more discrete layers of chasmoliths. In much of the beachrock studied the chasmolith layer contained filamentous forms (*Lyngbya* sp., *Schizothrix* spp.). A thin black sulphide zone occurred sometimes at the interface between the surface of the beachrock and the bottom of the mat (Fig. 21, h).

Abundance

Values of standing crop are within the range 5·93 to 55·1 μg chl. *a* cm^{-3} (0·0244 to 2·73 μg phaeo *a* cm^{-3}) (Table 3). Highest values are associated with *Scytonema* sp. and polygonal stromatolites of *Microcoleus chthonoplastes*. Highest percengages of phaeophytin *a* are found in thick mats of *Hydrocoleum* sp.–*Schizothrix* sp. and globular stromatolites of *Lyngbya* sp. Values of standing crop and percentage phaeophytin *a* are of the same order as those given by Sournia (1977) and lower than those of Krumbein *et al.* (1977).

Nitrogenase activity

Highest rates of acetylene reduction were associated with Rivulariaceae (*Gardnerula*, *Isactis*, *Rivularia*) from the upper littoral of the Gulf, lowest values with stromatolites of *Microcoleus chthonoplastes* from the sabkha area (Table 4). The nitrogenase activity of non-heterocystous communities in coastal pools is significantly lower than that found for heterocystous communities in the intertidal and around the same magnitude for non-heterocystous communities in the intertidal.

Taxonomic diversity

Species of blue-green algae exclusive to the intertidal of the Gulf and particular coastal pools are

TABLE 3. Standing crop of blue-green algal communities. x = mean, s = standard deviation, % = percentage phaeophytin *a* (phaeo *a*) of total pigments (chl *a* + phaeo *a*)

Environment	Community	μg chl *a* cm^{-3}		μg phaeo *a* cm^{-3}		%
		$\bar{x}$	$\bar{s}$	$\bar{x}$	$\bar{s}$	
Beachrock, intertidal	Rivulariaceae	32·7	9·11	0·722	2·03	2·15
Beachrock, intertidal	Rivulariaceae	17·8	8·22	0·321	0·505	1·77
Beachrock, intertidal	Rivulariaceae	15·3	4·13	0·201	0·491	1·29
Mangroves, Fig. 13, zone 4	*Scytonema*	43·6	12·4	0·567	0·231	1·30
Mangroves, Fig. 13, zone 4	*Calothrix*	26·2	4·92	0·486	0·659	1·82
Mangroves, Fig. 13, zone 4	*Kyrtuthrix*	31·6	15·9	0·165	0·330	0·518
Mangroves, Fig. 13, zone 4	*Lyngbya*	5·93	1·99	0·578	1·00	8·88
Mangroves, Fig. 13, zone 5	*Schizothrix*	30·3	15·5	1·77	2·99	5·54
Mangroves, Fig. 13, zone 5	*Hydrocoleum*	21·6	4·78	2·73	5·47	11·2
Mangroves, Fig. 14, zone 4	*Hyella*	20·6	8·57	0·644	1·35	3·00
Mangroves, Fig. 14, zone 5	*Lyngbya*	13·8	1·10	0·419	0·464	2·92
Sabkha, zone B (Table 2)	*Chlorophyta*	24·6	12·7	0·0244	0·0423	0·0990
Sabkha, zone C (Table 2)	*Pleurocapsa*	45·6	3·14	0·270	0·234	0·588
Sabkha, zone H (Table 2)	*Microcoleus*	55·1	3·28	0·662	0·624	1·19
Mangroves, Sournia (1977)	Sediment	10 to 20				10
Solar Lake, Krumbein *et al.* (1977)	Shallow water mat	260				50

listed in Tables 5 and 6 respectively. Species recorded from the intertidal as well as from pools totalled thirteen. Of the total fifty-five species recorded from the intertidal zone, forty-two are found exclusively here, 38% of these being heterocystous forms (Table 7). No heterocystous forms occur in coastal pools. In order of increasing species diversity in the different environments, the sabkha area at Nabq has fewest species, followed by the Ras Muhammad Pool, fish pond, the Solar Lake and finally the intertidal zone of the Gulf. A number of species characterize certain environments, for example *Johannesbaptistia* spp. (Fig. 18) are found only in coastal pools, *Dactylococcopsis* sp. is exclusive to the water column of the Solar Lake and a wide form of *Synechococcus* (Fig. 16) typifies zone F of the Ras Muhammad Pool (Table 2). The flora of the fish pond is quite different from other coastal pools, *Gomphosphaeria* spp. particularly, and a number of Oscillatoriaceae being distinctive. Generally the Solar Lake and the fishpond resemble one another in terms of species composition, as do the sabkha area at Nabq and the Ras Muhammad Pool.

Discussion

A relatively diverse flora of blue-green algae occurs along the shores of the Gulf of Elat (Aqaba) and in

TABLE 4. Nitrogenase activity (nM C_2H_4 produced cm^{-2} min^{-1}) associated with communities of blue-green algae. L = light rate, D = dark rate, G = rate with 0·1 g l^{-1} glucose, DCMU = rate in presence of DCMU (10^{-5} M). h = presence or absence of heterocysts. ND = not determined

Environment	Community	h	L		D		G		DCMU	
			$\bar{x}$	$\bar{s}$	$\bar{x}$	$\bar{s}$	$\bar{x}$	$\bar{s}$	$\bar{x}$	-
Sabkha, gypsum nodules, Table 2, zone C	*Pleurocapsa*	–	0·12	0·054	0·13	0·038	ND		ND	
Sabkha, polygonal mats, Table 2, zone H	*Microcoleus*	–	0·076	0·038	0·065	0·029	ND		ND	
Solar Lake thick mats, Table 2, zone H	*Microcoleus*	–	0·28	0·0040	0·27	0·016	0·33	0·062	0·31	0·029
Beachrock, intertidal	*Isactis*	+	0·86	0·0055	0·87	0·0071	1·0	0·0039	0·96	0·0039
Mangroves, Fig. 10, Fig. 14, zone 5	*Gardnerula*	+	0·43	0·10	0·14	0·0082	0·19	0·0087	0·19	0·011
Mangroves, Fig. 13, zone 3	*Lyngbya*	–	0·090	0·032	0·071	0·023	0·10	0·043	0·075	0·025

TABLE 5. Species of blue-green algae exclusive to the intertidal zone of the Gulf of Elat

Species	Habitat
Aphanocapsa sp. >8 μm; 8·2 μm	Epiphytic on pneumatophores
Brachytrichia sp.; 4·8 μm	Endolithic in beachrock
Calothrix aeruginea (Kützing) Thuret	Among other algae on pneumatophores
C. pilosa Harvey	Forming black felts over beachrock
C. scopulorum (Weber *et* Mohr) Agardh *ex* Bornet *et* Flahault	On beachrock
Calothrix sp.; *C. crustacea* Thuret	Epiphytic on pneumatophores
Calothrix sp.; 5·8 μm	Epiphytic on pneumatophores
Chroococcus sp. >6≤8 μm, striated sheath; 7·8 μm	In beachrock communities
Chroococcus sp. >8≤16 μm, striated sheath; 13·5 μm	In beachrock communities
Chroococcus sp. >8≤16 μm, non-striated sheath; 12·3 μm	On pneumatophores
Gardnerula corymbosa (Harvey) J. De Toni	Only at Ras Muhammad; calcifying
Gloeocapsa sp. >2≤4 μm, non-striated colourless sheath; 2·45 μm	In *Rivularia* colonies; epiphytic on pneumatophores
Gloeothece sp.; 12·3 μm	Bright blue-green films over mangrove sediment
Hormathonema luteo-brunneum Ercegović	Upper littoral
Hydrocoleum sp. ≤4 μm; 3·7 μm	In crusts over beachrock
Hydrocoleum sp. >6≤8 μm; 6·8 μm	Thick crusts over beachrock
Hyella caespitosa Bornet *et* Flahault	In lithified rock under sediment
Isactis sp.; 8·5 μm; *Isactis plana* Thuret	Epilith on beachrock
Kyrtuthrix dalmatica Ercegović	Endolith in beachrock
K. maculans (Gomont) Umezaki	Epilith on beachrock at Dahab
Lyngbya allorgei Frémy	On beachrock and pneumatophores
L. kützingii Schmidle	Among other algae
L. martensiana Meneghini	On pneumatophores
Lyngbya sp. ≤1 μm	Among other algae
Lyngbya sp. >1≤2 μm; 1·5 μm	On beachrock
Lyngbya sp.; 7·3 μm	Globular stromatolites in mangroves of Shura el Manqata
Lyngbya sp. >6≤8 μm; 7·3 μm	On beachrock
Mastigocoleus testarum Lagerheim	Endolithic in beachrock; lower littoral
Microcoleus sp. ≤2 μm; 1·3 μm	Among other algae
Microcoleus sp. >2≤4 μm; 2·3 μm; *M. tenerrimus* Gomont	In stratiform stromatolites at Ras Muhammad
Microcystis reinboldii Richter	Epipsammic on carbonate sand
Oscillatoria sp.; >12≤16 μm; 14·6 μm; *O. sancta* (Kützing) Gomont	On beachrock near Elat
Pleurocapsa sp.; 3·8 μm	On pneumatophores
Rivularia sp.; 12·3 μm	Epiphytic on pneumatophores, Shura el Manqata
Rivularia sp.; 9·8 μm	Epiphytic on pneumatophores, Ras Muhammad
Rivularia sp.; 14·7 μm	Epilithic on beachrock
Schizothrix calcicola (Agardh) Gomont	Epilithic and chasmolithic; beachrock
Schizothrix sp. ≤1 μm	Among other algae
Scytonema sp.; >8 μm, laminated sheath	In algal mats over beachrock
Scytonema sp.; 15·9 μm	Collars on pneumatophores
Sirocoleus sp.; 2·45 μm	Gelatinous mat over beachrock
Solentia stratosa Ercegović	Endolithic in rocks, lower littoral

adjacent hypersaline pools. Many communities are visually conspicuous, abundant and often clearly zoned, and seem suited ideally to comparative studies.

The most obvious differences between the flora of the intertidal zone and the coastal pools is the absence of heterocystous forms in the latter. This may reflect a greater cycling of nutrients within the pools and an increased availability of limiting nutrients, for example nitrogen. Nevertheless, nitrogenase activity was still detected in the pools, of the same magnitude as found in non-heterocystous communities in the intertidal zone. This suggests that nitrogen may still be a limiting factor in the pools. The effects of increased nutrient levels within the intertidal were visible clearly close to Elat. Input of phosphorus in the vicinity of a phosphate-loading port and nitrogen from the drainage waters of fishponds, leads to sporadic blooms of Chlorophyta and a replacement of the typical Rivulariaceae community by one dominated by *Oscillatoria* sp. It is probable that in coastal pools and mangrove

TABLE 6. Species of blue-green algae found exclusively in one coastal pool

1. Sabkha area at Nabq	None
2. Ras Muhammad Pool	*Phormidium* sp.; $>2 \leq 4$ μm; 2·5 μm; in red films over sediment *Synechococcus* sp.; 12·3 μm; gelatinous films over sediment
3 Solar Lake	*Aphanocapsa* sp.; $>1 \leq 2$ μm; 1·58 μm; *A. concharum* Hansgirg; chasmolith *Aphanothece stagnina* (Spreng.) A. Braun; in green layer below phototrophic bacteria *Aphanothece* sp.; $>6 \leq 8$ μm; 7·35 μm; *A. pallida* (Kützing) Rabenhorst; chasmolith in gypsum crusts *Dactylococcopsis* sp.; 3·7 μm; *D. acicularis* Lemmermann; plankton in epilimnion *Gloeothece* sp.; >4 μm; 4·9 μm; in shallow water mats *Johannesbaptistia* sp.; 7·4 μm; in shallow water mats *Lyngbya confervoides* Agardh; in mats at margins *Oscillatoria redekei* Van Goor; in shallow water mats *Pseudanabaena catenata* Lauterborn; chasmolith in salt crusts
4. Fish pond	*Gomphosphaeria aponina* Kützing; in green gelatinous mats on bottom *G. lacustris* Chodat; in green mats on bottom *Gomphosphaeria* sp.; in green mats on bottom *Oscillatoria formosa* Bory; in green films around sides *O. limosa* Agardh; in green films on bottom and sides *O. nigro-viridis* Thwaites; in green felts on bottom *Oscillatoria* sp. ≤ 1 μm; among other algae *Oscillatoria* sp. $>1 \leq 2$ μm; 1·3 μm; among other algae *Phormidium* sp. $>1 \leq 2$ μm; 2 μm; *P. foveolarum* Gomont; among other algae
Species found in two or more pools	*Aphanothece* sp. $>4 \leq 6$ μm; 4·9 μm; *A. microscopica* Nägeli; Solar Lake, Ras Muhammad Pool *Aphanothece* sp.; 8·2 μm; Sabkha, Ras Muhammad Pool, Solar Lake *Johannesbaptistia pellucida* (Dickie) Taylor *et* Drouet; Solar Lake, Sabkha, Ras Muhammad Pool, fish pond *Lyngyba digueti* Gomont; Solar Lake, Ras Muhammad Pool *Oscillatoria geminata* Meneghini; Ras Muhammad Pool, fish pond *Oscillatoria* sp. $>2 \leq 4$ μm; 3·3 μm; Solar Lake, fish pond *Spirulina* sp. ≤ 1 μm; Ras Muhammad Pool, fish pond *Synechococcus* sp. $>4 \leq 6$ μm; Ras Muhammad Pool, Solar Lake, fish pond

sediments, the greater availability of nutrients determines the development of an exclusive non-heterocystous flora. In a previous paper (Potts, 1979a), it was observed that in the Sinai mangroves, an exclusive nitrogen-fixing flora of Rivulariaceae occurs on aerial pneumatophores in contact with nutrient-poor Gulf waters, whereas an exclusive nitrogen-fixing non-heterocystous flora dominates the surface of sediments. Increased availability of nutrients may in part explain some of the observed zonation patterns on pneumatophores but it is clear that the dominant controlling factors are desiccation, salinity and wetting frequency. In pools, distinct horizontal zonation occurred along gradients of salinity and water depth. Where conditions were most extreme, epiliths were absent and blue-green algae occurred only as a chasmolith layer in the sediment. With increased wetting, thicker mats appear, dominated by filamentous forms. In similar environments of the Laguna Mormona, a closed lagoon on the Baja California (Mexico), the morphology of *Lyngbya aestuarii* mats is determined by overall filament abundance, flooding-history and water depth (Horodyski, 1977). Certain of the structures found here were compared to '*Conophyton*', a fossil stromatolite. These are very similar to the thick mats of *L. aestuarii* found in the Ras Muhammad Pool. In the mangroves of the Shura el

TABLE 7. Numbers of species of blue-green algae found in different environments. h = presence or absence of heterocysts, n = total number

Environment	Total			Exclusive		
	n	−h	+h	n	−h	+h
Intertidal zone	55	39	16	42	26	16
Solar Lake	24	24	—	9	9	—
Fish pond	18	18	—	9	9	—
Ras Muhammad Pool	13	13	—	2	2	—
Sabkha area at Nabq	6	6	—	—	—	—
Intertidal zone and coastal pools	84	68	16	13	13	—
Coastal pools	41	41	—	29	29	—

Manqata, overall gross morphology of *L. aestuarii* mats depends on position in the intertidal, ranging from buckled crusts when wetted intermittantly, to thick leathery mats close to the mangrove lagoon.

A range of surface structures are produced in pools, many of which are close to one another and formed by communities which are otherwise identical in terms of dominant species and species composition. A range of strikingly similar globular stromatolites have been described from littoral environments of the Bahamas and Netherlands Antilles (Golubić & Focke, 1978). All are formed by *Phormidium hendersonii* Howe and the authors argue persuasively for a specific control over the form of the stromatolite by the species of blue-green alga. Similar globular stromatolites are found in the Sinai mangroves, these are formed by a much wider species of *Lyngbya*. It seems here that oncolitic structures can form without any overriding influence from a species of alga. This is suggested from the large cartilaginous balls of dead sheath material found in the mangrove thickets. Dor (1975) describes these as a separate genus *Cyanohydnum* although they bear little resemblance to the figures and description given by Copeland (1936) for material found in Yellowstone National Park.

The striking stratification patterns observed in many mat communities indicate extreme vertical gradients in environmental parameters such as pH, Eh, wetting-frequency, light intensity and quality, and evaporation and precipitation. Preliminary studies of mats in coastal pools indicate that values of light intensity drop from 3000 μE m^{-2} sec^{-1} at the surface, to less than 10 μE m^{-2} sec^{-1} below the first layer of phototrophic bacteria (Krumbein & Potts, 1978). Maximum penetration in these mats varies between 1·44 and 40 mm. If the photosynthetic pigments are removed with organic solvents, light penetration increases one hundred to a thousand fold. The widespread occurrence of orange pigmented surface layers in intertidal mats, suggests a screening function (Conover, 1962; Golubić, 1973b). Values of light intensity at the surface of the mats are no doubt suboptimal as seen by the vivid green layer of active cells below the surface. It is perhaps not surprising that this layer is always characterized by filamentous forms capable of gliding motility in response to different light regimes. A study of several of the figures of mats (Figs 21–23) indicates that some forms may move up and down through dead layers, using these as 'screens'. It is harder to explain the chasmolithic layer of blue-green algae in beachrock, sometimes at a depth of 10 mm and overlaid by thick mats. Either these forms are capable of photosynthesis at extremely low light levels, or they are heterotrophs.

Another feature common to thicker mats of the mangroves and coastal pools was the obvious lower layers of phototrophic bacteria and bottom layers of sulphide. It was suggested previously that several non-heterocystous forms found in marine mats were capable of using anoxygenic photosynthesis to support nitrogen fixation (Potts, 1979a; Potts *et al.*, 1978). Non-heterocystous forms in the Solar Lake are known to fix CO_2 through either oxygenic or anoxygenic photosynthesis (Cohen *et al.*, 1977b). Filamentous forms in thick mats from coastal pools and intertidal environments may be at an advantage over non-filamentous coccoid forms in being able to respond to diurnal fluctuations in the vertical distribution of O_2 and H_2S, by carrying out either oxygenic or anoxygenic photosynthesis. Sulphide will therefore be another factor influencing the distribution of forms throughout the mats. The importance of sulphide in determining the zonation and stratification of photosynthetic microbial communities on a sandy beach in the lagoon of Aldabra Atoll has been demonstrated recently (Potts & Whitton, 1979).

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Effects of Water Stress on Cryptoendolithic Cyanobacteria from Hot Desert Rocks

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Abstract. Four strains of *Chroococcidiopsis* and one *Chroococcus*, all isolated from extreme arid desert rocks, and one marine *Chroococcus*, were subjected to water stress using both matric and osmotic control methods. For all *Chroococcidiopsis* strains, photosynthetic rates decreased with decreasing water potential. After 24 h preincubation the decrease was linear but after 72 h there was a sharp drop below -3400 kPa ($a_w \triangleq 0.976$). In contrast, the two *Chroococcus* strains showed optimum photosynthesis between -3000 and -4000 KPa. It appears, therefore, that *Chroococcidiopsis* in deserts may have a different survival strategy in response to aridity than *Chroococcus* (rare in deserts).

Absolute rates of $^{14}CO_2$ uptake were higher in matric than in osmotic control systems. It is suggested that, in a matric experimental system, the water status is more representative of the natural conditions in arid environments.

The consistent differences between different strains in their response to water stress suggest that this character in Cyanobacteria may be of taxonomic significance.

Key words: Cyanobacteria – *Chroococcidiopsis* – *Chroococcus* – Water stress – Photosynthesis – Endolithic – Matric – Osmotic – Taxonomy

Cyanobacteria occur in a wide variety of environments that include freshwater, highly saline and extreme dry habitats. In the latter, probably exemplified best by hot and cold deserts, rock-inhabiting forms exist in a non-aquatic environment where liquid water is only seldom present (Friedmann 1971; Friedmann et al. 1967). However, drought-tolerance is not limited to these desert forms and many Cyanobacteria are resistant to desiccation, for example *Nostoc commune* (Bewley 1979). Yet there are few data on water relations in Cyanobacteria. Most of these concern the effects of water stress on the nitrogenase activity of free-living forms (Jones 1977; Stewart 1974; Whitton et al. 1979) and the formation and germination of akinetes (Braune and Sanke 1979), responses to different osmotic concentrations (Batterton and Van Baalen 1971; Nordin and Stein 1980; Stam and Holleman 1975; Tel-Or 1980; Yopp et al. 1978), and the control of intracellular osmotic pressure (Allison and Walsby 1981; Borowitzka 1980).

This paper deals with the effects of water stress on Cyanobacteria isolated from rocks in hot deserts. Presumably, these organisms are adapted for survival under conditions of acute water stress. A marine strain, morphologically indistinguishable from one of the desert strains was included in the study for comparison.

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Material and Methods

Organisms. Axenic strains of desert Cyanobacteria used in this study were obtained from Dr. R. Ocampo-Friedmann. They form part of the Culture Collection of Microorganisms from Extreme Environments maintained currently in the Department of Biological Science, Florida State University, Tallahassee. Four strains are species of *Chroococcidiopsis* (or a related genus; the taxonomy of this group is uncertain), and two strains agree, in their morphological characteristics, with the diagnosis for *Chroococcus turgidus* (Kütz.) Näg. Desert strains were isolated from cryptoendolithic (inside rocks) or hypolithic (under stones) habitats (Table 1). They have been described and illustrated by Ocampo (1973). Descriptions of the lithophytic desert microbial habitats are given by Friedmann (1971, 1980; Friedmann and Galun 1973).

Cultures were grown photoautotrophically at 34° C. Liquid BG 11, a "freshwater" medium (Rippka et al. 1979), was used for *Chroococcidiopsis*, and MN, a sea water modification of BG 11 (Stanier & Cohen-Bazire 1977) for *Chroococcus*.

Control of Water Potential

Theory. Water potential (ψ) is the difference in free energy between the system under study and a "pool" of pure water at the same temperature.

$$\psi = 1065 \cdot T \cdot \log p/p_0$$

where T = temperature (°K), p = vapor pressure of the solution, p_0 = vapor pressure of pure water at the same temperature (Griffin and Luard 1979); p/p_0 = water activity (a_w) and corresponds to relative humidity/100 (Table 2).

Values of ψ are expressed in kilopascals (negative values):

100 kPa = 1 bar = 10^6 dyne cm^{-2}.

The water potential of a system may be controlled either through the direct addition of solutes to the medium, e.g. NaCl (osmotic control), or by equilibration above a solution of defined water potential (isopiestic or matric control; Harris et al. 1970). Data on water potentials of solutions of NaCl can be found in a paper by Lang (1967) and in the CRC Handbook of Chemistry and Physics (59th Edition).

Matric Control System. Sodium chloride was added to liquid BG 11 in different molal concentrations (Table 2). The concentrations correspond approximately to the following water

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Table 1. Strains of Cyanobacteria used in the study

Strain no.	Name	Origin[a]
N6904	*Chroococcidiopsis* sp.	cryptoendolithic; sandstone
N6904N	*Chroococcidiopsis* sp.	cryptoendolithic; sandstone
N6909A_1	*Chroococcidiopsis* sp.	hypolithic; flint pebbles
N6911A_6	*Chroococcidiopsis* sp.	hypolithic; limestone pebbles
N41	*Chroococcus turgidus* (Kütz.) Näg.	cryptoendolithic; sandstone
S24	*Chroococcus turgidus* (Kütz.) Näg.	intertidal rocks; Sinai Peninsula

[a] All in Negev Desert, Israel, except *Chroococcus* S24

Table 2. The water potentials of different molal concentrations of NaCl

Molality of NaCl (in BG11)$_{34\ C}$	$a_w (p/p_0)$[a]	ψ(kPa)
0 (BG11)	0.9994	−90
0.2	0.9928	− 1.033
0.4	0.9862	− 1.970
0.6	0.9796	− 2.924
0.7	0.9763	− 3.407
0.8	0.9729	− 3.896
1.0	0.9662	− 4.889
1.5	0.9487	− 7.477
2.0	0.9312	−10.217
4.0	0.8498	−23.103

[a] a_w = water activity = relative humidity 100
Note: $35^0{}_{00}$ seawater ≙ −2733 kPa at 34 C
MN medium ≙ −2230 kPa at 34 C

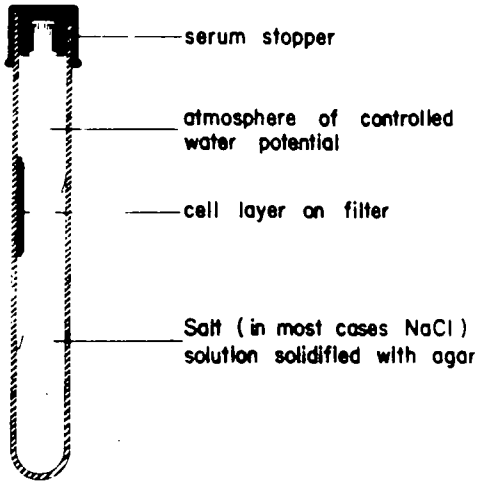

Fig. 1. The matric control system. A filter, supporting a film of cells, was positioned in the glass tube and curled around its inner surface in close proximity to the agar

potentials: −90, −1000, −2000, −3000, −3500, −4000, −5000, −7500, −10,000 and −23,000 kPa. These solutions were supplemented with 2% agar (Harris et al. 1970). Four ml aliquots of the agar were introduced into nine ml capacity culture tubes and the tubes sealed with rubber serum stoppers.

Cultures in the logarithmic phase of growth were filtered (usually two to three ml) through 25 mm membrane filters (pore size 0.45 µm), to yield a layer of cells containing *c* 30 µg total protein. The filters were then placed in incubation tubes and equilibrated at 34 C (Fig. 1).

Osmotic Control System. A 500 ml culture in the logarithmic phase of growth was concentrated by low-speed centrifugation and the pellet resuspended in 10 ml fresh medium. One milliliter aliquots of the cell suspension were inoculated into a range of molal concentrations of NaCl in liquid BG11 (see Table 2) to give a final cell density approximately equal to that of the original culture. After pre-incubation (24 or 72 h), 4 ml aliquots were introduced to 9 ml culture tubes and the tubes then sealed with rubber serum stoppers.

Generation of $^{14}CO_2$. The method of Belly and Brock (1974) and Brock (1975a, b) was used. Two milliliter $NaH^{14}CO_3$ solution (19.2 µCi ml^{-1}; specific activity 6.2 µCi µM^{-1}; New England Nuclear, Boston, MA) were introduced into a 60 ml glass serum bottle. The bottle was sealed with a rubber serum stopper coated with high vacuum silicone grease. A 2 ml volume of air was removed from the bottle with a gas-tight syringe and replaced with 2 ml 5 M H_2SO_4. After the bottle had stood for 30 min at 34 C, the activity of the generated gas was measured by removing 200 µl aliquots and injecting these into 10 ml scintillation fluid containing 1 ml 97% phenethylamine (to trap CO_2). In most cases, the activity was 150 – 200 × 10^3 cpm 200 µl^{-1}.

Assays for $^{14}CO_2$ Uptake. 200 µl aliquots of the air/$^{14}CO_2$ mixture were injected into incubation tubes containing either filters (matric assays) or cell suspensions (osmotic assays). Incubation was at 34 C at a photon flux density of 62 µE m^{-2} s^{-1} for either 60 min (most experiments) or 24 h (see Fig. 1f). Experiments were terminated by injecting either 0.3 ml 8% formaldehyde into cell suspensions, or 1 ml 8% formaldehyde over the surface of a filter – after a period of 2 min it was possible to remove all the cells from a filter by gentle pumping and agitation with a syringe.

Measurement of $^{14}CO_2$ Uptake. Fixed cell suspensions were filtered through 0.45 µm membrane filters and kept over HCl overnight in a desiccator. The filters were then added to 10 ml scintillation cocktail made up of: 500 ml Triton X-100, 1000 ml toluene, 8.25 g l^{-1} PPO and 0.25 g l^{-1} Bis-MSB. Radioactivity was measured in a Beckmann LS8000 Series Liquid Scintillation System.

Protein Determination. Cell protein was extracted and estimated by the methods of Kochert (1978a, b), using Bovine Serum Albumin as a standard (American Monitor Corporation, Indianapolis, IN).

Results

Matric Control of ψ

Chroococcidiopsis Strains (Figs. 2a, b)

Photosynthetic efficiency decreased with decreasing water potential. The pattern of inhibition was generally the same for

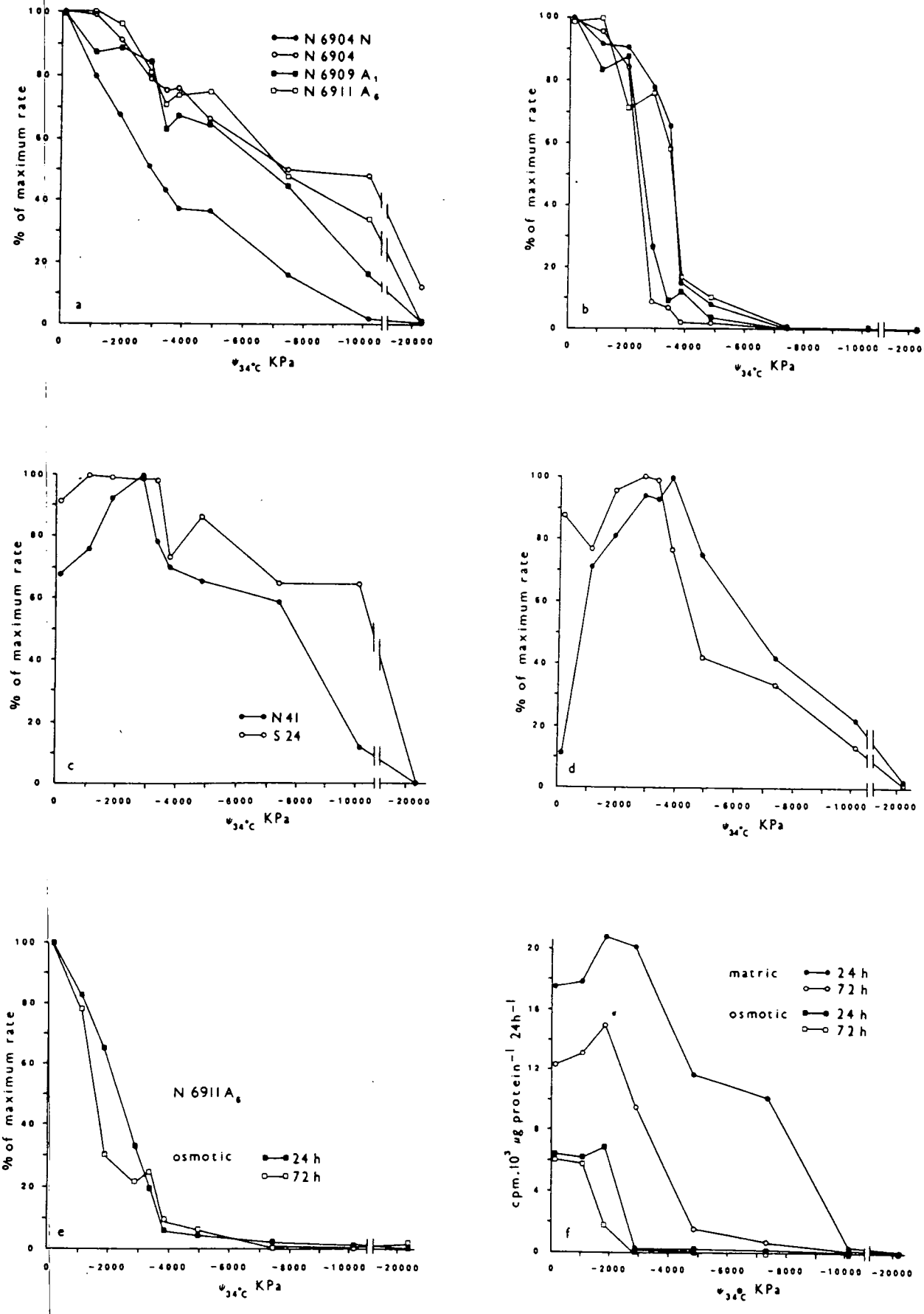

Fig. 2a—f. Rates of $^{14}CO_2$ uptake by different strains of Cyanobacteria at different water potentials. **a—e** Expressed as % of maximum rate. **f** as absolute rates. **a** and **b** matric system; four strains of *Chroococcidiopsis*, 60 min assay, pre-incubation **a** = 24 h, **b** = 72 h. **c** and **d** matric system; *Chroococcus* N 41 and S 24, 60 min assay, pre-incubation **c** = 24 h, **d** = 72 h. **e** osmotic system; *Chroococcidiopsis* N 6911 A_6, 60 min assay, 24 and 72 h pre-incubation. **f** osmotic and matric systems; *Chroococcidiopsis* N 6911 A_6, 24 h assay, 24 and 72 h pre-incubation

each strain. After 24 h pre-incubation the decrease was approximately linear (Fig. 2a). Below −20,000 kPa only N6904 showed an appreciable uptake of CO_2. After 72 h pre-incubation, there was a sharp decrease in CO_2 uptake between −2000 and −4000 kPa, and uptake was negligible at −7500 kPa and below (Fig. 2b). In all cases, however, the rates of CO_2 uptake at these low water potentials were higher than controls (cells killed with formaldehyde). Longer pre-incubation resulted in lower absolute rates of CO_2 uptake. Thus, after 24 h pre-incubation, the maximum rates of $^{14}CO_2$ uptake for strains N6904N, N6904, N6909A_1 and N6911A_6 were 70.4, 53.9, 77.6 and 55.3 cpm μg protein^{-1} min^{-1} respectively, while after 72 h the corresponding rates were 50.4, 47.2, 23.5 and 42.5 cpm μg protein^{-1} min^{-1}.

Chroococcus "turgidus" Strains[1] (Figs. 2c, d)

The two *Chroococcus* strains do not grow in freshwater media (Ocampo-Friedmann, pers. comm.). In both strains maximal uptake of CO_2 occurred around −3000 to −4000 kPa (near the water potential of seawater). This effect was much more evident after 72 h pre-incubation. In *Chroococcus* N41 CO_2 uptake was inhibited strongly at −90 kPa (freshwater) and reached a maximum at −4000 kPa. S24 was somewhat less inhibited at −90 kPa with a peak in photosynthesis at −3000 kPa. In both strains the decrease in the rate of photosynthesis with decreasing water potential was more or less linear even after 72 h pre-incubation.

Osmotic Control of ψ (Figs. 2e, f)

Experiments using osmotic control were carried out with *Chroococcidiopsis* N6911A_6. The pattern of CO_2 uptake after both 24 h and 72 h pre-incubation (Fig. 2e) was similar to that found using matric control after 72 h pre-incubation (Fig. 2b). Absolute rates of $^{14}CO_2$ uptake were significantly higher in matric assays than in osmotic assays (Fig. 2f). In this series of experiments, assay time was increased from 60 min to 24 h and CO_2 uptake reached a maximum at −2000 kPa under both matric and osmotic conditions.

At low water potentials, CO_2 uptake was generally greater under matric conditions than under osmotic conditions. This was evident from both 60 min and 24 h assays (see Figs. 2a, 2e, 2f).

The Color of Cyanobacteria — Observations and Comments

The color of the Cyanobacteria used in experiments changed at different water potentials and these changes were persistent for cells both in suspension (osmotic system) and on supporting filters (matric system). In liquid cultures (−90 kPa), as well as at potentials down to −1000 kPa, the cells appeared dark blue-green, between −1000 kPa and −4000 kPa the cells were olive-green, and below −4000 kPa they appeared bright blue-green. Changes in absorbances (at 665 nm, 440 nm and 410 nm) of crude methanol extracts of cell suspensions of *Chroococcidiopsis* N9611A_6 grown for 10 days at different water potentials indicate these color changes may be associated with different pigment concentrations. The absorbances at 665 nm and 440 nm correspond to chlorophyll *a*; the absorbance at 410 nm corresponds to phaeophytin *a*. Compared with the control at −90 kPa (BG11), cells grown at −1000 kPa and −3000 kPa showed a pronounced drop in absorbance at all three wavelengths. Cells kept at −10.000 kPa showed only a slight decrease in absorbance at 440 nm and 410 nm, and no decrease at 665 nm. The ratio A_{440} A_{410} was similar for all extracts (1.1 to 1.2) while the ratio A_{665}/A_{440} was similar for cells grown at −90 kPa, −1000 kPa or −3000 kPa (0.27 to 0.29) and significantly higher for −10.000 kPa (0.54).

Discussion

Chroococcidiopsis (and probably related genera) is dominant among the extreme xerophytic Cyanobacteria which inhabit rocks in deserts (Friedmann 1980). Representatives of this group have been isolated from rocks in more than 170 different localities — comprising several hot desert regions of the world (Ocampo 1973, and unpublished data). It is significant, therefore, that the *Chroococcidiopsis* strains in our experiments were not particularly resistant to low water potentials. *Microcoleus* sp., a dominant cyanobacterium in desert soil crusts, is, according to Brock (1975a), even more sensitive than the strains tested here. Although these two genera do not seem especially adapted for growth and photosynthesis at low water potentials, they show a considerable ability to tolerate severe drought conditions. Further, desiccated cells of *Chroococcidiopsis* (dried over fused KOH) resume photosynthesis within five minutes of rewetting (unpublished data). This adaptation to desert conditions through an extreme resistance to drying and rapid activation of photosynthesis is similar to that shown by desert lichens (see Kappen 1973; Kappen et al. 1980; Smith 1979). Such a rapid activation of metabolic activity may be compared with the activation of nitrogenase activity in rewetted cells of certain heterocystous Cyanobacteria (Paul et al. 1971; Potts 1979; Stewart et al. 1978).

Chroococcus N41 does not grow at low solute concentrations in fresh-water media and, in its response to water stress, is similar to *Chroococcus* S24, a marine strain. Optimum photosynthetic efficiency of both *Chroococcus* strains occurred near the water potential at which the *Chroococcidiopsis* strains were inhibited completely. For *Chroococcus* N41, it is possible that the adaptation to desert conditions is the ability to photosynthesize at rather lower water potentials (quite different from the situation with *Chroococcidiopsis*). Lange (1969) has demonstrated that lichens in hot deserts are able to utilize water vapor and photosynthesize at a relative humidity of 80% (−30 MPa), a value considerably lower than the minimum necessary to support photosynthesis by the Cyanobacteria investigated in this study. Responses of microorganisms to water stress may thus be radically different.

In an extreme xeric desert environment, the availability of water may be infrequent. The possibility exists, therefore, that growth in liquid (or even agar) culture selects against those strains adapted to non-aquatic conditions. While this eventuality cannot be dismissed completely, it is considered unlikely. Were such adaptations to aridity prevalent, at least some forms would survive in water and be able to photosynthesize at lower water potentials. Furthermore, the experiments of Brock (1975a), with *Microcoleus* mats collected from nature, showed no evidence of such adaptations in Cyanobacteria.

1 Geitler (1932) concluded that *Chroococcus turgidus* was polymorphic and regarded it as "eine Sammelspezies," i.e. *C. "turgidus"* includes a number of forms which may or may not belong to different species

The significant and consistent differences in response to water stress suggest that this may be a physiological property of Cyanobacteria, characteristic of different strains – it may prove to be of taxonomic value in this group.

A comparison of the results in matric and osmotic control experiments shows that the pattern of sensitivity is generally similar in both systems. Differences were apparent when the absolute rates of $^{14}CO_2$ uptake were compared. Possible causes of lower rates in osmotic systems include the toxic effects of some ions in high concentrations, interference when organisms may have a specific requirement for a particular ion (e.g. Na^+), the possibility of fermentation when sugars are used (e.g. sucrose), retardation of gas exchange in concentrated solutions, and the inevitable dilution and "lag" effects following the inoculation of the culture. The higher values obtained under matric conditions may also be due to a more rapid uptake of $^{14}CO_2$ by the film of cells with a large surface area and a greater dilution of the radioisotope in the osmotic system.

For microorganisms that live in a rock or soil, a matric system is probably more representative than an osmotic system of the environmental conditions prevailing in nature.

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Control of matric water potential (Ψ_m) in immobilised cultures of cyanobacteria

(Water stress; nitrogenase; *Nostoc*; downshift; ATP)

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1. SUMMARY

Three simple methods are described that permit cells of cyanobacteria, immobilised on filter supports, to be subjected to matric water stress that leads to a downshift in nitrogenase activity. In *Nostoc commune*, a desiccation-tolerant form, nitrogenase activity is more sensitive to water stress than the intracellular ATP pool. When it is dried rapidly to −99.5 MPa, nitrogenase activity ceases within 30 min while the ATP pool is maintained at 16.07 pmol ATP · μg protein^{-1}. During short-term incubation, decreasing Ψ_m from −0.10 to −23.1 MPa may result in an increased rate of CO_2 uptake.

2. INTRODUCTION

The water potential of a system may be controlled either through the direct addition of solutes, e.g., NaCl, to the medium (osmotic control) or by equilibration above a solution of defined water potential (isopiestic or matric control). Studies on the water relations of cyanobacteria have focussed, almost exclusively, on osmotic systems [1]. Drawbacks to the use of these systems include the retardation of gas exchange in concentrated solutions, the toxicity of certain ions, interference if the strain under investigation has a specific requirement for an ion, and the possibility of fermentation if sugars, for example sucrose, are used as the osmolyte.

Matric water potential $(\Psi_m) = 1065 \cdot T \cdot \log p/p_0$, where T = temperature (K), p = vapor pressure of the solution, p_0 = vapor pressure of pure water at the same temperature; p/p_0 = water activity (a_w) and corresponds to relative humidity/100. Values of Ψ_m are expressed in MPa; 0.1 MPa = 1 bar = 10^6 dyne · cm^{-2}. Previous studies have demonstrated the use of matric systems in the study of desiccation-tolerant cyanobacteria [2,3]. This paper describes the utility of three matric methods developed for the study of water-induced upshifts and downshifts in the N_2-fixing cyanobacterium *Nostoc commune*.

3. MATERIALS AND METHODS

3.1. Cyanobacteria

Axenic cultures of *N. commune* UTEX 584 were grown in Fernbach flasks containing 1 l MB_0 medium (a modified form of BG-11 with elevated concentrations of cations, see [4]). Cultures were incubated without forced aeration on a rotary

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shaker (100 rpm), at 25 °C with an incident photon flux density of approx. 35 μmol photons $\cdot m^{-2} \cdot s^{-1}$.

3.2. Immobilisation of cells

Cells were harvested by filtration and collected on 1 of 3 different inert supports: 25-mm Millipore filters (pore size, 0.45 μm); 25-mm cotton gauze filters (four layers, approx. 2 mm thick, approximate pore size 0.25–1 mm^2); 25-mm nylon fabric (0.13 mm thick, pore size 0.04 mm^2). Supports were used immediately after filtration or placed on a layer of dry cotton gauze or filter paper (for 10 s) to remove excess liquid.

3.3. Regulation of Ψ_m; desiccation of cells

Some features of the first system in its unmodified form have been described briefly [2]. Aliquots (9 ml) of molten agar containing a particular concentration of sodium chloride were dispensed into 22-ml culture tubes to form slants. A single support (with immobilised cells) was positioned in each tube and curled around its inner surface in close proximity to the slant. The tube was sealed with a septum and the cells were allowed to equilibrate under the desired incubation conditions. In certain experiments, the gas phase was replaced at regular intervals with two vols of air of the same Ψ_m.

In the second system the supports were placed in a glass tube (3 × 15 cm) with tapered ends. Tygon tubing (type r-3603) was used to connect the chamber, in a closed circuit, with a peristaltic pump (Varistaltic junior model, Manostat, USA) and a 1-l glass desiccator containing approx. 200 ml of a sodium chloride solution of the desired concentration. The air was circulated through the chamber at approx. 1 $l \cdot min^{-1}$, and its water potential was measured continuously via a psychrometer probe (HMP 21 U, Vaisala, MA) inserted directly into the chamber through an airtight port. The complete system was maintained at the same constant light and temperature.

In the third system, rapid desiccation of cells was achieved by placement of the supports, immediately after filtration, on cotton gauze and then on a glass plate in a constant light and temperature incubator (Ψ_m maintained at approx. −95.5 MPa; 0.5 a_w).

3.4. Nitrogenase activity

Supports were removed from the various systems at different times during equilibration and assayed for nitrogenase activity using the acetylene reduction assay technique [5]. In the tube system, some assays were performed directly in situ.

3.5. Intracellular ATP pool

Intracellular ATP was measured using the luciferin-luciferase assay [6].

3.6. Chlorophyll a

Chlorophyll *a* was extracted from cells in 95% methanol at 4 °C in the dark. Absorbance was read at A_{665} and chlorophyll *a* was estimated using an absorption coefficient of 78.74 $g^{-1} \cdot cm^{-1}$ [7].

3.7. Total protein

Total protein was extracted and measured using the methods of Kochert [8,9].

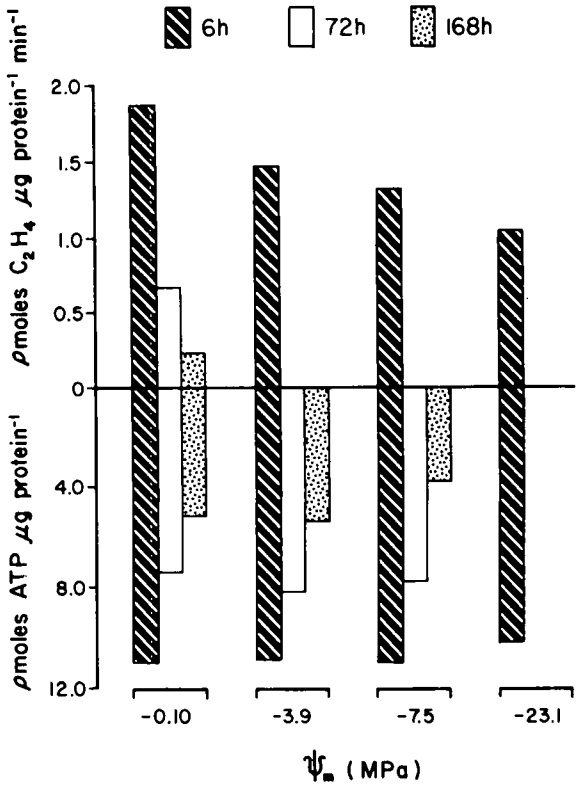

Fig. 1. Effect of water stress on nitrogenase activity and the intracellular ATP pool in cells incubated in the tube system. Cells were incubated for 6, 72 or 168 h.

4. RESULTS AND DISCUSSION

In *N. commune*, nitrogenase activity is restricted to a narrow range of water potential and is more sensitive to matric water stress than the intracellular ATP pool (Fig. 1). The methods provide a means for subjecting cells of cyanobacteria to different rates of drying and varying degrees of matric water stress (Figs. 1, 3; Table 1). Depending on the method used, cells may be subjected to either slow (days), fast (h), or rapid (min) equilibration.

Nitrogenase activity and levels of intracellular ATP both fell with decreasing water potential and with increased incubation time at a given water potential (Fig. 1). This was true even at −0.10 MPa, equivalent in terms of water potential to that of a liquid culture. Stimulation of nitrogenase activity by flushing with air suggests that CO_2 limitation and/or increased O_2 levels from photosynthetic activity may be significant constraints during long-term equilibration in the tube system (Fig. 2). More significant, however, is the marked inverse correlation between decreasing water potential and the stimulation of nitrogenase activity in response to flushing. This correlation suggests that one response of *N. commune* to short-term water stress may be an increased rate of CO_2 uptake. This increased rate may be a consequence of a need to synthesize some osmolyte to regulate intracellular water potential. An accumulation of polyglucosyl granules has been observed in unicellular cyanobacteria exposed to acute water stress [3]. The slight depression in ATP levels with flushing at lower water potentials reflects, presumably, increased diversion of ATP to nitrogen fixation and other ATP-consuming processes.

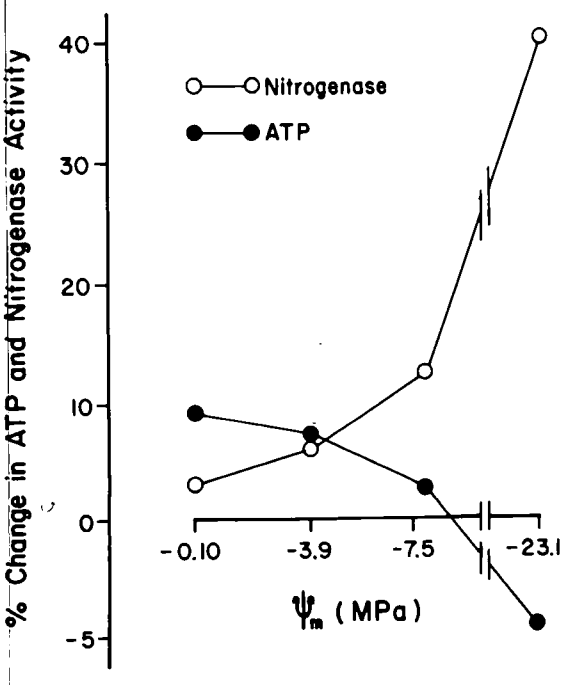

Fig. 2. Response of nitrogenase activity and intracellular ATP pools to flushing. Cells were incubated in tubes at the various water potentials for 6 h, and the gas phase was replaced every 2 h. The ATP pools of cells in liquid culture, immediately after filtration, and at −0.10 MPa after 6 h (flushed; maximum value) were 11.04, 10.97, and 11.71 pmol ATP·μg protein^{-1}, respectively. The equivalent rates of acetylene reduction were 1.39, 1.77, and 1.92 pmol C_2H_4·μg protein^{-1}·min^{-1}, respectively.

At −23.1 MPa in the chamber system, equilibration is more rapid and nitrogenase activity is inhibited completely within 3–5 h (Table 1). One major disadvantage of this system is that only a limited number of supports may be processed simultaneously at one particular water potential, and observed rates of nitrogenase activity were more variable than in the other two systems.

When blotted dry, cells cease nitrogenase activity with 30 min at −99.5 MPa (Fig. 3). The intracellular ATP pool remains constant during the first 9 h but is undetectable in cells after overnight drying.

The size of the extractable ATP pool in liquid cultures of *N. commune* was 16.07 ± 7.34 pmol ATP·μg protein^{-1}. The average protein : chlorophyll *a* ratio in the cells was 10.15 ± 3.64, so the ATP pool is comparable to the 165 ± 35 pmol

Table 1

Comparison of equilibration times required to suppress nitrogenase activity in the tube system and chamber system. Rates are expressed as pmol C_2H_4·μg protein^{-1}·min^{-1}

Incubation (h) at −23.1 MPa	Nitrogenase activity	
	Chamber	Tube
3	1.65 ± 1.99	4.70 ± 0.584
5.5	0	2.79 ± 0.280

Activity (pre-blotting) = 6.095 ± 0.527.
Activity (post-blotting) = 4.99 ± 0.516.

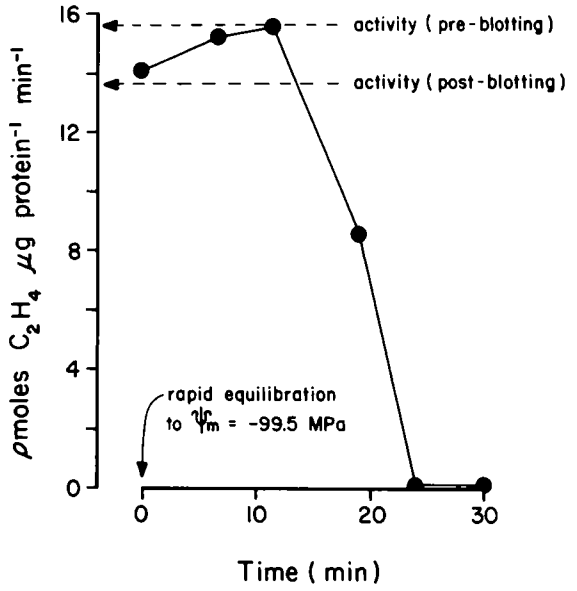

Fig. 3. Nitrogenase activity of cells dried rapidly to −99.5 MPa. Rates of acetylene reduction pre- and post-blotting are indicated by arrows. Levels of intracellular ATP remained constant during this time and for the following 6 h.

ATP · µg chlorophyll a^{-1} found for *Anabaena cylindrica* [10]. The capacity of the cells to maintain their ATP pool during short-term water stress was marked, although there was a tendency for a transient drop in the size of the pool upon filtration of cells.

Of the different supports used to immobilise cells, the Millipore filters were of least use. These filters became brittle and friable upon drying and were unsuitable for the filtration of cultures with significant amounts of mucilage. The cotton gauze and nylon filters were equally effective for all manipulations.

The methods described here provide an inexpensive and easy means for subjecting nitrogen-fixing cyanobacteria to matric water stress. *N. commune* is particularly tolerant of desiccation [11], yet nitrogenase activity is restricted to a narrow range of water potential. These data resemble those obtained for $^{14}CO_2$ uptake in other desiccation-tolerant forms [2]. It is significant that in response to osmotic (NaCl) stress, the relative sensitivities of nitrogenase and photosynthesis in nitrogen-fixing cyanobacteria resemble the findings reported here [12]. The consequences of water stress in these microorganisms may, therefore, be similar in osmotic and matric systems.

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Sensitivity of *Nostoc commune* UTEX 584 (Cyanobacteria) to water stress

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Abstract. Cells of *Nostoc commune* UTEX 584 from liquid cultures expressed an upshift in nitrogenase activity when immobilised on inert supports and exposed to matric water potentials between −0.10 and −99.5 MPa. Cells incubated at −0.10 MPa ($a_w = c\ 1.0$) maintained increased activity for at least 48 h following immobilization. At water potentials below −23.1 MPa ($a_w = 0.85$), the upshift was transitory. Nitrogenase activity decreased rapidly when immobilised cells were incubated at lower values of ψ_m.

Desiccated cells stored at −99.5 MPa ($a_w = 0.50$) underwent an upshift in nitrogenase activity, and in the size of the intracellular ATP pool, when rewetted with either distilled water or liquid MB_0 medium ($\psi_o = -0.18$ MPa). The upshift in nitrogenase activity was chloramphenicol-sensitive and was preceeded by a lag. The duration of the lag depended on the time taken to equilibrate cells to −99.5 MPa, the time desiccated, and the conditions of storage and rewetting. Cells that had no, or very low, nitrogenase activity when rewetted in air, showed a marked stimulation of nitrogenase activity in the presence of 5% v/v CO_2 under both aerobic and anerobic conditions.

When rewetted in the presence of 1% w/v glucose ($\psi_o = -0.14$ MPa), vegetative cells remained intact, but heterocysts underwent autolysis and nitrogenase activity was not detected, even in the presence of 5% v/v CO_2.

Key words: Cyanobacteria – Immobilised cells – Desiccation – Water stress – Nitrogenase – ATP pool – Photooxidation

A capacity to tolerate water stress and desiccation is a feature of many cyanobacteria of both marine and terrestrial origin (Potts and Friedmann 1981; Potts et al. 1983; Whitton et al. 1979; Whitton and Potts 1982). Studies concerned with the water relations of cyanobacteria have, however, dealt primarily with the response of these microorganisms to elevated solute concentrations (see Walsby 1982). An understanding of desiccation tolerance, and its basis at the biochemical and molecular level, is lacking.

The capacity of dried colonies of *Nostoc commune* to recover nitrogenase activity upon rewetting has been demonstrated in materials collected from a variety of field locations (Coxson and Kershaw 1983; Rodgers 1977), in some cases after desiccation periods in excess of 1 year (Scherer et al. 1984; Whitton et al. 1979).

We have developed methods to subject immobilised axenic cultures of *Nostoc commune* UTEX 584 to controlled matric water stress (Potts et al., 1984). Nitrogenase activity of this strain was found to be more sensitive to water stress than the intracellular ATP pool (Potts and Morrison, in press). When immobilised cultures were dried rapidly to a matric water potential of −99.5 MPa ($a_w = 0.50$), nitrogenase activity ceased within 30 min while the ATP pool was maintained at 16.07 pmoles ATP μg protein^{-1} for several hours.

In the present study we investigated the factors that influence the capacity of desiccated cells of this strain to recover, upon rewetting, from storage at −99.5 MPa (desiccation).

Materials and methods

Conditions for the growth of *Nostoc commune*, and three methods for the control of matric water potential (ψ_m) in immobilised cultures, are described by Potts et al. (1984). In the present study, cells in a 4-ml aliquot of a liquid culture were immobilised on a circular nylon filter support of 2-cm diameter (pore size 0.04 mm^2). These filters were superior to Millipore, cotton-gauze, and GF/C discs (Whatman) in that filament aggregates were retained efficiently yet excess liquid could be removed rapidly by blotting. In addition, desiccated material was easily removed with a sterile spatula. Immobilised cells were subjected to varying rates of equilibration to the desired water potential (Potts et al., 1984; Potts and Friedmann 1981). Cells were dried rapidly at −99.5 MPa by placement of the supports, immediately following filtration, in a constant light/temperature/relative humidity incubator.

Storage and rewetting of cells

Cells were stored (desiccated) either in continuous light (35 μmol photons m^{-2} s^{-1}), or in the dark, at −23.1 or −99.5 MPa. The gas phase during storage was either air, air/CO_2 (95/5 v/v), Ar/CO_2 (95/5 v/v) or nitrogen (100%).

Rewetting was accomplished by placing the supports on a layer of cotton-gauze saturated with sterile distilled water or MB_0 (Potts et al., 1984). Rewetted cells were incubated in continuous light, under a gas phase of either air, air/CO_2 (95/5 v/v) or Ar/CO_2 (95/5 v/v).

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Abbreviations. TTC, 2,3,5-triphenyl-2-tetrazolium chloride; ψ_m, matric water potential; ψ_o, osmotic water potential; a_w, water activity

Nitrogenase activity

The acetylene reduction assay technique was used under routine conditions unless stated otherwise (Stewart et al. 1968). Ethylene evolution was determined using acetylene as an internal standard and an ethylene (in helium) external standard. Rates of ethylene production are expressed in relation to the total protein content of the sample in question.

Chlorophyll a

Chlorophyll *a* was extracted in 95% methanol, at 4° C, in the dark. Absorbance was read at OD_{665}, and chlorophyll *a* was estimated using an absorption coefficient of 78.74 g^{-1} cm^{-1} (Meeks and Castenholz 1971).

Total protein

Total protein was extracted and measured using the methods of Kochert (1978a, b).

Intracellular ATP

The intracellular ATP pool was extracted in perchloric acid and ATP luminescence was measured using the luciferin-luciferase assay and a Packard Tri-Carb Scintillation Counter (Bottomley and Stewart 1976). Perchlorate caused quenching of ATP luminescence. For this reason, calibration curves were obtained using purified ATP standards prepared in 3 M perchloric acid, neutralized to pH 7.4 with Hepes-$MgSO_4$ buffer. ATP samples were stable in perchloric acid.

Results

Drying of immobilised cells

In aerobically-grown, liquid cultures, nitrogenase activities varied between 0.1 and 13 pmol C_2H_4 μg protein^{-1} min^{-1}. An elevated level of nitrogenase activity was detected after filtration and immobilization of cells on solid supports. This upshift was maintained for at least 48 h when immobilised cells were incubated at a matric water potential of −0.10 MPa. During this 48 h the cells maintained an intracellular ATP pool equivalent in size to that of cells growing exponentially in liquid culture (*c* 16 pmol ATP μg protein^{-1}). Upon short-term incubation at −5.63 MPa (a_w = 0.96), acetylene reduction increased, and a plot of rate versus time gave a sigmoid curve. The specific activity had more than doubled after 3 h (Table 1). Fluctuations in water potential between −5.63 and −8.5 MPa (a_w = 0.94) did not influence the increase in the rate of acetylene reduction. In contrast, acetylene reduction activity was depressed more than 70% when immobilised cells were incubated for the same time interval at −23.1 MPa (a_w = −0.85); acetylene reduction was not detectable after 5 h incubation at ψ_m = −23.1 MPa. The increase observed when immobilised cells were incubated at −99.5 MPa was transient, and acetylene reduction ceased within 20−30 min of incubation at this water potential.

Storage of desiccated cells

Cell material assumed a blue-grey color within 72 h when immobilised on nylon supports and stored at −99.5 MPa, in the light, under air. Material stored in the dark under the same conditions retained a green color during this time but eventually became blue-grey, and finally pale purple, after prolonged storage (months). A substantial loss of chlorophyll *a* accompanied the bleaching of cells immobilised for 15 days at −99.5 MPa, in the light under air (Table 2). The loss in chlorophyll *a* from the same material when it was stored in the dark under anaerobic conditions was far smaller. Bleaching was prevented under these conditions when the gas phase was 100% nitrogen.

Table 1. Increase in nitrogenase activity after immobilisation of cells and equilibration to ψ_m = −7.1 MPa (a_w = 0.95)

Incubation time[a] (min)	ψ_m (MPa)[b]	p/p_o[c]	Specific[d] activity	Comments
	− 0.18	0.99	9.80	liquid culture
0	−14.5	0.90	13.5	immobilised cells
40	− 5.6	0.96	15.5	immobilised cells
80	− 8.5	0.94	26.9	immobilised cells
100	− 7.1	0.95	30.3	immobilised cells
180	− 5.6	0.96	33.0	immobilised cells

[a] Cells grown in liquid MB_0 medium (k = 0.39 d^{-1}; 0.56 doublings per day). Immediately following filtration and immobilisation, the cells (on supports), were introduced into a chamber system (see Potts et al., 1984) through which air of −7.1 MPa was circulated

[b] $\psi_m = 1065 \cdot T \cdot \log p/p_o$ (Potts and Friedmann 1981)

[c] The water activity (p/p_o) of the atmosphere in the chamber was monitored continuously with a digital-readout psychrometer probe

[d] Nitrogenase activity (acetylene reduction) is expressed in pmol C_2H_4 produced μg protein^{-1} min^{-1}

Table 2. Factors influencing the degradation of chlorophyll *a* during storage of immobilised cells at −99.5 MPa, and upshift in nitrogenase activity and the intracellular ATP pool upon rewetting

Storage conditions[a]	% loss of chlorophyll *a*	Rewetting conditions[b]	ATP pool size[c]	nitrogenase activity[d]
Light Air/5% CO_2	93.1	Light Air/5% CO_2	2.9	0.0342
Light Ar/5% CO_2	82.7	Light Air/5% CO_2	0.70	0.0936
Dark Air/5% CO_2	48.7	Light Air/5% CO_2	40	2.28
Dark Ar/5% CO_2	33.0	Light Air/5% CO_2	70	30.7

[a] Immediately following immobilisation of cells, each filter supported 31.2 μg chlorophyll *a* filter^{-1} (5089 pmol ATP). Cells were incubated at −99.5 MPa, under the conditions specified, for 15 days

[b] Cells were rewetted with sterile distilled water for 48 h under the conditions specified, prior to assays for nitrogenase activity and extraction of the ATP pool

[c] The extractable ATP pool (pmol ATP μg chlorophyll a^{-1}) is expressed as a percentage of that extracted from cells prior to desiccation (163 pmol ATP μg chlorophyll a^{-1}). No ATP was detected in desiccated material stored for 15 days at −99.55 MPa

[d] As chlorophyll *a*, and protein (data not shown) are both degraded upon desiccation, nitrogenase activity in this case is expressed as total pmol C_2H_4 produced filter^{-1} min^{-1} after 48 h of rewetting

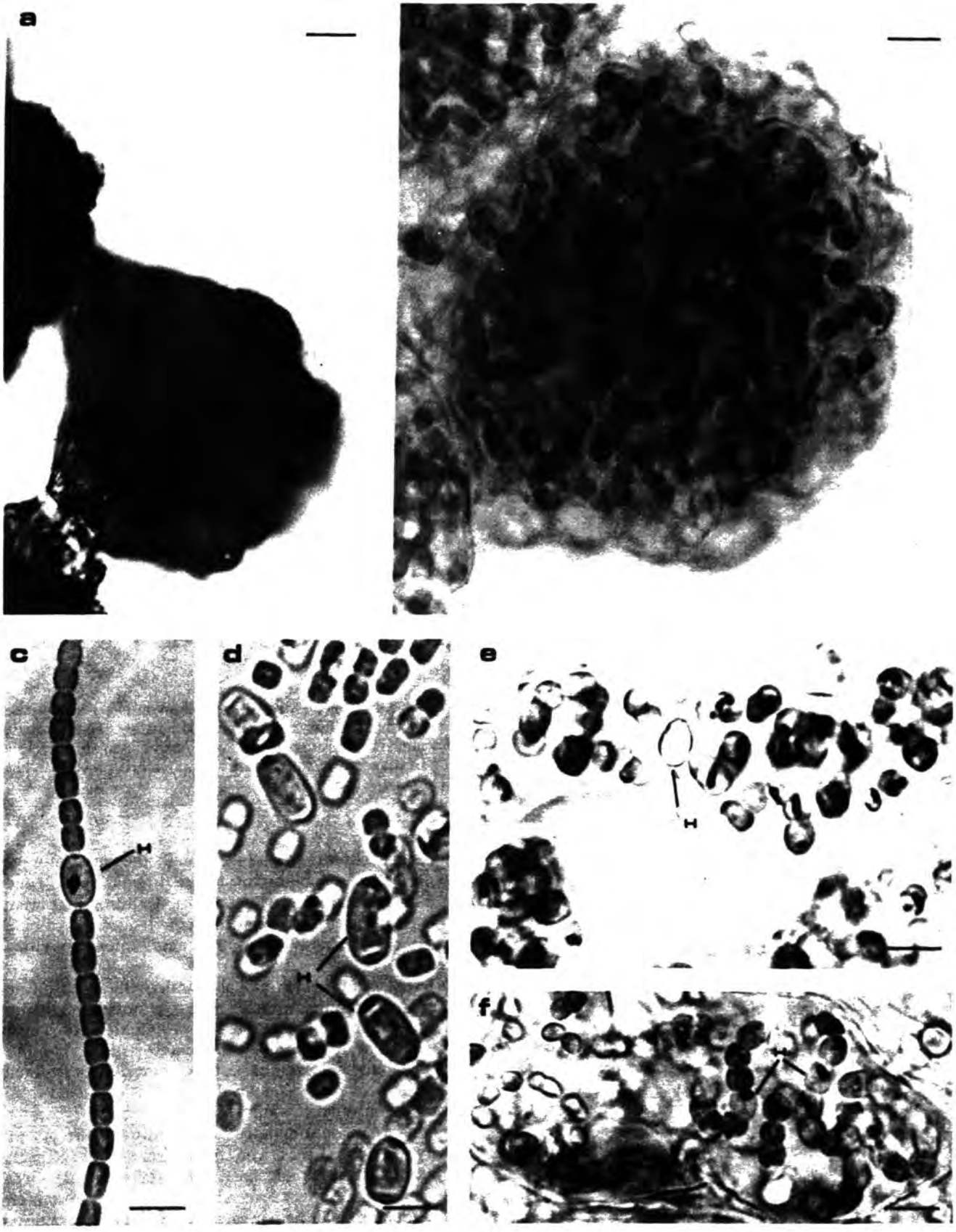


Fig. 1. **a** Desiccated aseriate cell mass dried for 15 days at −23.1 MPa. **b** Same material as **a**, rewetted with distilled water for 1 min. **c** Filaments from a liquid culture growing exponentially; stained for 15 min with 0.1% w/v TTC. Formazan deposition is associated only with heterocysts. Immobilised cells were dried at −99.5 MPa for 5 days, rewetted for 60 min and stained with TTC for 60 min: **d** Filaments (seriate stages) fragment when pressure is applied and no formazan deposition occurs. **e** Aseriate cell masses from the same material. Note lack of formazan deposition in "external" heterocysts and heavy deposition in vegetative cells. **f** Cell material dried slowly, stored at −99.5 MPa in the dark (aeorbically), for 15 weeks, rewetted for 60 min in the presence of TTC. Formazan crystals appear first in heterocysts and then in vegetative cells. Scale bar = 10 μm; *H* = heterocyst

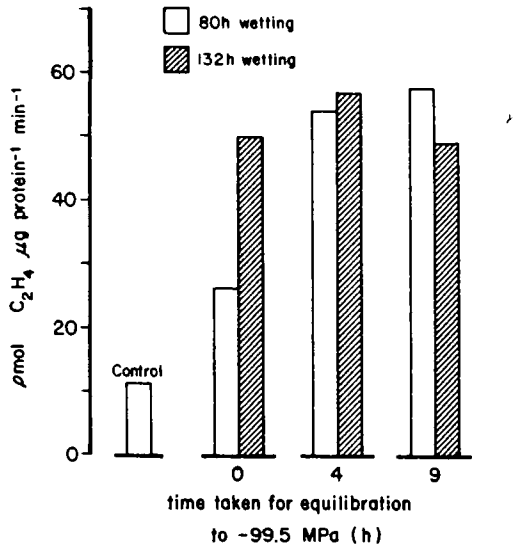


Fig. 2. Influence of initial drying time on the recovery of nitrogenase activity in rewetted material. Immobilised cells were brought to equilibrium at −99.5 MPa at different rates (0 equilibration time = rapid drying) and rewetted in air/5% CO_2

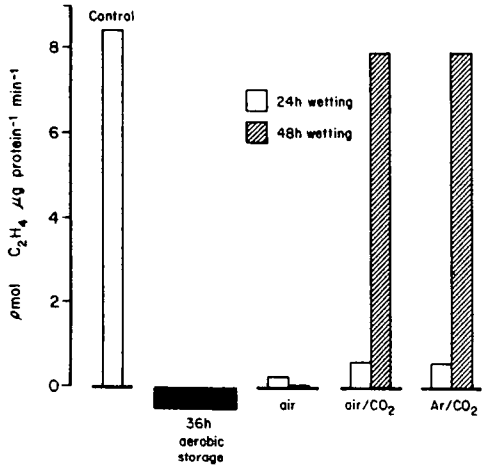
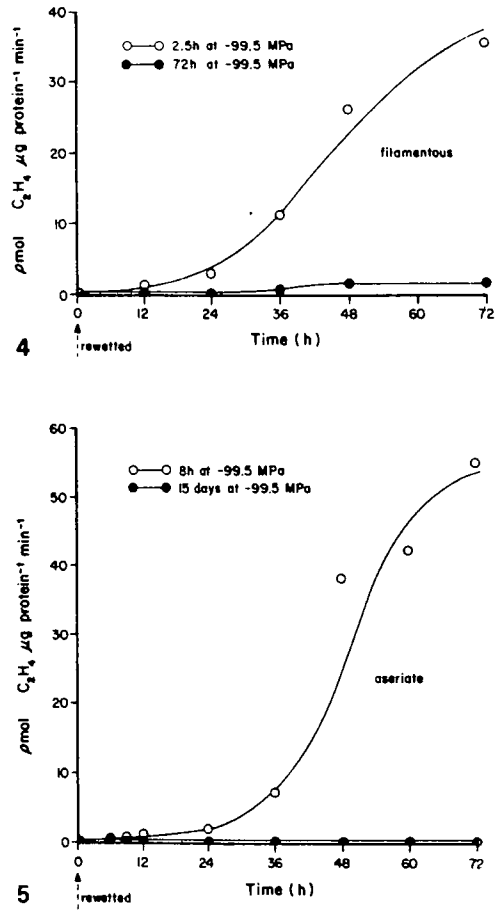


Fig. 3. Stimulation of upshift in nitrogenase by 5% CO_2. Immobilised cells were dried rapidly and stored at −99.5 MPa for 36 h before rewetting under different gas phases

Rewetting of desiccated cells

Within 15 s of rewetting, desiccated material (Fig. 1a, b) assumed a morphology that was characteristic of cells in liquid culture. Immobilised filaments of the "filamentous" morphotype (Fig. 1c) that were stored either in the light or dark, for 5 days at −99.5 MPa, and then rewetted were susceptible to fragmentation (when pressure was applied with a glass coverslip), but individual cells remained intact (Fig. 1d). In some cases, filaments stored for prolonged periods had a "bead-shaped" appearance after rewetting, with narrow, drawn-out junction connecting individual cells. Dried aseriate cell material appeared, upon rewetting, the same as that observed in liquid culture (Fig. 1b, e, f). Cell lysis was not observed upon rewetting with either sterile distilled water or MB_0.

Upon rewetting, a lag ensued before acetylene reduction was detected. The duration of the lag depended on the time



Figs. 4 and 5. Influence of dry storage time on upshift in nitrogenase upon rewetting. Cultures composed predominantly of either seriate (filamentous) or aseriate cell stages were immobilised, dried rapidly to −99.5 MPa, stored for the times indicated and rewetted in air/5% CO_2, in the light

taken to dry cells prior to storage (Fig. 2), the time of storage (Figs. 3, 4, 5), the conditions of storage (Fig. 6a; Table 2) and conditions of rewetting (Figs. 5, 6b). Cultures that were composed primarily of aseriate material tended to exhibit a more rapid recovery of nitrogenase upon rewetting (Figs. 3, 4).

The extractable ATP pool also underwent an upshift in size upon rewetting. An ATP pool was not detectable in dried cells that had been stored at −99.5 MPa for longer than 48 h. The ATP pool was detectable within 15 min of rewetting in the light, but at least 24 h wetting was required before the pool size was equivalent to that extracted from the cells prior to desiccation.

Carbon dioxide stimulated significantly the nitrogenase activity of rewetted cells. The stimulation was of the same magnitude in aerobic and anaerobic assays (Fig. 3). Cells that showed no, or very low, nitrogenase activity when rewetted for 72 h in air had an intracellular ATP pool equivalent in size to that of cells in liquid culture. Chloramphenicol prevented the upshift in nitrogenase activity when desiccated cells were rewetted.

To test whether rewetted cells could utilize an organic carbon source, desiccated material was rehydrated in the presence of 1% w/v glucose ($\psi_o = -0.14$ MPa). Cell supernatants assumed a blue-green color within 24 h

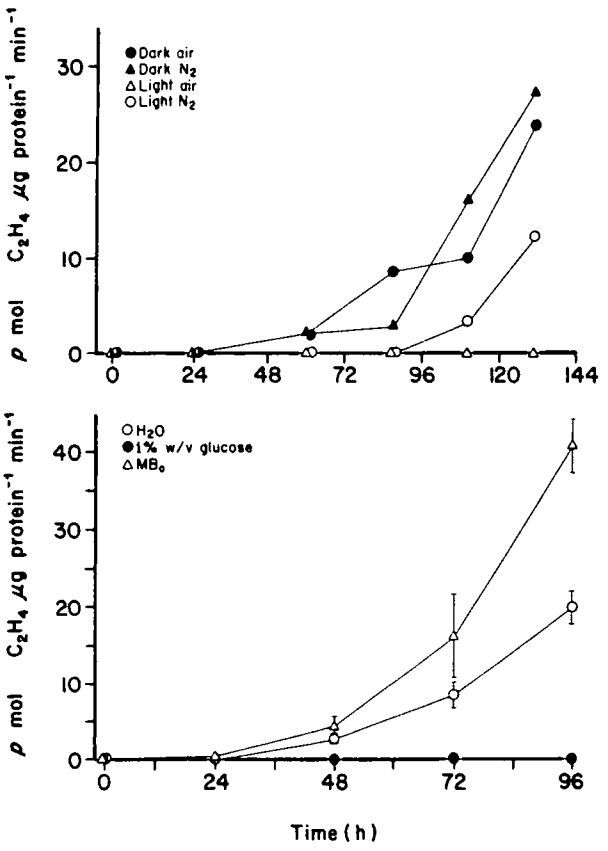


Fig. 6. a Effects of dry storage conditions on upshift in nitrogenase upon rewetting. Cells were immobilised, dried and stored at −99.5 MPa for 72 h under the conditions indicated. Cells were rewetted in air/5% CO_2, in the light. **b** Upshift in nitrogenase activity when rewetting occurs in osmotica of different water potentials. Cells were immobilised, dried rapidly and stored at −99.5 MPa before rewetting in air/5% CO_2, in the light

(absorption max. = 614 nm), and no acetylene reduction was detected (Fig. 6b). Microscopic examination revealed significant numbers of intact vegetative cells but an almost complete lack of heterocysts. In contrast, significant nitrogenase activity and a high frequency of heterocysts were observed in the same material rewetted for 24 h with either distilled water or MB_0 medium ($\psi_o = -0.18$ MPa).

The increases observed in nitrogenase activity and the size of the intracellular ATP pool during the first 48 h of rewetting cells (Table 2) were not accompanied by any detectable increase in cell numbers.

No deposition of formazan was detected in free heterocysts and vegetative cells of the "filamentous" morphotype following storage at −99.5 MPa in the light, rewetting for 6 h, fragmentation of filaments and staining with TTC (Fig. 1d). In the same material, formazan crystals were deposited predominantly in the vegetative cells, and very rarely in the heterocysts, of aseriate stages (Fig. 1e). Often, crystal deposition was diffuse and associated only with the central regions of large aseriate cell masses such as the one shown in Fig. 1b. Material (with significant amounts of sheath material) that had been subjected first to a slow rate of drying to −99.5 MPa and then to a 15 week period of storage in the dark at this water potential showed, upon rewetting, the same pattern – and kinetics, formazan crystals appeared in heterocysts within 10 min – of TTC reduction as a liquid culture in exponential growth (Fig. 1f; compare with Fig. 1c). The same results were obtained when desiccated materiall was either rehydrated in the presence of TTC or wetted for 24 h prior to the addition of TTC.

Discussion

Although a number of studies have investigated the physiological responses of dried field materials of cyanobacteria to rewetting, there have been few attempts to define systems for the study of matric water stress on axenic cultures, with a view to a more detailed biochemical investigation of the nature of desiccation tolerance (Potts et al., 1984). In the present study, cultures of *Nostoc commune* were subjected, in sequence, to water stress by drying of immobilised cells (matric stress), prolonged storage and desiccation at a low water potential (matric stress), and rewetting of desiccated cells (osmotic stress). Axenic, immobilised cells of this strain clearly show a capacity to withstand acute water stress, despite the fact that certain of the conditions imposed in this study may have been more extreme than those experienced by communities growing in situ – for example, the constant light regime, very thin layers of immobilised cells, and rapid drying during exponential growth phase. It is not surprising, therefore, that the conditions that diminished the capacity of immobilised cultures of *Nostoc commune* to recover from desiccation, i.e., to undergo an upshift in nitrogenase activity upon rewetting, included some that are known to enhance photooxidation in other cyanobacteria. In *Anacystis nidulans* L-1402-1, photooxidation in light and air leads to a sequential loss of photosystem II activity, chlorophyll *a* and photosystem I (Schmetterer et al. 1983; Schmetterer and Pescheck 1981). A renewed synthesis of chlorophyll (also seen in the present study) and growth of intracytoplasmic membranes accompanied the transfer of cells of *Anacystis* to suitable growth conditions. Under natural conditions, however, it is hard to assess the potential significance of photooxidation during the desiccation of communities that may incorporate substantial amounts of heavily, pigmented, cartilaginous sheath material (see Potts et al. 1983; Scherer et al. 1984). Optimal recovery of axenic, immobilised *Nostoc commune* did, nevertheless, occur following storage under nonphotooxidative conditions. In addition, the demonstration of a capacity for nitrogenase activity (in the presence of 5% v/v CO_2/air) in material that did not reduce acetylene in air is significant. The stimulation by CO_2 may reflect simply an increased supply of reductant or a secondary, protective effect at the time the cells may be, for one or more reasons, particularly susceptible to photooxidation.

Aseriate stages appear more tolerant of desiccation than filamentous ones, as evidenced by the greater recovery of aseriate material upon rewetting. In addition, the results of staining with TTC suggest that vegetative cells of aseriate stages have a capacity to maintain an intact electron-transport chain during prolonged desiccation in the dark. The greater resistance of aseriate stages is related, presumably, to the possession of thicker cell wall and sheath layers around cell masses (see Fig. 1). In view of the thickened envelope present in heterocysts, the apparent sensitivity of these cells when they are rewetted in 1% w/v glucose is puzzling. The water potential of this solution is, in fact, higher than that of MB_0, a medium with high concentrations of divalent cations used routinely to grow the cells in liquid culture (Potts et al., 1984). A similar sensitivity of

heterocysts of *Nostoc muscorum* to nonionic osmotica was demonstrated by Blumwald and Tel-Or (1982). Heterocysts collapsed in 0.2 M mannitol (c −0.46 MPa) while vegetative cells remained intact. The suggestion was made that vegetative cells were able to perform osmoregulation whereas heterocysts were not. The heterocysts did not collapse, however, in 0.2 M NaCl (ψ_o = −0.92 MPa), and, in our study, the increase in absorbance at 614 nm (C phycocyanin) of cell suspensions when *Nostoc commune* was rewetted in 1% w/v glucose suggests that some vegetative cells were also lysed, although the percentage appeared to be very small as judged from microscopic examination.

De novo protein synthesis must account, in part, for the lag noted upon upshift in nitrogenase upon rewetting. A similar lag in nitrogenase upshift (sensitive to chloramphenicol) has been shown for desiccated marine *Scytonema* crusts (Potts 1979) and also in frozen and rethawed *Nostoc commune* (Dubois and Kapustka 1983).

In this study, for *Nostoc commune*, a lag was noted when cells were dried rapidly and then rewetted either immediately or after prolonged storage. For cells rewetted immediately, it seems likely that the lag represents some short-term, reversible change, independent of osmolyte accumulation or photooxidation, and perhaps involving a breakdown of nitrogenase itself or other proteins involved in nitrogen fixation. Current studies on the stability of mRNA's and DNA during drying and desiccation and the identification of those proteins synthesized upon rewetting should permit a clearer appraisal of the significance of this lag and the events that precede it.

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Shifts in the intracellular ATP pools of immobilised *Nostoc* cells (Cyanobacteria) induced by water stress

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Key words Cyanobacteria Desiccation *Nostoc* Intracellular ATP Water stress

Summary Immobilised, desiccated cells of *Nostoc commune* UTEX 584 have the capacity to increase the size of their extractable intracellular ATP pool upon rewetting. The time taken to recover the pool size depends on the conditions of storage at a particular water potential and the duration of storage. Under the conditions employed, the rewetting of cells induced an increase in ATP pool size at the expense of photophosphorylation or electron transport (oxidative) phosphorylation. The rise in the ATP pool size was instantaneous and was shown to be due to ATP synthesis. This increase did not occur when cells were rewetted in the presence of sodium azide (10 mmol/l), while a partial inhibition was observed with CCCP (carbonyl cyanide *m*-chlorophenylhydrazone; 2 μmol/l). For cells dried at more extreme water potentials, the lag of *c* 48 h observed before the ATP pool reached control values is of similar duration to that observed in the recovery of nitrogenase upon rewetting. Chloramphenicol (10 μmol/l) stimulated significantly the upshift in the size of the ATP pool of *Nostoc* cells upon rewetting, yet inhibited completely the rise in nitrogenase activity.

Introduction

The water relations of cyanobacteria are poorly understood in contrast to other aspects of the physiology and biochemistry of these photoautotrophs. The majority of studies have been concerned with osmotic systems[1,2,9,14,19,20,21], while matric systems have largely been ignored[5,12,13]. As a result, an understanding of desiccation tolerance is lacking, despite the potential significance of this trait in determining the distribution and activities of nitrogen-fixing forms and in the selection of strains for use in managed agriculture.

Nostoc commune, in particular, is the dominant nitrogen-fixing form in terrestrial environments subject to repeated cycles of wetting and drying, *e.g.*, upland limestone areas of NE England and China, the Burren (Eire), Aldabra Atoll (Indian Ocean), and the coastal lowland of Antarctica (for references see reference 22 and 15). We have selected *Nostoc commune* UTEX 584 for a detailed investigation of the basis for desiccation tolerance.

Methods for subjecting immobilised axenic cultures of cyanobacteria to matric water stress have been developed[12,13] particularly for use with *Nostoc commune* UTEX 584[11]. A previous study demonstrated that the intracellular ATP pool of *Nostoc commune* was much less

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sensitive to matric water stress than nitrogenase activity[11]. In addition, the size of the intracellular ATP pool in *N. commune* was shown to be equivalent in size to that of *Anabaena variabilis*[3]. In a recent study, Scherer *et al.*[15] demonstrated that, for field material of three *Nostoc* species dried for two years, the sequence of reactivation upon rewetting was respiration, photosynthesis, then nitrogen fixation. The present study is concerned with the effects of water stress (rapid drying, desiccation, rewetting) on the extractable intracellular ATP pool of *N. commune* UTEX 584.

Materials and methods

Growth of Cyanobacteria

Nostoc commune UTEX 584 was grown in liquid MB_0 medium, at 25°C, under a photon flux density of 35 $\mu E\ m^{-2}\ s^{-1}$ [11].

Immobilization of cells

Cells from liquid cultures were immobilised on circular nylon supports of 2 cm diameter (pore size 0.04 mm^2). Immobilised cells were subjected to varying rates of equilibration to the desired water potential in a constant light/temperature/relative humidity incubator[11]. In certain experiments, the methods of Potts and Friedmann[12] were used to control water potential.

Storage and rewetting of cells

Cells were stored either in continuous light (35 $\mu E\ m^{-2}\ s^{-1}$) or in the dark, at −23.1 or −99.1 MPa. The gas phase during storage was either air, air/CO_2 (95/5 v/v), Ar/CO_2 (95/5 v/v) or nitrogen (100%).

Rewetting was accomplished by placement of the supports on a layer of cotton-gauze saturated with sterile distilled water or MB_0[11]. Rewetted cells were incubated either in the light or in the dark, in air.

Chlorophyll a

Chlorophyll *a* was extracted in 95% methanol, at 4°C, in the dark. Absorbance was measured at 665 nm, and chlorophyll *a* was estimated using an absorption coefficient of 78.74 $g^{-1}\ cm^{-1}$ [10].

Total protein

Total protein was extracted and measured using the methods of Kochert[7,8].

Intracellular ATP

The intracellular ATP pool was extracted in perchloric acid and measured using the luciferin-luciferase assay[4].

Acetylene reduction assay technique

Nitrogenase activity was measured using the acetylene reduction assay technique[18].

Results

Sensitivity of the ATP pool to matric water stress

Nitrogenase activity was more sensitive to matric water stress than was the intracellular ATP pool[11]. Filtration of exponentially growing

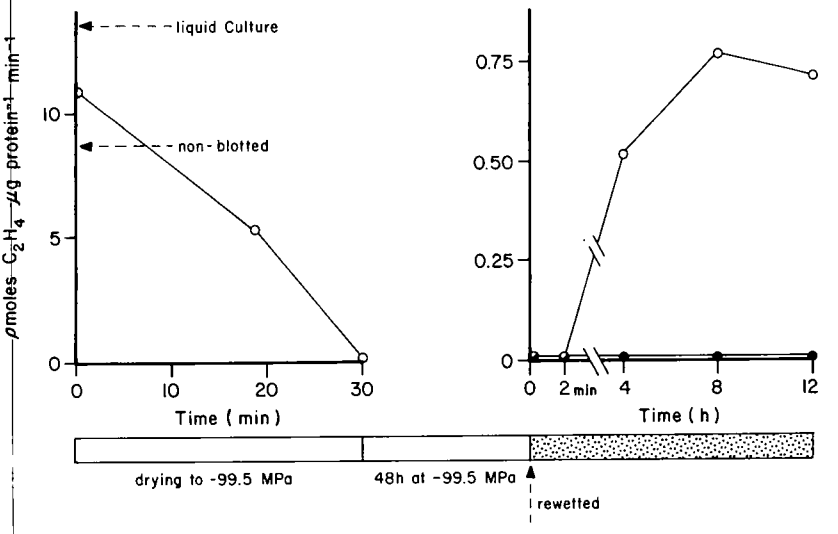

Fig. 1. Influence of rapid drying, desiccation, and rewetting on nitrogenase activity of *Nostoc commune*. Nitrogenase activities of cells in liquid culture and those immobilised but not dried are indicated. CAM = chloramphenicol. ○, control; ●, + 10^{-5} mol/l CAM.

cultures, followed by rapid drying at −99.5 MPa, led to complete suppression of nitrogenase activity within 30 min (Fig. 1). Dried cells stored in the light for 48 h at this water potential resumed nitrogenase activity within 4 h of rewetting although 48 to 72 h rewetting was required for activities to reach control levels. The onset of nitrogenase activity upon rewetting was chloramphenicol-sensitive (Fig. 1).

An intracellular ATP pool of *ca* 16 μmoles ATP μg protein^{-1} was maintained during the time that nitrogenase activity was inhibited completely by water stress. Although significantly lowered, an ATP pool was detectable after 48 to 78 h storage at −99.5 MPa. No ATP pool was detected in immobilised cells stored for longer periods at this water potential (Fig. 2). Intracellular ATP was detected within minutes of rewetting of immobilised cells, but more than 48 h were required for the pool size to reach control values. The increase in the size of the intracellular ATP pool upon rewetting was influenced markedly by conditions of illumination during drying of cells and the time the cells were maintained in the air-dry state (data not shown).

Sensitivity of ATP pool to cycles of wetting and drying

The data in Fig. 2 can be considered representative for cells immobilised for extended periods. After 3 h rewetting the pool represented only 10% of the control value (that of the cells before drying). Exposure of the cells to a 30 min period of drying after this 3 h wetting

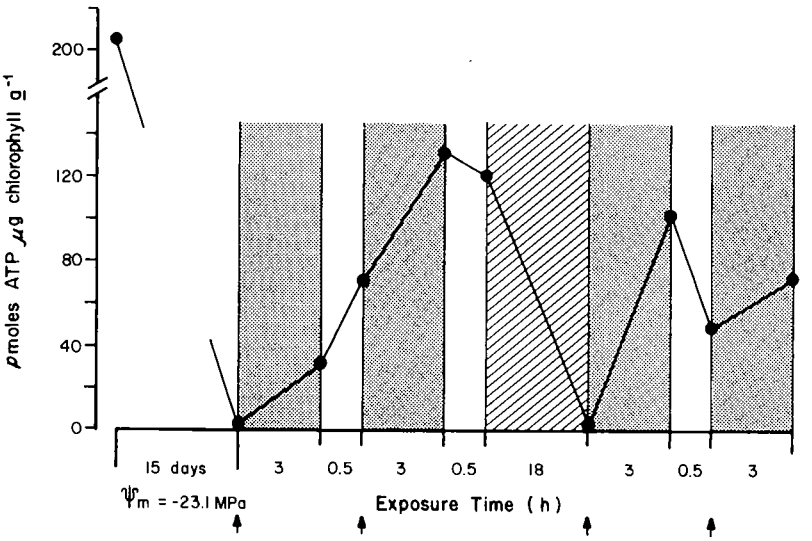

Fig. 2. Influence of cycles of wetting and drying on the size of the extractable ATP pool of *N. commune*. The intraceullular ATP pools of cells prior to drying (in liquid culture) was 205 μmoles ATP μg chlorophyll a^{-1}. Times of rewetting are indicated by arrows. ▨, rewetted ψm = 0 MPa (3 hours); ▭, dried ψm = −99.5 MPa (30 minutes); ▨, dried ψm = −99.5 MPa (18 hours).

period did not prevent the increase in the size of the pool, but, during subsequent cycles of rewetting and drying, the size of the ATP pool decreased during periods of water stress (Fig. 2). As is shown below, the rise in the size of the ATP pool upon rewetting was more rapid when cells were dried for shorter periods.

Influence of chloramphenicol on ATP pool size

A stimulation in the rise of the ATP pool size, upon rewetting, was observed when chloramphenicol was incorporated in the rehydration medium (Fig. 3). Similarly, chloramphenicol induced an increase in the size of the ATP pool when it was added to a suspension of cells rewetted previously for 6 h.

Effects of inhibitors on increase in ATP pool size

After storage of immobilised cells at −99.5 MPa for 24 h, an intracellular ATP pool was still detected. For this material, sodium azide suppressed completely the rise in the ATP pool upon rewetting (Fig. 4). The increase was also inhibited in the presence of CCCP (Figs. 4, 5). although a general trend in increase of the pool was still detected. The effects of CCCP during the first 1 to 3 h of rewetting of this

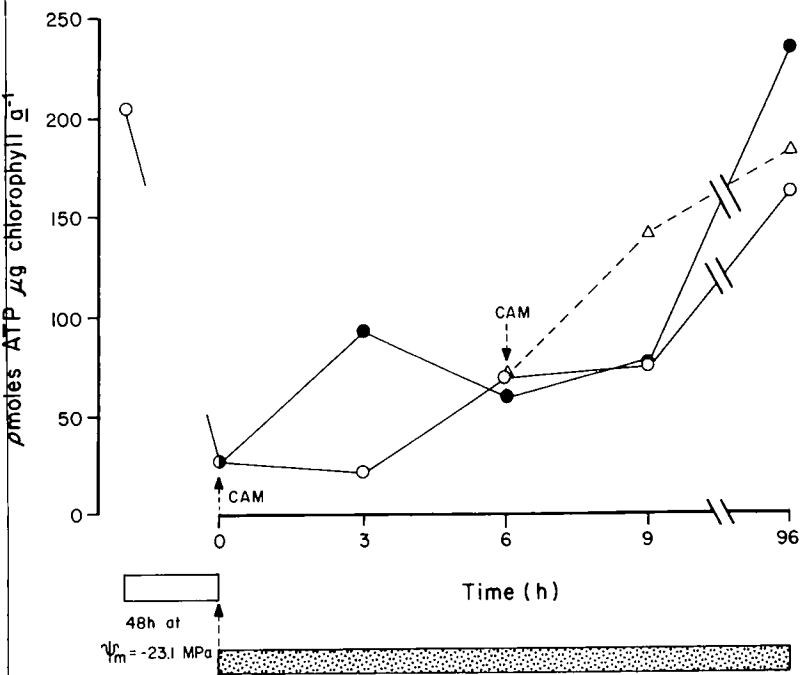

Fig. 3. Cells were immobilised in the tube system[12] for 48 h, in the light, in air, at −23.1 MPa. Cells were rewetted with MB_0 medium, in the light, in air. Chloramphenicol was incorporated in the rehydration medium or was added after 6 h of rewetting at −99.5 MPa. ○, Control; ●, + 10^5 mol/l CAM (added at time 0); △, + 10^5 mol/l CAM (added at 6 h).

material were variable while marked inhibition with CCCP was observed using cell material dried for either 72 h or longer (Fig. 6).

Increase in the ATP pool in the light and the dark

When the tube system was used to control matric water potential[12], an intracellular ATP pool was detected in cells incubated for 48 h, in the light at either −0.1 or −23.1 MPa (Fig. 7a), in the dark at −0.1 MPa, but not at −23.1 MPa in the dark (Fig. 7b). After 24 h rewetting in continuous light, the ATP pool of cells stored previously at −0.1 MPa was significantly higher than that of those kept at −23.1 MPa (Fig. 7a). A similar response was observed in material stored and rewetted in the dark (Fig. 7b). Under these conditions, there was a lag of *c* 60 min before a rapid rise in the size of the pool was measured. After 24 h rewetting, no ATP pool could be detected in cells stored previously at −23.1 MPa, while the pool size for cells stored at −0.1 MPa remained at control values (Fig. 7b).

After immobilization for 72 h in the tube system, only cells incubated

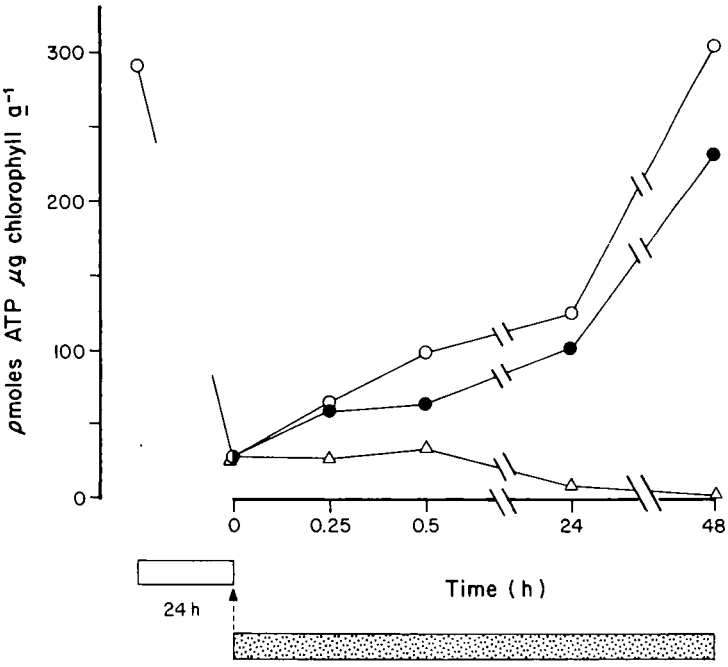

Fig. 4. Cells were dried rapidly at −99.5 MPa and stored in the light, in air for 24 h. Sodium azide (10 mmol/l) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 2 μmol/l) were incorporated in the rehydration medium. The concentration of the latter inhibitor is that expected to give half the maximal effect at pH 7.8[6]. Cells were rewetted in the light, in air. ○, Control; ●, + CCCP; △, + Na N_3.

at −0.1 MPa in the light maintained the size of their extractable ATP pool (Fig. 8a). This pool size was maintained following a 3 h wetting period and after rapid drying and then incubation at −0.1 MPa for 93 h. In cells that were immobilised previously for 72 h at −23.1 MPa, the intracellular ATP pool underwent more pronounced fluctuations in response to cycles of wetting and drying (Fig. 8a). No rapid increase in the size of the ATP pool was measured in cells that were rewetted in the dark for 3 h after a previous 72 h immobilisation in the dark (at −0.1 or −99.5 MPa; Fig. 8b). Rapid drying of this material, and then incubation at either −0.1 or −23.1 MPa in the light, led to a rapid rise in the size of the ATP pool of cells at −0.1 MPa but not in those at −23.1 MPa (Fig. 8b).

Discussion

Immobilised cells of *Nostoc commune* have the capacity both to maintain the size of their extractable ATP pools during short-term

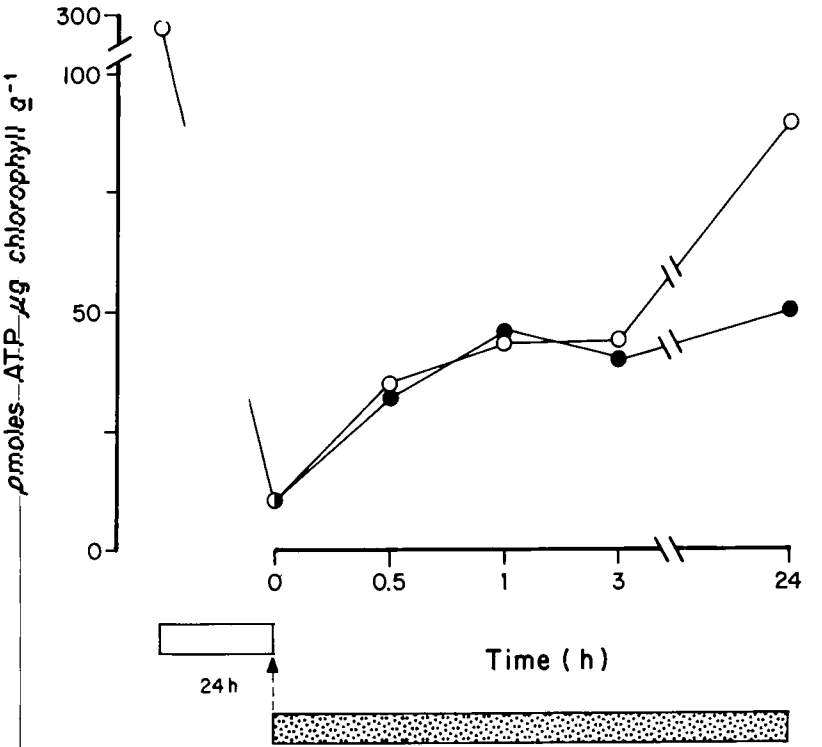

Fig. 5. Same conditions as indicated in Figure 4 with the exception that cells were dried rapidly under reduced illumination. ○, Control; ●, + CCCP.

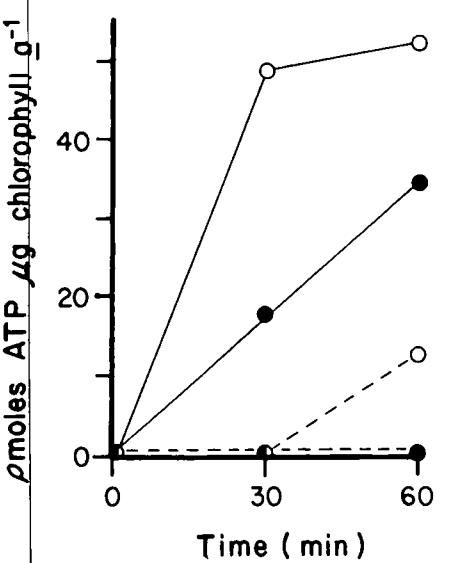

Fig. 6. Cells were immobilised and stored at −99.5 MPa, in the light, in air, for either 72 h or 8 days. Cells were then rewetted in the presence of CCCP, in the light, in air. 72 h dry; ○——○, Control; ●——● + CCCP; 8 days dry; ○------○, Control; ●------● + CCCP.

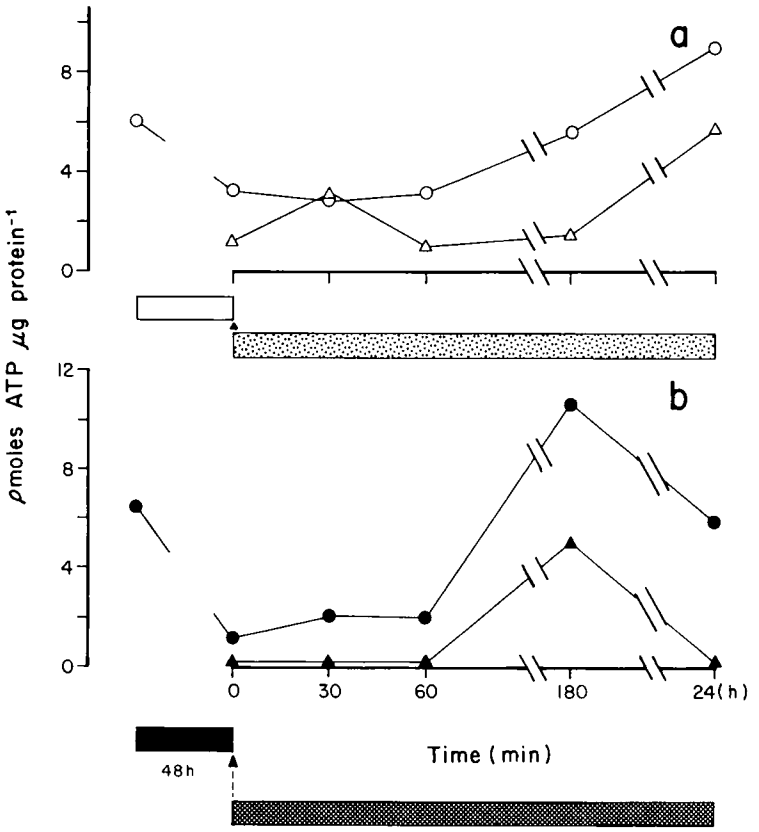

Fig. 7. Cells were immobilised and incubated at either −0.1 MPa or −23.1 MPa, in the light or the dark, in air using the tube system[12]. a. Cells were immobilised for 48 h in the light and then rewetted in the light, in air. Storage ψm. ○, −0.1 MPa light; △, −23.1 MPa light. b. Cells were immobilised for 48 h in the dark and then rewetted in the dark, in air. Storage ψm. ●, −0.1 MPa dark; ▲, −23.1 MPa dark.

water stress and to return that pool size to control levels following 48 h of rewetting, even though ATP is undetectable in the cells when they are in a state of desiccation. The increase, or rise, in the size of the pool upon rewetting is similar, in some respects to that observed in heterotrophic eubacterial spores undergoing germination[16,17]. Our data show that this increase in *N. commune* upon rewetting is due to *de novo* synthesis of ATP and not 'carry over'. The data from experiments with inhibitors suggest that apparently intact electron transport systems function during rewetting. This conclusion is also supported by the observation that, upon rewetting, desiccated cells reduce TTC (2,3,5-triphenyl-2-tetrazolium chloride) with the same characteristics and kinetics as do cells growing exponentially in liquid culture (unpublished data).

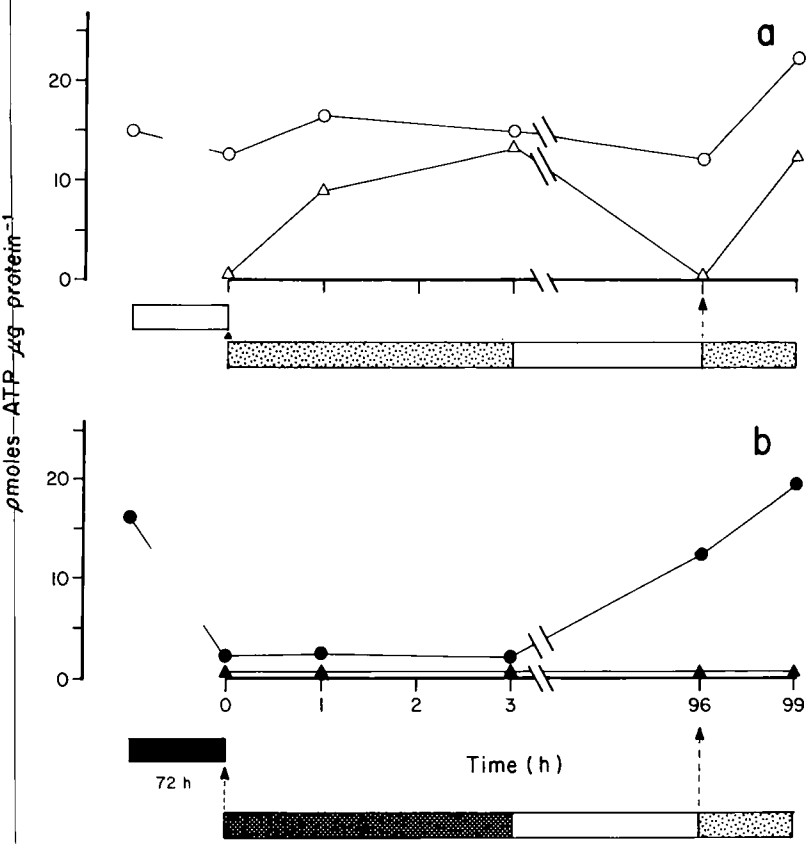

Fig. 8. Cells were immobilised and incubated at either −0.1 or −23.1 MPa, in the light or the dark, in air using the tube system. a. Cells were immobilised for 72 h in the light and then rewetted in the light, in air. After 3 h the cells were dried rapidly at −99.5 MPa and then incubated at −0.1 MPa for 93 h, in the light, in air. Cells were again rewetted, in the light, in air. Storage ψm. ○, −0.1 MPa light; △, −23.1 MPa light. b. Cells were immobilised for 72 h in the dark and then rewetted in the dark, in air. After 3 h the cells were dried rapidly in the light at −99.5 MPa, and then incubated at −23.1 MPa, for 93 h, in the light, in air. Cells were then rewetted, in the light, in air. Storage ψ_m. ●, −0.1 MPa dark; ▲, −23.1 MPa dark.

The 48 h of rewetting (lag) during which ATP levels rise is similar to the lag observed for the rise in nitrogenase activity at photosynthesis[15] (M. Potts and M. A. Bowman, unpublished data). As chloramphenicol prevents the rise in nitrogenase but stimulates the increase in the size of the ATP pool the 48 h lag represents, presumably, a period of active ATP-requiring *de novo* protein synthesis. The rapid increase in ATP synthesis upon rewetting of immobilised cells supports previous observations on the marked capacity of cyanobacteria to maintain their pool size when undergoing various shifts in metabolism. For example, in *Anabaena variabilis* optimum pool size can be maintained, at least for a period, without significant change in total biomass

by total phosphorylation and oxidative (electron transport) phosphorylation[3]. Substrate level phosphorylation was shown to be unable to maintain the ATP pool and is considered to be of limited significance in cyanobacteria (see reference 3 for references). For *A. variabilis*, oxidative phosphorylation was shown to be unimportant in the light. In the present study, the incomplete inhibition of ATP synthesis by CCCP suggests either inefficient entry of the inhibitor into cells, insufficient concentrations of CCCP, or possibly short-term synthesis at the expense of a collapsed membrane potential. It is significant that high respiration rates were observed immediately after rewetting of desiccated field colonies of *N. commune*, and oxygen uptake in the dark was abolished completely by KCN[15]. A further consequence of our results is that the enzymes required for ATP synthesis must be intact in the immobilized dried cells (72 h old).

Data shown in Figs. 2, 7 and 8 demonstrate that the rise in the ATP pool upon rewetting depends greatly on the water potential at which cells are stored and the duration of storage. The burst in ATP synthesis in the dark (aerobic; Fig. 7b) demonstrates the capacity of cells to recover rapidly and to maintain, at least in the short term, an ATP pool of normal size solely at the expense of oxidative (electron transport) phosphorylation (if one assumes substrate level phosphorylation is insignificant). Clearly, the build-up of endogenous reductant in cells prior to desiccation influences significantly the capacity of cells rewetted in the dark to return their ATP pool to normal size. Other factors must, however, be involved as cells stored for longer than 72 h did not show a capacity to perform dark oxidative electron transport (Fig. 8b). In this material the size of the ATP pool was recovered most likely through total photophosphorylation. Accumulations of polyglucosyl-like bodies have been observed in coccoid cyanobacteria exposed to matric water stress[13], and *Nostoc muscorum* is known to accumulate sucrose in response to osmotic water stress[2]. In addition, increased CO_2 uptake has been observed for *N. commune*[11] and increased photosynthesis in *N. muscorum*[2] when the cyanobacteria were exposed to matric and osmotic stress, respectively. The initial response of cells to water stress may thus be similar under osmotic and matric conditions.

A consequence of our results is that the enzymes required for ATP synthesis must remain intact in immobilized cells dried for at least 72 h in the light. A source of reductant for ATP synthesis would be particularly significant in desiccated cells with a reduced capacity for photophosphorylation as a consequence of damage to thylakoid membranes or phycobilisomes.

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Purification of polysomes from a lysozyme-resistant desiccation-tolerant cyanobacterium

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Summary

Six different techniques were compared for the extraction and purification of polysomes from cells of the desiccation-tolerant cyanobacterium *Nostoc commune* UTEX 584. Cells resisted treatment with lysozyme, and methods which relied upon 'gentle lysis' resulted in inefficient cell breakage and poor yields of polysomes. In contrast, the passage of cells through a French Pressure Cell achieved complete disruption of even the most resistant cell aggregates but only monosomes and ribosomal subunits were recovered. The grinding of cells with glass beads in the presence of neutral detergents was the most successful of all the methods tested and resulted in efficient cell lysis with high yields of polysomes. Treatment of the cells with acetone, at 0° C, prior to homogenization, also resulted in good yields of polysomes although the degree of cell breakage was less than when cells were ground. The choice of the grinding material, and the extent of the grinding, were both critical for polysome extraction. Grinding of cells with alumina and sterile sand gave very efficient cell breakage but no polysomes were recovered. Excessive grinding with glass beads led to a progressive loss of intact polysomes and concomitant increase in 70 S monosomes and subunits in cell extracts.

This study provides data on various physical treatments and buffer compositions which may be used effectively in the isolation and purification of polysomal RNA from highly resistant bacterial cells. A method which relies upon the grinding of cells in the presence of neutral detergents will permit further studies of gene expression in cells which resist methods of 'gentle lysis'.

Key words: RNA; Polysomes; Water stress, Cyanobacteria

Introduction

The fine structure and macromolecular composition of the cyanobacterial cell wall has been found to be similar to that of gram-negative bacteria [1]. The peptidoglycan layer of most cyanobacteria is, however, thicker than that of most gram-negative bacteria, and varies between 1 and 10 nm, or it may reach 200 nm. One or more

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additional layers, usually of considerable thickness surround the cell wall. The compositions of these external layers and their synthesis are poorly studied [1, 2].

For many strains of cyanobacteria, efficient lysis is often difficult to achieve with commonly-used methods of cell disintegration. In some strains the peptidoglycan layer resists treatment with 4% sodium dodecylsulfate at 100 °C [3], and cells may withstand incubation with lysozyme and other wall-specific degradative enzymes [2, 4]. These characteristics produce technical problems which pose obstacles to the molecular analysis of these particular strains. *Nostoc commune* is a lysozyme-resistant, filamentous cyanobacterium with a marked capacity for desiccation tolerance [5, 6]. Immobilized cells in the desiccated state are especially difficult to disintegrate. To permit further analysis of gene expression in *N. commune* it became necessary to isolate polysomal RNA. This communication describes and compares several techniques for the isolation and purification of free and membrane-bound polysomes from cells of this cyanobacterium.

Materials and Methods

Microorganisms and growth conditions

Nostoc commune UTEX 584 was grown in axenic culture at 32 °C, with aeration, and under a photon flux density of 50 μmol photons m^{-2} s^{-1} with a BRL 1.8 l air-lift fermentation system [6]. All equipment, reagent solutions and buffers were sterilized prior to use; where possible items were baked overnight at 240 °C and chemicals (e.g., sucrose) were RNAse free.

Harvesting of cells

The following procedures were performed as rapidly as possible. Several 150 ml aliquots of the cell suspension were removed from the fermentation vessel under positive pressure and were collected directly in 250 ml centrifuge bottles. The bottles were kept at −70 °C before use and contained either ca. 20 ml of liquid nitrogen or 50 ml of frozen sterile water. This procedure served to chill the cell suspension rapidly. The cells were collected by centrifugation at 12000×g for 5 min at 4 °C, and the pellets were used immediately for the isolation of polysomes. Usually, about 4 g (wet weight) of cells were used for each extraction of polysomes.

Extraction of polysomes

Unless stated otherwise, all procedures were performed at 0–4 °C. Different methods were compared to determine those conditions for cell lysis which would give optimum recovery of intact polysomes. These methods included the freezing and thawing of cells on ice in the presence of lysozyme [7], grinding of cell material under liquid nitrogen [8], grinding with coarse sand, grinding with glass beads (0.5 mm dia.), grinding with alumina (Sigma type 305), agitation of cells in a teflon-homogenizer with or without the addition of a small volume of acetone [9], and passage of cells through a French Pressure Cell at 120 MPa. Cells were removed periodically during the various extraction procedures for microscopic examination to determine the extent of lysis.

Preparation of cell lysates: lysozyme treatment

The method described by Gupta and Carr [7] was used with minor modifications. The cells were frozen with liquid nitrogen in a sterile mortar and ground to a powder. The final concentrations of reagents after lysis were 79 mM Tris-HCl, pH 8.1, 10% w/v sucrose, 1.0 mg lysozyme ml^{-1}, 0.8 mM EDTA, 100 mM EGTA, 1% Sodium deoxycholate (DOC), 1% Triton X-100, 1% Brij 35, 4.8 mM DTT, and 37.5 mM $MgCl_2$.

Grinding with sterile sand, glass beads or alumina

Cells were frozen under liquid nitrogen and ground to a powder with either sterile sand or glass beads (0.5 mm diameter). Cells were allowed to warm to ca. 4 °C, buffers 1 and 2 (Table I, column A) were added and the mixture was ground

TABLE I

COMPOSITION OF STOCK BUFFERS AND OPTIONAL COMPONENTS USED IN POLYSOME EXTRACTIONS

All buffers and optional components were prepared in sterile deionized water (pH_2O). For buffers 1 to 11, the volume (ml) of buffer or component used per 10 ml of the final buffer mixture is indicated. Buffer 12 was not considered part of the lysis mixture and was usually added after all other components were combined. VRC, vanadyl ribonucleoside complex; CAM, chloramphenicol; DOC, sodium deoxycholate; DTT, dithiothreitol.

Buffer	A	B	C
1. 50% w/v sucrose 10 mM Tris, pH 8.1	2	-	-
2. 5% NP-40 5% Brij 35 5% Tween 20 150 mM $MgCl_2$ 250 mM Tris, pH 8.1	1	2	2
3. 40 mM EGTA 0.8 mM EDTA 1% NP-40 1% Brij 35 1% Tween 20 2% DOC 42 mM Tris, pH 8.1	5	-	-
4. 100 mM Tris, pH 8.1	-	5	5
5. 20% DOC	-	0.5	0.5
6. 1 M DTT	-	0.043	0.043
7. Triton X-100 100% autoclaved	-	0.1	-
8. 1 mg/ml CAM	-	1	1
9. 200 mM VRC	-	0.22	0.22
10. 1 mg/ml DNAse (RNAse free)	-	-	-
11. Sterile pH_2O	2	1.14	1.24
Total volume	10	10	10
12. 4 M KCl 200 mM Tris, pH 8.1	1.1	1.1	1.1

occasionally for 10 min before the addition of buffers 3 and 11 (Table 1, column A). The slurry was ground occasionally for an additional 20 min and then the correct volume of buffer 12 was added. Occasionally, a complete mixture of buffers (all components Table 1, column A) was added to the remaining cell debris; the mixture was ground vigorously and the supernatant was combined with the first extract. Typically, the unbroken cells which remained were highly resistant aseriate stages.

The grinding of cells with alumina was performed in a similar manner except that liquid nitrogen was not used. Sterile alumina (siliconized or non-siliconized) was added to the wet cells (2 g alumina/g cells), components 4, 6, 8 and 9 (Table 1, column B) were added and the mixture was ground to a paste before the addition of the remaining buffers (Table 1, column B). The concentrations of the various components were adjusted to achieve a final ratio of 4 ml total buffer mixture/g cells.

Homogenization

This method was developed in view of a previous report that lysozyme-resistant bacteria (*Staphylococcus aureus, Bacillus cereus*) could be weakened with acetone prior to the extraction of cell proteins [9]. *Nostoc* cells were resuspended in acetone (200 μl/g cells) for 10 min in a 12 cm dia. chilled mortar which was held on ice. During this 10 min the cells were stirred slowly with a pestle. Buffers 1 and 2 (Table 1, column A) were added, the suspension was stirred for 30 s and then transferred to a glass homogenizer. The piston of the homogenizer was made of teflon. The solution was homogenized vigorously for 10 min, buffer 3 was added and the mixture was subjected to one stroke with the piston every 5 min. After 45 to 60 min the solution appeared dark brown in color at which time the appropriate amount of buffer 12 was added to the lysate to achieve a final ratio of 10 ml total buffer mixture/g cells.

French Pressure Cell

Cells were suspended in a buffer that contained 50 mM Tris-HCl, pH 8.1, DTT, VRC, CAM and buffer 4 (Table 1, column B). The mixture was vortexed briefly and then passed twice through a chilled French Pressure Cell, at 100 to 120 MPa. The brie was collected directly in a tube which contained a mixture of detergents (buffers 2, 5, 7 and component 11; Table 1, column B). Component 12 could be added either with the mixture that was used in the French press or it could be added to the solution after lysis. The time at which component 12 was added to mixture did not appear to be critical. The brie was vortexed intermittently for 10 min, at ~ 0 °C, after the addition of detergents. A modified buffer mixture was used in experiments when Triton X-100 was omitted (Table 1, column C). The buffers were used at 4–6 ml total buffer mixture/g cells.

In all of the above procedures, cell debris was removed from cell lysates by centrifugation at 12000 × *g* for 25 min, at 0–4 °C. The brown supernatant was layered carefully over 3 ml of a 1.5/2.2 M sucrose step cushion (1 ml of 1.5 M sucrose over 2 ml of 2.2 M sucrose in polysome buffer: 40 mM Tris-HCl, pH 8.1, 400 mM KCl, 15 mM $MgCl_2$, 5 mM EGTA) and centrifuged for 21 h at 113000 × *g*, at 4 °C. After removal of the supernatant by aspiration, the insides of the tube were

washed carefully with sterile distilled water and wiped clean with a sterile cotton-tip applicator. The clear opalescent pellet was then resuspended in gradient buffer (GB; 40 mM Tris, pH 8.1, 10 mM $MgCl_2$, 60 mM KCl; usually 300 μl per pellet). In some cases, the pellet was homogenized gently with the end of a sterile sealed pasteur pipette. Around 200–300 μl of the resuspended pellet were applied carefully to the surface of a 12 ml sucrose density gradient (35, 25, 15, 10% w/v sucrose in GB; 4.5, 3.2, 2.3, 1.5 ml respectively) which was then centrifuged at $75000 \times g$ for 3 h (SW 27.1 rotor, Beckman). Gradients were analyzed with an ISCO gradient fraction collector (ISCO type 6 optical unit, model D density gradient fractionator, UA-5 absorbance fluorescence detector and Bausch & Lomb digital electronic readout indicator) at 254 nm. Gradients were collected at 0.5 or 1.0 ml min^{-1}. Background absorbance was measured simultaneously in control gradients.

Results

Treatment of cells with lysozyme

Cells of *Nostoc commune* UTEX 584 show a marked resistance to treatment with lysozyme and lysis is difficult to achieve without physical disintegration of the cells. Microscopic examination of cell suspensions during their incubation with lysozyme indicated only a small degree of lysis and a large fraction of aseriate cell aggregates were left intact by this treatment. A typical gradient profile of the polysome extracts obtained with the method of Gupta and Carr [7] is shown in Fig. 1A. Polysomes aggregated in the central region of the gradient and appeared as a single broad peak (P) in gradient traces. Smaller peaks, in the upper portion of traces were attributed to ribosomal material with average Svedberg values of 70 S (possibly monosomes, ribosomes and subunits). It was not possible to discriminate between polysome multimers (dimers, trimers, etc.). It was difficult to obtain consistent results with this method, and the addition of DTT to the sucrose gradient buffer (GB) caused high background absorbance at 254 nm (data not shown). As a consequence, DTT was omitted from GB in subsequent trials.

Grinding of cells

Grinding of cells with sterile glass beads gave, consistently, efficient cell lysis and improved the yield of polysomes (Fig. 1B). Lysis was enhanced further if the cells were frozen in liquid nitrogen prior to grinding them. A proportion of cells always resisted the grinding procedure despite the use of glass beads of different sizes and different durations of grinding. The addition of fresh buffer to this fraction and further grinding did not lead to further cell lysis. Excessive grinding led to polysome degradation as indicated by the concomitant decrease in the polysome peak and increase in the amounts of ribosomal material at the top of gradients (Fig. 1B).

Grinding of cells with sterile carbonate sand gave efficient lysis although no polysomes, ribosomes or subunits were ever obtained even in the presence of one or more of the following components: VRC, DNAse, CAM, lysozyme.

Grinding with alumina, which gave the most efficient cell lysis of the three methods, did not yield polysomes or ribosomes. With siliconized alumina, only a small amount of UV-absorbing material was detected at the surface of gradients (Fig. 1C).

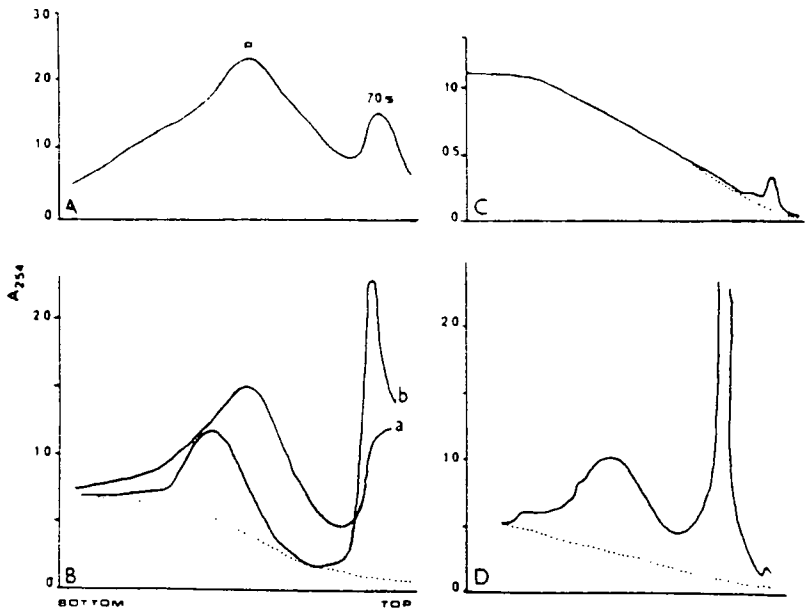

Fig. 1. Gradient analyses of polysome preparations obtained with different techniques. Background absorbance is indicated by a dashed line. A. Lysozyme procedure [7]; P, polysome. B. Grinding with glass beads; a, one extraction; b, two extractions; supernatants combined. C. Grinding technique (with siliconized alumina). D. Homogenization technique (with acetone). Direct extraction of the nucleic acid from the central peak (P) of gradients indicated the presence of 23 S, 16 S and 5 S RNA [16].

Homogenization

The addition of acetone improved the degree of cell lysis during homogenization and the polysome profiles which were obtained with this method were similar to those obtained when cells were ground with glass beads (Fig. 1D). It was not essential to add the buffer components in a stepwise fashion, and a single buffer mixture could be used to treat the cells directly in the homogenizer. However, a large portion of cells still resisted lysis. The use of higher concentrations of acetone, longer incubation times, and more vigorous homogenization, all failed to increase lysis.

French press

Two passages through a chilled French Pressure Cell lead to complete disruption of all cell types. However, no polysomes were recovered with this method and only monosomes, ribosomes and subunits were detected in gradients (Fig. 2A). A similar result was obtained when Triton X-100 was included in the buffers (Fig. 2B).

Discussion

The methods which we have compared to extract polysomes from cells of *Nostoc* include a number which have proven successful with lysozyme-sensitive eubacteria and cyanobacteria, as well as higher plants and eukaryotic cells [7, 10–15]. The ma-

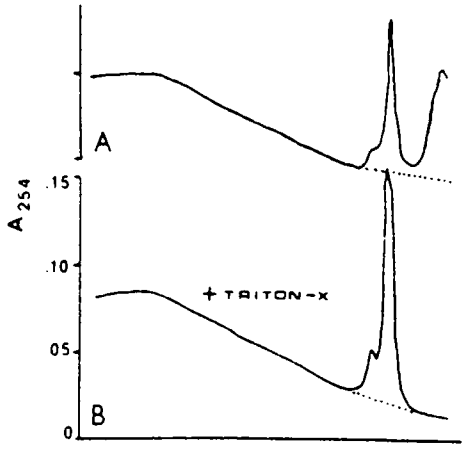

Fig. 2. Gradient analyses of polysome preparations obtained with the use of a French Pressure Cell. Background absorbance is indicated by a dashed line. A. In the absence of Triton X-100. B. In the presence of Triton X-100.

jor feature which distinguishes the different methods is the severity of the physical disruption technique. Methods which relied upon 'gentle lysis' – such as the technique of Gupta and Carr [7] which was used successfully with another strain of *Nostoc* – led to the lysis of only a small fraction of the cells. These sensitive cells represented, as judged by microscopic analysis, seriate (filamentous) stages which possess comparatively thin cell wall layers. Aseriate stages, which possess thick additonal wall layers, persisted throughout all stages of this gentle lysis procedure. Typically, it was aseriate cell aggregates which dominated the unbroken cell fraction in all those methods where cell disruption was found to be incomplete. Grinding of the cells with liquid nitrogen markedly improved the subsequent lysis of all cell types and increased the ultimate yield of polysomes. However, it was clear that prolonged grinding led to disruption of polysomes with a resultant increase in the amount of ribosomal material. The extent of grinding must be determined empirically as it is expected that the time required for efficient cell lysis and optimum polysome yield will differ considerably among different cell types.

Of the three materials which were used to grind cells, glass beads proved to be the most successful. Alumina, as we have since demonstrated, appears to bind nuclei acid fractions effectively and this accounts, presumably, for the lack of success with this technique. It should be noted, however, that very efficient cell breakage was achieved through the use of alumina. The reason for the lack of success when grinding with sterile sand is not readily apparent. However, the sand did contain a substantial fraction of coral-derived calcium carbonate which, through dissolution of calcium, may have enhanced the activity of RNAse's. Pretreatment of cells with acetone did improve the degree of cell lysis and this appears to be a useful means to weaken such resistant cells. Polysome yields after acetone treatment and homogenization were, nevertheless, equivalent to or less than those which were obtained by grinding with glass beads.

Although the passage of cells through a French Pressure Cell led to complete breakage of cells, this method of disruption is clearly too severe to permit the recovery of intact polysomes.

Grinding of cells with glass beads, in the presence of a mixture of neutral detergents is, therefore, the method of choice to achieve efficient lysis of *Nostoc* cells. This method has proved successful in the study of gene expression in *Nostoc* in response to water stress [16] and it is expected that it may have utility with other cell types which resist the action of lysozyme and other wall specific degradative enzymes. Despite the success of this method, total yields of RNA from these cells were low in comparison with those which have been obtained from other cell types with different extraction procedures. Direct extraction of nucleic acids from pellets and supernatants at different stages of the extraction procedure gave good yields of RNA [16]. This suggests that the low recoveries of polysomal RNA reflect the formation of nucleic acid-membrane aggregates rather than high RNAse activity. Similar problems have been found with the extraction of DNA from *Nostoc* and have been traced to the formation of detergent-resistant membrane and carbohydrate complexes (Xie, Jäger and Potts, unpublished data). We are currently developing techniques for the efficient extraction of cyanobacterial RNA.

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Polysome Turnover in Immobilized Cells of *Nostoc commune* (Cyanobacteria) Exposed to Water Stress

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Water stress induced changes in the polysome content of immobilized cells of the desiccation-tolerant cyanobacterium *Nostoc commune* UTEX 584. Cells maintained an intact protein synthesis complex during 2 h of drying at −99.5 MPa. Polysomes were not recovered from cells subjected to extended periods of desiccation.

Desiccation tolerance is more prevalent in procaryotes than in eucaryotes (2). Of the procaryotes that have this ability, the cyanobacteria warrant particular attention in view of their biochemical and phylogenetic affinity with the higher-plant chloroplast and their ecological significance (4). The filamentous, heterocystous cyanobacterium *Nostoc commune* shows a marked capacity for desiccation tolerance—a feature which contributes, no doubt, to its persistence in environments subject to extremes of drought (12, 18). When cells of *N. commune* UTEX 584 are immobilized and then dried rapidly at a matric water potential (ψ_m) of −99.5 MPa (a_w = ~0.5), they maintain high levels of chloramphenicol-sensitive protein synthesis for more than 90 min (11). Extensive proteolysis is detected after prolonged desiccation, and phycobilisomes become dissociated in both light- and dark-stored desiccated material. Nitrogen fixation is particularly susceptible to even short periods of drying (13), but the *nifH* product (nitrogenase reductase [5, 15]) has been detected by immunoblotting in immobilized cells, desiccated cells, and dried cells subjected to rehydration (M. Potts, unpublished data). When desiccated cells are rewetted, there is a rapid turnover of protein, and lags are measured before the onset of nitrogenase activity, ATP synthesis, photosynthesis, and respiration (12, 14, 17). To permit further analysis of the response of gene expression in *N. commune* UTEX 584 to water stress, we investigated the turnover of polysomal RNA in immobilized cells which were subject to drying, desiccation, and rehydration.

N. commune UTEX 584 was grown in liquid BG-11_0 medium (16) in an airlift fermentation system (11). Cells in the exponential phase of growth were harvested directly from the fermentor and collected in liquid nitrogen for polysome analysis, or they were immobilized and dried on hydrophobic nylon supports as described previously (12). Conditions of desiccation were a matric water potential (ψ_m) of −99.5 MPa, with a photon flux density of 50 μmol of photons m^{-2} s^{-1}, at 32°C. Desiccated material was rehydrated with sterile water under the same conditions of light and temperature. Cells of *N. commune* UTEX 584, particularly aseriate aggregates, resist treatment with lysozyme and methods of gentle lysis that rely on detergents, such as those used in protocols for plasmid extraction from other cyanobacteria (9). As a consequence, a lysozyme-EDTA procedure which has been used to isolate polysomes from another strain of *Nostoc* (6) gave poor yields with *N. commune*. Many methods were compared, therefore, to permit efficient disintegration of cells and optimal recovery of polysomes. The most successful of these methods is described below. When possible, all procedures were carried out at 0 to 4°C, and RNase-free chemicals (e.g., sucrose) were used. All buffer and reagent solutions were sterile, and glassware was baked overnight at 240°C or autoclaved prior to use. Cell material, either as a pellet from the fermentor or as immobilized crusts, was immersed in liquid nitrogen and ground in a chilled mortar. The powder was allowed to warm to ca. 4°C, after which the cells were suspended in a mixture of sucrose buffer (2 ml/g of cells; 10 mM Tris hydrochloride, pH 8.1, 25% [wt/vol] sucrose, 12 mM dithiothreitol) and a detergent solution (1 ml/g of cells; 250 mM Tris, [pH 8.1], 10 mM vanadyl ribonucleoside complex [VRC], 5% Nonidet P-40, 5% Brij 35, 5% Tween 20, 150 mM $MgCl_2$, 50 μg of chloramphenicol per ml). Sterile glass beads (0.5 mm diameter) were added, and the mixture was ground for 10 min. This grinding was essential and increased the susceptibility of the cells to detergents. Lytic buffer (5 ml/g of cells; 42 mM Tris hydrochloride [pH 8.1], 40 mM EDTA, 0.8 mM EGTA, 1% Nonidet P-40, 1% Tween 20, 1% Brij 35, 2% sodium deoxycholate) was added, and the slurry was mixed only occasionally for a further 30 min, during which extensive lysis took place. A solution of 4 M KCl (1.1 ml/g of cells; 200 mM Tris, pH 8.1) was then added to the lysate, which was usually brown or yellow at this stage. After the removal of cell debris through centrifugation (12,000 × *g* for 30 min at 4°C), the slightly viscous brown supernatant was placed on a 3-ml sucrose cushion (1 ml of 1.5 M sucrose placed over 2 ml of 2.2 M sucrose in 40 mM Tris hydrochloride [pH 8.1], 400 mM KCl, 15 mM $MgCl_2$, 5 mM EGTA) and centrifuged at 113,000 × *g* for 21 h at 4°C. The brown supernatant and sucrose cushions were removed by aspiration, and the sides of the tubes were rinsed carefully with distilled water and then dried with cotton-tip applicators. The opalescent pellet was suspended in 300 μl of gradient buffer (GB [40 mM Tris hydrochloride (pH 8.1), 10 mM $MgCl_2$, 60 mM KCl] and a minimum of 200 μl of this suspension was transferred to the surface of a sucrose density gradient. Gradients were made from steps of 35, 25, 15, and 10% (wt/vol) sucrose (4.5, 3.2, 2.3, and 1.5 ml, respectively, in GB and centrifuged at 75,000 × *g* for 3 h at 4°C (SW27.1 rotor; Beckman Instruments). The gradients were analyzed with a gradient fraction collector (ISCO type 6 optical unit, model D density gradient fractionator, UA-5 absorbance fluorescence detector, and Bausch & Lomb digital electronic readout indicator) at 254 nm. Polysomes sedimented in the central region of sucrose gradients and were detected as a single peak in gradient

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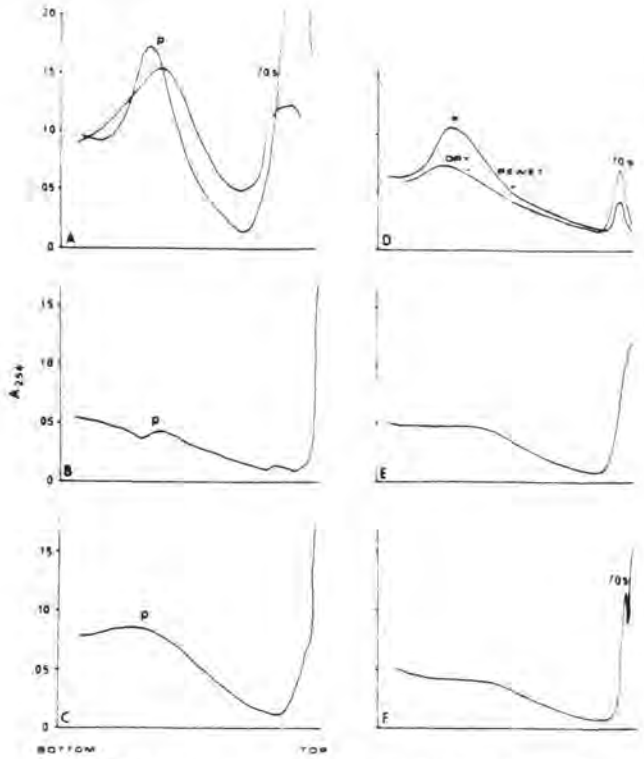

FIG. 1. Gradient analyses of polysome profiles from cells subjected to different conditions of water stress. The background absorbance (measured simultaneously in control gradients) is indicated by a dashed line. P, Polysomes. (A) Two preparations obtained from cells in exponential growth phase in liquid culture. (B through D) Cells from liquid culture which were immobilized on an inert support, dried rapidly, and incubated at −99.5 MPa for 2 h (B) or 16 h (C) or incubated for 2 h and rehydrated for 2 h (D). (E and F) Cells stored at −99.5 MPa for 14 days in the light and then rehydrated for 2 h (E) or 24 h (F).

traces. Two representative traces are illustrated in Fig. 1A. Fractions were collected from different regions of the sucrose density gradients directly in phenol (equilibrated previously with aqueous buffer; 0.1 M Tris hydrochloride, 0.2% [vol/vol] β-mercaptoethanol). After further deproteinization with chloroform-isoamyl alcohol (24:1), RNA was precipitated with ethanol (95%, vol/vol) at −70°C, suspended in TE buffer (10 mM Tris hydrochloride, pH 8.0, 1 mM EDTA), and analyzed by agarose gel (2%, wt/vol) electrophoresis (Fig. 2A) under nondenaturing conditions (10).

To assess the purity of the polysome preparations, the effectiveness of the different fractionation procedures, and the potential for activity of RNases, and to identify various fractions, total RNA was isolated rapidly from cells in exponential growth phase with a French press (at 4°C) in a lytic buffer which contained VRC (20 mM) and β-mercaptoethanol (120 mM). Total RNA fractions contained genomic DNA, tRNA, 23S, 16S, and 5S rRNA, and two distinct fragments, F1 and F2 (Fig. 2B, lanes 1 and 2). rRNAs were identified by comparing their mobilities with "purified" *Escherichia coli* RNA markers (Fig. 2B, lane 3). The presence of F1 and F2 was demonstrated in total RNA preparations of *N. commune* UTEX 584 obtained by a range of different extraction procedures, under conditions in which RNase activity was inhibited. The fragments were detected in both nondenaturing and agarose-glyoxal gels. The identities of F1 and F2 are presently under investigation. While it is possible these fractions represent breakdown products from 23S and 16S RNA, RNase activity was not considered significant under these conditions, as the total RNA preparations drive significant rates of in vitro translation (K. Jäger and M. Potts, unpublished data).

Direct extraction of the nucleic acid from the central peak region of analytical gradients (Fig. 1A) indicated the presence of 23S, 16S, and 5S RNA and fragments F1 and F2 (Fig. 2A, lane 1). After centrifugation, ribosomal material (which had an average Svedberg value of 70S and probably consisted of monosomes, ribosomes, and subunits) remained at the surface of the gradients (Fig. 1A), together with tRNA, DNA, and thylakoid membrane complexes (Fig. 2A, lane 2). Gentle homogenization and brief centrifugation of the crude polysome pellets (prior to gradient analysis) led to the removal of these membrane complexes. Without this brief centrifugation step, a small green pellet was sometimes present at the bottom of the tubes after centrifugation of the gradients. These pellets were shown to contain 23S, 16S, and 5S RNA as well as F1 and F2 and presumably contained thylakoid-bound ribosomes. Membrane vesicles were not detected when gradient fractions were negatively stained and examined by electron microscopy. With the method of

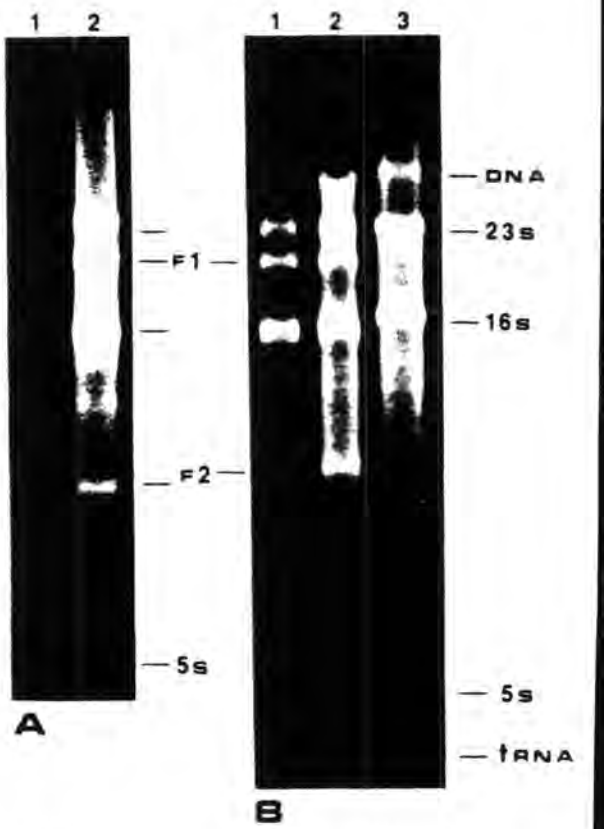

FIG. 2. Agarose gel electrophoresis of RNA fractions in 2% (wt/vol) agarose gels under nondenaturing conditions (10). The time of electrophoresis was slightly different for each of the gels. Gels were stained with ethidium bromide and viewed under UV illumination to visualize bands of RNA. Fragments F1 and F2 are indicated. (A) Fractions from polysome preparations. Lanes: 1, RNA present in the polysome fraction obtained from analytical sucrose gradients; 2, nucleic acids present at the surface of analytical sucrose gradients after high-speed centrifugation. (B) Total RNA preparations from cells of *N. commune* UTEX 584 in exponential growth phase. Lanes: 1, 1.39 μg of total RNA; 2, 4.17 μg of total RNA; 3, *E. coli* RNA standards (Promega Biotech, Madison, Wis.).

cell disruption described here, the ratio of polysomes to 70S material was generally equal to or less than 2:1. This ratio could be improved if the grinding step with glass beads was omitted, although the efficiency of cell lysis was reduced considerably, aseriate aggregates—which are more tolerant of desiccation—remained unbroken, and polysome yields were low.

The polysome content of cells which were immobilized and incubated at −99.5 MPa for 2 h (rapid drying) prior to polysome extraction was significantly lower than that in control material. Under these conditions the cells reached equilibrium at −99.5 MPa within 80 to 90 min, and in a previous study (11) this period of drying was shown to arrest chloramphenicol-sensitive protein synthesis. The decrease in the amount of polysomes was accompanied by an increase in ribosomal material at the surface of sucrose gradients (Fig. 1B), where a clear distinction between monosomes, ribosomes, and subunits was not always apparent. A similarly low recovery of polysomes was obtained from cells that had been dried for 16 h under the same conditions (Fig. 1C). Proteolysis and changes in the protein index occur in cells after drying for this time (11). In gradient analyses of samples taken from cells subjected to 72 h of desiccation, UV-absorbing material was associated almost exclusively with the surface of gradients (data not shown). Immobilized cells which were subjected to slow drying for 2 h also gave a significantly lower yield of both polysomes and monosomes than cells in liquid culture. A marked increase in polysomes was detected after the rehydration of cells which had been dried rapidly, immobilized, and then incubated at −99.5 MPa for 2 h (Fig. 1D). This increase in the polysome peak was accompanied by a decrease in the size of the 70S peak. Rehydration of the cells also led to a resumption of protein synthesis. Incorporation experiments demonstrated that the amount of ^{35}S-labeled protein in the cells increased after rewetting, reached a maximum after 30 to 60 min, and then began to decline. Two-dimensional electrophoretic analysis indicated that there was a marked turnover of labeled proteins during rewetting (M. Potts, unpublished data). When the time of desiccation was extended to 14 days, there was no increase in the polysome content of the immobilized material after either 2 or 24 h of rewetting (Fig. 1E and F, respectively). In the latter case, gradient analyses of the extracts showed a 70S peak with only a small amount of UV-absorbing material in the central region of the gradient (Fig. 1F).

Water stress (drying, desiccation, and rewetting) induces marked changes in the extractable polysome level of immobilized *N. commune* cells. In view of the rapid recovery of polysome levels, which occurred after the rewetting of material which had been dried for 2 h, it is clear that cells have the ability to tolerate these changes. This ability of rehydrated cells to recover rapidly appears to be lost in laboratory-grown cultures when the cells are desiccated for extended periods, a fact which may explain the long delays which have been observed in the recovery of physiological functions, for example nitrogen fixation, when desiccated cells are rewetted (12, 17). Photo-oxidative protein degradation, which occurs during prolonged desiccation of immobilized cells in the light, may contribute to the loss of this ability, although, as we have discussed previously, it is questionable whether this would occur in field populations which have a number of photoprotective mechanisms, such as thick heavily pigmented sheaths (11, 12).

The fact that polysome levels decrease after drying and increase rapidly after rewetting, considered with previous findings on the effects of water stress on protein synthesis (11), suggests that the protein-synthesizing system is maintained, at least during short-term desiccation, in an intact form. This is the case for desiccation-tolerant plants, such as the moss *Tortula ruralis* (1, 3, 8). Furthermore, studies suggest that there is neither a temporal nor a quantitative correlation between the levels of RNase in desiccated cells and the depletion of the protein synthesis complex (3), and the observed decrease in polysome levels after drying is considered to be due to runoff and not to enzymatic degradation. The relationship of these findings to the ability of cells to tolerate desiccation remains poorly understood. It has been suggested that stress-induced reductions of polyribosome levels and cell ψ are largely independent processes, but they are highly correlated with each other in growing cells because early steps in both processes are regulated by the same signal that modifies control molecules located at the plasma membrane (7).

Under natural conditions, communities of *N. commune* are subjected to repeated cycles of drying, desiccation, and rewetting (18). Water stress imposes considerable restrictions on the nitrogen-fixing activities of *Nostoc* spp. (12, 13), yet the rates of acetylene reduction recorded for some terrestrial communities are among the highest in the literature for in situ studies of cyanobacteria (18). While the molecular basis for desiccation tolerance is poorly understood, an ability to maintain the protein synthesis complex intact during short-term extremes of water stress is one factor which must contribute to this trait.

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Protein Synthesis and Proteolysis in Immobilized Cells of the Cyanobacterium *Nostoc commune* UTEX 584 Exposed to Matric Water Stress

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Cells of the cyanobacterium *Nostoc commune* UTEX 584 in exponential growth were subjected to acute water stress by immobilizing them on solid supports and drying them at a matric water potential (ψ_m) of −99.5 MPa. Cells which had been grown in the presence of $Na_2{}^{35}SO_4$ before immobilization and rapid drying continued to incorporate ^{35}S into protein for 90 min. This incorporation was inhibited by chloramphenicol. No unique proteins appeared to be synthesized during this time. Upon further drying, the level of incorporation of ^{35}S in protein began to decrease. In contrast, there was an apparent increase in the level of certain phycobiliprotein subunits in solubilized protein extracts of these cells. Extensive proteolysis was detected after prolonged desiccation (17 days) of the cells in the light, although they still remained intact. Phycobilisomes became dissociated in both light- and dark-stored desiccated material.

Recognition of the marked and often complex changes in enzyme biosynthesis that take place within cells of *Escherichia coli* when they are subjected to a shift in growth conditions has prompted interest in stimulons, that is, sets of genes that become active and produce their protein products in response to particular environmental stimuli (2, 10, 20). For example, a change in the osmotic water potential of the growth medium can influence the differential synthesis of two outer membrane proteins OmpC and OmpF (12, 18). A model has also been proposed whereby a 95K protein, the *kdpD* product, interacts with the promoter of the *kdp* operon in response to a rise in turgor pressure. Induction of the *hop* regulon, in response to a decrease in the osmotic water potential of the growth medium, leads to an elevated rate of synthesis of more than three major proteins. In this last case, the response is considered to be a true adaptation to a change in environmental conditions rather than a general response to cell damage (8). It is expected that studies such as these will reveal novel patterns, if not novel mechanisms, of gene regulation (14).

Clearly, our current knowledge and understanding of gene expression in procaryotes derives almost exclusively from investigations of the growth of heterotrophic eubacteria, particularly *Escherichia coli*. In contrast, the molecular biology of cyanobacteria is poorly understood despite the utility of these procaryotes as model systems for the study of nitrogen fixation, differentiation, and photosynthesis (11, 13).

Many cyanobacteria express a marked tolerance of water stress and desiccation (6, 25, 27, 28, 32). In preliminary studies with immobilized cells of the filamentous, nitrogen-fixing strain *Nostoc commune* UTEX 584, we described upshifts and downshifts in both nitrogenase activity and the size of the intracellular ATP pool in response to matric water stress (25, 26; M. Potts, N. S. Morrison, *Proceedings of the 3rd International Symposium on N_2 Fixation With Non-Legumes*, Helsinki, in press).

This study focuses on protein turnover in immobilized cells of *N. commune* UTEX 584 when they are subjected to acute water stress (rapid drying at a matric water potential [ψ_m] of −99.5 MPa).

MATERIALS AND METHODS

Growth of cells. *N. commune* UTEX 584 was grown in BG-11_o (31) medium in continuous axenic culture with an air-lift fermentation system (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The volume of the reactor vessel was 1.8 liter. Cells were grown under a continuous photon flux density of 300 μmol of photons m^{-2} s^{-1} at 32°C and at a pH of 8.7.

Labeling of cells and measurement of protein synthesis. Cells were harvested directly from the fermentor, washed extensively in BG-11_{o-s} (BG-11_o that lacked combined sulfur), homogenized briefly to disperse any filament aggregates, and suspended in the same medium to a cell density of approximately 60 μg of total protein ml^{-1}. The cell suspension (4 ml) was then aerated vigorously under a photon flux density of 300 μmol of photons m^{-2} s^{-1} at 32°C. After equilibration for 15 to 30 min, 160 μCi of $Na_2{}^{35}SO_4$ (80 μl, carrier free; specific activity, 1,498 Ci $mmol^{-1}$) was added to the suspension, and the incubation was continued. Samples of 50 μl were removed periodically during the incubation and transferred to Whatman no. 3MM filter disks (23-mm diameter). The incorporation of ^{35}S into protein was measured by the method of Mans and Novelli (19).

The incorporation of ^{35}S into protein during subjection of the cells to matric water stress was determined after the addition of label to a cell suspension either 60 min or immediately before immobilization and rapid drying of the cells at −99.5 MPa (see below).

Immobilization of cells and control of matric water potential. After a period of labeling, samples (50 or 100 μl) of a cell suspension were transferred to Whatman 3MM filter disks (23-mm diameter) which were supported on steel pins. Depending on the experiment, the filters, at the time of cell immobilization, were either dry, saturated with BG-11_o, or saturated with BG-11_{o-s}. Filters were incubated at 32°C under a photon flux density of 300 μmol photons m^{-2} s^{-1}, at a matric water potential of −99.5 MPa (26). The filters were

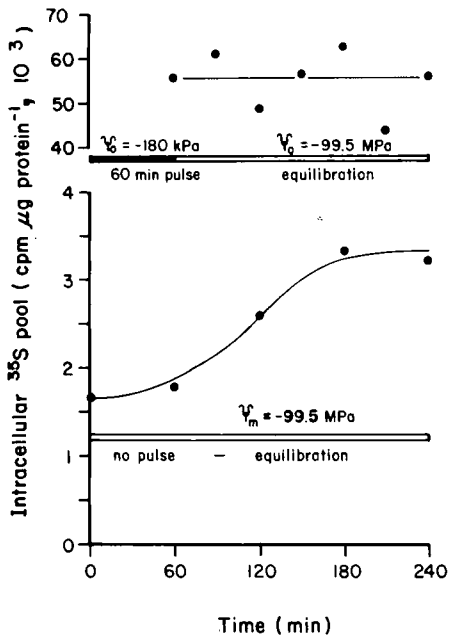

FIG. 1. Change in the size of the total intracellular pool of ^{35}S after immobilization of cells. $^{35}SO_4$ was added to a suspension of cells (at a density of 69.9 μg of total protein ml^{-1}) in exponential growth. One series of samples (100 μl) was immobilized immediately after the addition of ^{35}S, and a second series was immobilized after the cell suspension had been incubated for 60 min in the presence of ^{35}S. In each case the filters were dry before the immobilization of the cells, and incubation was continued at −99.5 MPa. Periodically, filters were removed from the incubation chamber and then washed extensively in distilled water at 4°C. After drying, the filters were placed directly in scintillation fluid to measure radioactivity. All values were adjusted for quenching and nonspecific uptake of ^{35}S during washing.

allowed to equilibrate to this matric water potential, or they were moistened periodically with 50 μl of BG-11_o or BG-11_{o-s}.

After a period of drying and equilibration, the filters were processed to measure the incorporation of ^{35}S in protein (see above). When larger quantities of cell material were required, the total cell suspension was centrifuged at 12,000 × *g* for 5 min, and the pellet was spread as a thin veneer on a fine-mesh nylon filter support (26). The advantage of this support over filter disk supports was the efficiency of recovery of the desiccated cell material.

Measurement of the total uptake of ^{35}S by cells. The intracellular pool of ^{35}S in cells was measured after extensive washing of filters in ice-cold H_2O (in certain experiments, BG-11_o was used).

Radioactivity that remained on filters after the various manipulations was measured in Biofluor cocktail (New England Nuclear Corp., Boston Mass.) with a Packard Tri-Carb model 2405 liquid scintillation counter. Measurements were corrected for quenching and nonspecific adsorption of ^{35}S to filters.

Measurement of total protein. Total protein was extracted and measured by the methods of Kochert (15, 16).

Extraction of soluble proteins. Cells were ground with sterile sand in a chilled mortar at 0 to 4°C, and the soluble proteins were extracted in cold buffer [10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid [pH 7.2], 10 mM NaCl, 5mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaN_3). The extracts were cleared by centrifugation at 12,000 × *g* for 10 min, mixed with an equal volume of trichloroacetic acid (20%, wt/vol), and kept on ice for at least 45 min. The precipitates were collected by centrifugation and then suspended in trichloroacetic acid (5%, wt/vol). After washing in ice-cold acetone-water (50:50, vol/vol) and then ether, the pellets were dried under a stream of air and solubilized in cracking buffer (0.2 M Tris, [pH 8.8], 2% [wt/vol] sodium dodecyl sulfate, 1 M β-mercaptoethanol, 15% [vol/vol] glycerol, 0.01% [wt/vol] bromphenol blue) at 100°C for 3 min.

Electrophoresis of protein extracts. Samples were analyzed on either 10 or 15% (wt/vol) polyacrylamide gels supported on gel bond (FMC Corp., Rockland, Maine) with a vertical-gel apparatus (Bethesda Research Laboratories) and the buffer system of Laemmli (17). After fixing, gels were stained with either Coomassie brilliant blue R or silver stain, or they were prepared for fluorography by impregnation with En^{3}Hance solution, (New England Nuclear). In the last case, proteins were detected with Kodak X-Omat AR (XAR-5) film exposed at −70°C.

Extraction of phycobilisomes. Cells were ground with sand in cold grinding buffer (0.65 M NaH_2PO_4-K_2HPO_4 [pH 8.0], 1 mM β-mercaptoethanol). The extracts were incubated with 1.2% (vol/vol) Triton X-100 for 30 min at room temperature and were then centrifuged at 31,000 × *g* for 30 min. Purified phycobilisomes and additional fractions were obtained after centrifugation of the aqueous extracts through 0.2 to 1.0 M sucrose step gradients, at 23,000 × *g*, for 13 h in a Beckman SW27 rotor (37). The absorbance spectrum of phycobilisome preparations and other fractions isolated from gradients was measured with a Hewlett Packard model 8450A UV-visible spectrophotometer with ancilliary 7470 graphics plotter. The protein composition of these samples was analyzed after solubilization in cracking buffer (room temperature) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (see above).

RESULTS

Under the experimental conditions employed during incorporation experiments, the time required for immobilized cells to reach equilibrium, that is, the time after which no change in weight of the cells could be detected, depended on the volume and density of the cell suspension that was transferred to a filter and the physical nature of the solid support. Upon transfer of a 50-μl sample of well-dispersed cell suspension to a dry Whatman 3MM filter (23-mm diameter), the filaments were immobilized immediately within the confines of the upper matrix of the support and occupied a circular area approximately 8 mm in diameter. The liquid medium in association with the cells was absorbed rapidly and wetted the filter in a circular area approximately 1 cm in diameter. When the filter was then incubated at −99.5 MPa and illuminated (photon flux density of 300 μmol of photons m^{-2} s^{-1}) at 32°C, it was dry to the touch within less then 3 min. When the filter was saturated with liquid medium before the immobilization of the same quantity of cell suspension, the support appeared dry within 30 to 40 min. Generally, filters that supported different quantities of cell suspension (between 0.16 and 2.7 g [wet weight]) achieved a constant weight after 80 to 90 min of incubation at −99.5 MPa (data not shown).

Uptake of $^{35}SO_4$ by immobilized cells. The total intracellular pool of ^{35}S in cells that were immobilized immediately after the addition of $^{35}SO_4$ to a cell suspension increased during a drying period (ψ_m = −99.5 MPa) of 4 h (Fig. 1). The trend involved a slight increase in the size of the pool during

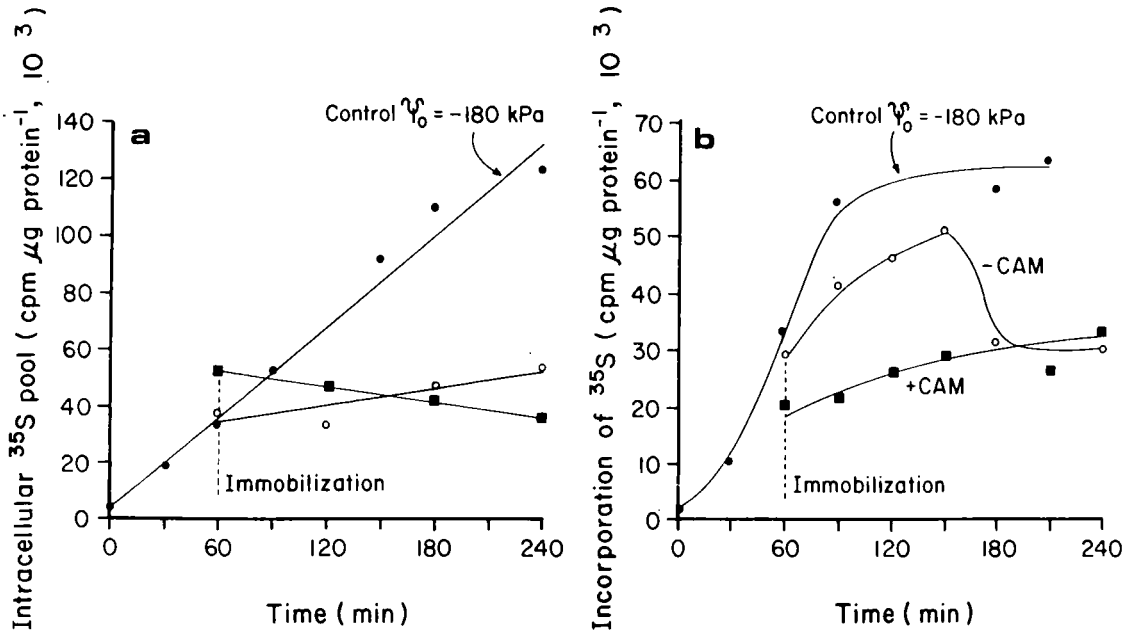

FIG. 2. (a) Change in the size of the total intracellular pool of ^{35}S after immobilization of cells at −99.5 MPa. (●) Increase in the total intracellular pool of ^{35}S in cells growing exponentially in liquid culture. (○) Size of the intracellular ^{35}S pool after 60 min of incubation in the presence of $^{35}SO_4$ and immobilization on dry filters at −99.5 MPa for different periods of time. Filters were washed extensively in BG-11_o (supplemented with 0.1 M $MgSO_4$). (■) Cells were immobilized on filters that were saturated with BG-11_o before immobilization of the cells. Filters were then washed extensively in BG-11_o (supplemented with 0.1 M $MgSO_4$) before measurements of radioactivity were taken. (b) Level of incorporation of ^{35}S in protein after immobilization of cells at −99.5 MPa. (●) Incorporation of ^{35}S by a suspension of cells in exponential growth. (○) Incorporation by cells after 60 min of incubation in the presence of ^{35}S and then immobilization on filters that were presoaked in BG-11_{o-s}. Incubation was at −99.5 MPa. (■) Cells were immobilized in the presence of 50 μg of chloramphenicol ml^{-1}.

the first 60 min of immobilization and then a linear increase during the following 2 h. After this time the pool size remained constant. After 3 h, the size of the pool was less than 10% of that measured in cells grown in liquid culture (ψ_o = −180 kPa) for 60 min before immobilization. In this case, the size of the pool was maintained after immobilization of the cells and during a 3-h period of drying at −99.5 MPa, although there was greater variability between measurements. The data illustrated in Fig. 1 were obtained when the size of the total intracellular pool of ^{35}S was measured after the cells had been washed extensively in distilled water at 4°C (see Materials and Methods). When BG-11_o (supplemented with 0.1 M $MgSO_4$) was used in the washing process, the variability in readings, particularly in cases where the pool size was large, was far less (Fig. 2a). In addition, an increase in the pool size was observed during drying when prelabeled cells were immobilized on dry filters, whereas the pool size decreased in the same material when the cells were immobilized on filters that were saturated previously with BG-11_o. In contrast to the pattern of $^{35}SO_4$ uptake shown by immobilized cells (Fig. 1) after the addition of $^{35}SO_4$ to a suspension of cells in exponential growth, there was a linear increase in the size of the intracellular pool of ^{35}S with no lag during a 4-h incubation (Fig. 2a).

Protein synthesis by prelabeled immobilized cells. When cells were grown in the presence of $^{35}SO_4$ for 60 min and then immobilized on dry filters and dried rapidly, the cells continued to incorporate ^{35}S into protein for approximately 90 min, after which time a net decrease in the level of incorporation was observed (Fig. 2b). This incorporation of ^{35}S into protein was inhibited by chloramphenicol. The maximum level of incorporation was detected in immobilized cells at a time when, as judged from dry weight measurements, they had lost the bulk of their associated water. In one experiment, the level of incorporation at time 150 min, that is, after 90 min of drying, was higher than that measured in cells grown in liquid culture for an equivalent period. The level of incorporation in the dried cells fell to preimmobilization levels after 130 min at −99.5 MPa. A high level of incorporation was still detectable after 24 h of drying.

Protein synthesis of unlabeled immobilized cells. When cells were immobilized immediately after the addition of $^{35}SO_4$ to the suspension and then subjected to a matric water potential (ψ_m) of −99.5 MPa, no significant incorporation of ^{35}S into protein was detected during a 4-h incubation period. A low level of incorporation was detected during the 60 min after immobilization of cells when the filter supports were kept moist with periodic additions of BG-11_{o-s} ($\psi_m + \psi_o$ = −180 kPa). After 60 min of incubation the level of incorporation then decreased. This initial increase in the level of incorporation represented only 7% of that detected during the same time period when the cells were incubated under osmotic conditions with aeration (i.e., in suspension; ψ_o = −180 kPa).

Proteolysis during equilibration of immobilized cells. In view of the results of incorporation experiments (see above), a qualitative electrophoretic analysis was made of the proteins present in immobilized cells during short- and long-term drying at −99.5 MPa.

Figure 3 illustrates the patterns which were obtained after electrophoresis of solubilized proteins extracted from control cells and material dried at −99.5 MPa for either 2 or 24 h. No obvious differences could be detected among the three samples. The most prominent proteins were the phycobiliprotein subunits located in the lowest region of the gel and a 50K protein. When identical gels were treated with silver stain, four bands, corresponding to 141, 158, 186, and 206K proteins, were obvious in the protein extract from cell

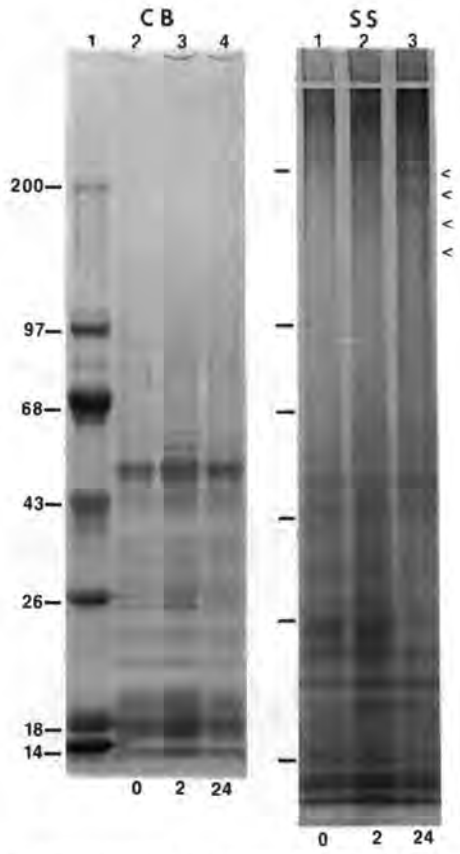

FIG. 3. Polyacrylamide gel electrophoretic analysis of solubilized protein extracts obtained from cells in liquid culture and the same material immobilized and dried at −99.5 MPa for either 2 or 24 h. Gels were stained with either Coomassie brilliant blue R (CB) or silver stain (SS). CB lanes: 1, molecular weight standards; 2, control; 3, cells dried for 2 h; 4, cells dried for 24 h. SS lanes: 1, control; 2, cells dried for 2 h; 3, cells dried for 24 h.

material desiccated for 24 h in the light. These bands were not apparent in protein extracts from control material or material desiccated for 2 h in the light. Streaking and smearing of bands were observed upon electrophoresis and fluorographic analysis of extracts obtained from cells that had been labeled with ^{35}S and then immobilized and dried at −99.5 MPa for 17 h (Fig. 4). Further, in comparison to the patterns obtained after electrophoretic analysis of control material, the following changes were observed: a decrease in intensity of a band at 59K, a decrease in mobility of a protein of 20K, the appearance of a new band at 46K, and an increase in intensity of a band that corresponds to a 17.4K biliprotein. Smearing and streaking in gel lanes were pronounced after the analysis of protein extracts taken from material that had been desiccated for 15 days (data not shown). In this material only some dozen proteins were apparent after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The majority of these proteins appeared to have decreased mobilities as judged from a comparison of the banding pattern with that obtained for control samples. The most obvious protein was a 60K protein.

Changes in phycobilisomes during drying of immobilized cells. As noted previously (25), visible differences in color were apparent in cells that had been stored under different conditions during desiccation. The colored phycobiliproteins represent a major fraction of the protein present in cyanobacterial cells. An analysis was made, therefore, of the effects of drying on the phycobilisome content of immobilized cells.

After cell disruption and treatment of the extracts with Triton X-100, supernatants from cells that were desiccated for 18 days, either in the light or the dark, appeared purple to grey-blue, in contrast to the supernatant of the control, which was brown-red. After centrifugation at 31,000 × *g* for 30 min, the supernatants from the control and dark-stored cells were both blue-purple in color, whereas the supernatant from the light-stored cells was bright pink.

The absorbtion spectrum of these samples revealed three major peaks at 564, 614, and 660 nm. These correspond to the absorption maxima of three phycobiliproteins: phycoerythrin, phycocyanin, and allophycocyanin, respectively. The content of phycobiliprotein in desiccated cells was lower than that found in control cells. In addition, the phycobiliprotein content of light-stored cells was lower than that of dark-stored cells.

The results obtained when these supernatants were subjected to sucrose density centrifugation are illustrated in Fig. 5a. A shallow green layer of solubilized membrane material was observed at the surface of the control gradient. This layer was less obvious at the surface of gradient D (cell material desiccated in the dark) and was not apparent in gradient L (cell material desiccated in the light). A series of four bright blue bands, corresponding to intact phycobilisomes, was observed in the 0.6 to 0.8 M sucrose region of the control gradient. Three bands were observed in gradient D; in relation to the control, the banding pattern showed a decrease in polydispersity and sedimentation rate. These blue bands were absent from gradient L. No obvious qualitative differences were observed between the electro-

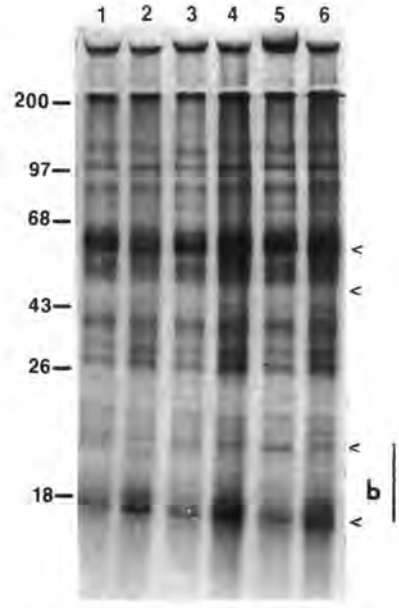

FIG. 4. Fluorographic analysis of solubilized protein extracts obtained from cells grown in suspension in the presence of ^{35}S for 4 h (26,816 cpm per μg of total protein) and from the same material after immobilization, rapid drying, and incubation at −99.5 MPa for 17 h. The biliprotein subunits are indicated (b). The amount of radioactivity loaded in the well of each lane is indicated. Control lanes: 1, 3, and 5; 51,351, 51,351, and 102, 702 cpm, respectively. Cells dried for 17 h: lanes 2, 4 and 6; 56,421, 112,842, and 112,842 cpm, respectively.

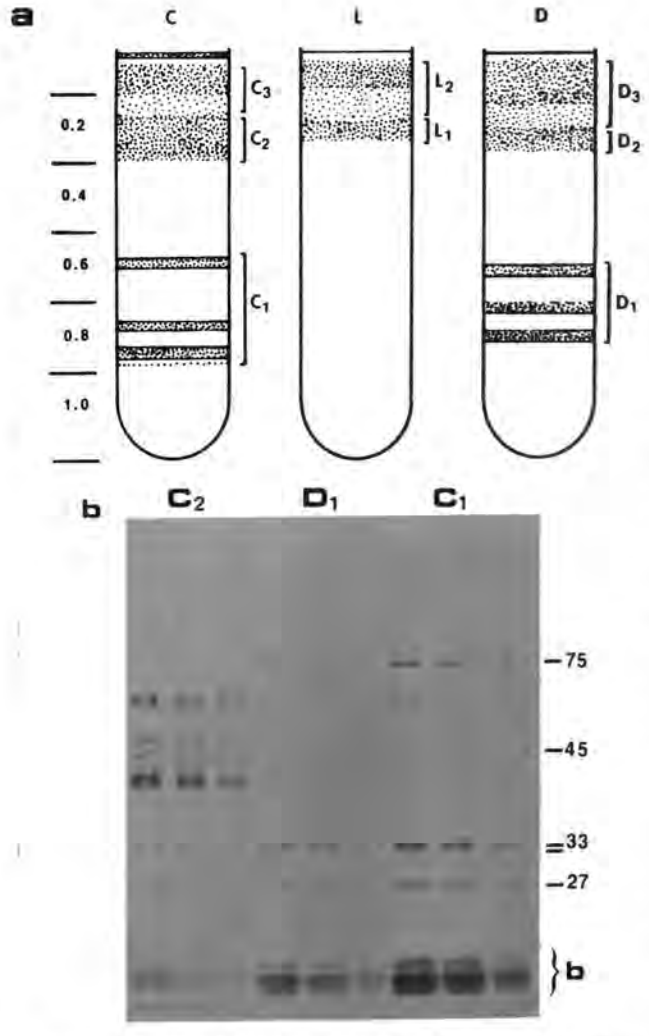

FIG. 5. Influence of desiccation on phycobilisomes. (a) Sucrose density gradient analysis of extracts: C, control material; L, cell material desiccated in the light; D, cell material desiccated in the dark. (b) Polyacrylamide gel electrophoretic analysis of fractions C_1, C_2, and D_1. Samples were analyzed on a 10% (wt/vol) acrylamide gel, and gels were stained with Coomassie brilliant blue R. The molecular masses (in kilodaltons) of the major phycobilisome linker polypeptides are indicated. The biliprotein subunits (b) are located in the lower portion of the gel photograph.

phoretic profiles of solubilized phycobilisomes isolated from gradient D (D_1) or the control (C_1; Fig. 5b).

A diffuse zone of free phycobiliproteins and some membrane material occupied the upper region of all three gradients. In each gradient this zone appeared to have two components: an upper portion which was pink (fractions C_3, L_2, D_3) and a lower portion which was purple-pink (fractions C_2, L_1, D_2). The lower limit of the zone corresponded approximately with the lower part of the 0.2 M sucrose layer (Fig. 5a). Fractions obtained from the lower portion of the zones in gradients L and D contained relatively large amounts of free phycobiliproteins (Fig. 6). The same fraction from the control gradient contained a very small proportion of phycoerythrin and phycocyanin, but approximately the same amount of allophycocyanin as seen in the equivalent fractions from the other two gradients (lane C_2, Fig. 5b). The upper region of the control gradient was enriched in allophycocyanin, whereas free phycoerythrin and phycocyanin dominated the upper region of gradients L and D.

DISCUSSION

When cells alter the synthesis of their proteins in response to a particular stimulus, it is essential to distinguish between changes that may be considered a consequence of cell damage or senescence and those that may represent a true adaptation of the cell (1, 36). In this respect, the influence of water stress on gene expression is poorly understood. Osmotic upshifts in *E. coli* have been shown to induce the *hop* regulon as well as cause changes in the abundance of membrane proteins; the hop products are distinct from the heat-shock proteins. The effects of osmotic water stress on cyanobacteria are currently the focus of extensive investigation (5, 22, 29, 30). Studies on the effects of matric water stress on these microorganisms are few (6, 25, 27). Desiccation tolerance and matric water relations of cells have been studied almost exclusively in eucaryotic systems and especially thoroughly by Bewley and co-workers (1, 23, 24, 34).

Whereas our previous work demonstrated the marked capacity of *N. commune* to tolerate desiccation and acute water stress, the observation of a relatively high rate of incorporation of ^{35}S into protein in cells that had been interrupted during logarithmic growth in liquid culture, immobilized, and then dried rapidly at −99.5 MPa was surprising. Superficially, this response would seem to typify the sluggish shift that these microorganisms often undergo in response to pronounced fluctuations in growth conditions (7, 11). One-dimensional electrophoresis could not detect any marked accumulation of specific proteins after 2 h of drying. In addition, two-dimensional fluorographic analysis indicates there are few qualitative changes in the pool of ^{35}S-labeled protein after rapid drying and incubation at −99.5 MPa for 30 min (unpublished data). These findings are similar to those observed for desiccation-tolerant mosses (24). Moss proteins are remarkably stable in vivo during desiccation and rehydration, with no selective loss as a consequence of the stress. However, a previous study demonstrated that nitrogenase activity in *N. commune* ceased within 20 min of immobilization (25, 26); heterocysts seem more sensitive to water stress than vegetative cells (3, 25). It is not known whether nitrogenase is destroyed or inactivated during rapid drying, or whether another component of the nitrogen-fixing complex is affected. In the case of a rapid-dried moss, drying may take 15 to 60 min, and this is thought to be insufficient time to permit the synthesis of new proteins (24). Nevertheless, it has been suggested that an accumulation of hydrophobic proteins (rich in -SH groups) may maintain a reduced environment within cells; this, in turn, may prevent protein aggregation or denaturation (35). The choice of isotope (^{35}S, ^{14}C, or ^{3}H) may therefore be an important consideration in the design of experiments to assess the occurrence of proteins that show either sensitivity or tolerance to matric water stress.

As shown in this study, sulfate uptake, an energy-dependent process, can occur after immobilization and during drying of cells, although at a markedly lower rate than measured in cell suspensions. In experiments where incorporation of ^{35}S into protein was shown to persist for some time after immobilization and rapid drying of the cells, it is clear that the ^{35}S was derived from the intracellular pool. As such, it is possible that the finite pool size of ^{35}S was one factor limiting the rate of incorporation of ^{35}S into protein during drying of the cells.

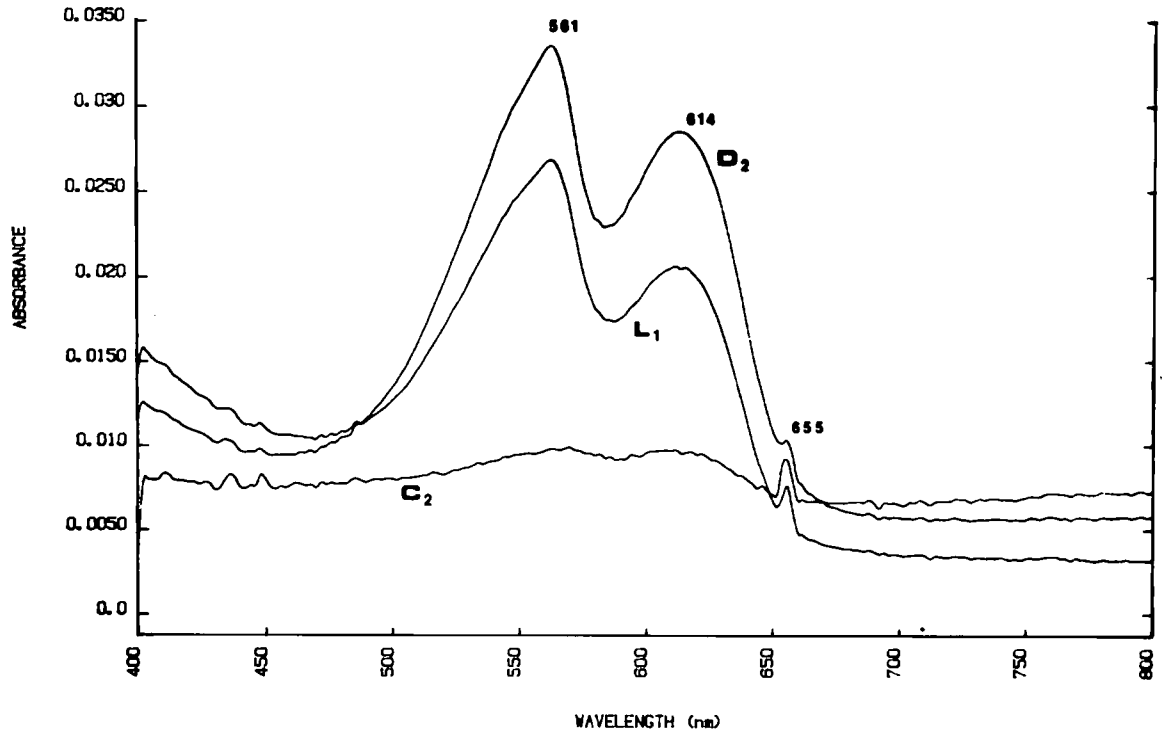

FIG. 6. Absorption spectra of fractions C_2, L_1, and D_2 obtained from sucrose density gradients (see legend to Fig. 5a).

After 90 min of drying the first indication of proteolysis—at least of ^{35}S-labeled protein—was observed. After this time it is difficult to assess the relative importance of proteolysis due to desiccation or proteolytic effects due to photooxidation. Clearly, extended desiccation leads to pronounced protein degradation in response to light-dependent effects, but, as pointed out above, it is hard to assess the potential significance of such findings when one considers the response of natural populations of cyanobacteria to desiccation (25, 32). In both light- and dark-stored cells there is an increase in free phycobiliproteins. After desiccation of cells for only a relatively short period, there is an obvious increase in the level of a 17K protein, possibly α-allophycocyanin (Fig. 4). Although intact phycobilisomes appeared to be sensitive to desiccation, relatively large quantities of phycobiliproteins were still detected in dark-desiccated cells. Previous studies have indicated that such cells also remain intact after extensive and prolonged photoxidation, and their activity (nitrogenase; ATP generation) is restored by rewetting and after a lag that involves protein synthesis (25, 33; Potts et al., in press).

The present study demonstrates that protein synthesis is maintained at a high level in *N. commune* during the time cells are subjected to acute water stress, but no unique class of proteins appears to be synthesized. In this latter respect the response of *N. commune* to matric water stress (a shock) is different from the responses shown to heat shock and osmotic shock by other microorganisms (4, 8, 9, 21). It should be noted, however, that this study was concerned with the effects of rapid drying on cells in logarithmic growth. In other systems, slow drying has been shown to have more damaging effects on cells (1, 23, 24).

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The protein index of *Nostoc commune* UTEX 584 (cyanobacteria): changes induced in immobilized cells by water stress

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Abstract. Two-dimensional gel electrophoresis was used to analyze the effects of water stress (immobilization and rapid drying, desiccation, rewetting) on the protein index of the desiccation-tolerant cyanobacterium *Nostoc commune* UTEX 584. Five major "landmark" protein constellations were detected in the protein index of control cells (in liquid culture) and were designated A (1 protein), B (7 proteins), C (8 proteins), D (3 proteins) and E (2 proteins). These included proteins which showed different sensitivities to water stress. Upon immobilization and rapid drying of the cells at a water potential (Ψ_m) of −99.5 MPa (a_w = 0.5) for 30 min, few changes took place in the index. Four conspicuous proteins and the majority of proteins in the size range 18 to 97 K diminished in abundance while most proteins of constellations A, B and C were detected in fluorographs with the same intensity as in the control. Although protein synthesis continued during this time of drying, no novel class of proteins was detected. The level of incorporation of ^{35}S in protein increased rapidly during the first 60 min of rehydration, and then decreased gradually for a further 2.5 h. Extant proteins that were hardly detectable after 24 h of drying, reappeared and increased in abundance upon rewetting of cells for 60 min while a number of proteins which disappeared after drying did not appear during this time. No novel class of proteins appeared upon rewetting. During further rehydration, extensive proteolysis was observed.

The *nifH* product (Fe protein of nitrogenase) was detected on Western blots – through cross-reaction with antibody – as an acidic polypeptide with a molecular mass of 33.8 K. Fe-protein was detected in immobilized cells after 30 min of drying, in desiccated material, and in rehydrated cells.

Key words: Immobilized cells – Gene expression – Regulon – *nifH* protein – Desiccation – *Nostoc commune*

In response to stress, both prokaryotic and eukaryotic cells may express a particular set of coregulated genes that share a common regulator molecule (Ingraham et al. 1983; Neidhardt and Phillips 1984; Epstein 1983; Clark and Parker 1984). The products of such coordinate multigene responses are either absent, or present at a very low level, when the cells are grown under 'normal' (balanced growth) conditions. A comparison of the protein catalogs of stressed and normal cells should, therefore, make it possible to identify the numbers and types of gene products involved in a particular stress response. The technique of 2-dimensional gel electrophoresis has made it possible to analyze such complex mixtures of proteins with a high degree of resolution (O'Farrell 1975). Through the use of this technique, protein catalogs have been compiled for both eukaryotic and prokaryotic cell types (Neidhardt and Phillips 1984; Bravo and Celis 1984). The catalog of *Escherichia coli* is particularly comprehensive and the construction of a gene-protein index now seems feasible (Neidhardt et al. 1983). With respect to "stress" responses of cells, considerable data have accumulated on the heat-shock response (Grossman et al. 1985), a response which has been described in both eukaryotic and prokaryotic systems. The further study of the heat-shock regulon is likely to yield valuable information on the nature of gene expression *per se*.

Water availability limits the distribution and activities of all prokaryotes and eukaryotes, and it is clear that water stress can influence gene expression significantly (Bewley and Oliver 1983). Of those prokaryotes which express a marked capacity to withstand extremes of matric and osmotic water stress, the cyanobacteria warrant particular attention in view of their ecological and evolutionary significance (Carr and Whitton 1982). Recently, there have been considerable advances in the study of the molecular biology of these microorganisms – particularly of those forms which lend themselves readily to cultivation under "defined" laboratory conditions (Kuhlemeier et al. 1983, 1984; Golden et al. 1985; Tomioka et al. 1981; Pilot and Fox 1984; Curtis and Haselkorn 1984). The regulation of gene expression in cyanobacteria remains, however, poorly understood in comparison with the heterotrophic Eubacteria.

In previous studies we have described methods for the immobilization and desiccation of different strains of cyanobacteria (Potts and Friedmann 1981; Potts et al. 1983, 1984) and have investigated the effects of acute water stress on nitrogen fixation (Potts and Bowman 1985), intracellular ATP levels (Potts and Morrison 1986) and protein synthesis (Potts 1985). These studies suggested that the drying and rewetting of immobilized cells could be used with effectiveness in the study of gene expression in cyanobacteria. This communication analyses changes in the protein index of the desiccation-tolerant, nitrogen-fixing strain *Nostoc commune* UTEX 584, in response to acute water stress.

Materials and methods

Conditions for the growth of cells, the labelling of proteins in vivo and the immobilization of cells are described in the

Abbreviations. PMSF, Phenylmethylsulfonyl fluoride; IEF, Isoelectric focussing

primary publications (Potts 1985; Potts and Bowman 1985; Potts et al. 1984; Potts and Morrison 1986). A quantitative analysis of the kinetics of protein turnover in immobilized cells subjected to matric water stress is given in Potts (1985).

Antibodies

Serum antibodies directed against component II (Fe protein) of the nitrogenase from *Rhodospirillum rubrum* were provided kindly by Dr. Paul Ludden, University of Wisconsin-Madison.

Breakage of Cells

Cells of *Nostoc commune* UTEX 584, especially when dried, resist many of the general methods used for the physical or chemical disruption of cells. Of the many methods we have tested, only the grinding of cells in a pestle and mortar with alumina (type 305; Sigma Chemical Co.) achieves efficient breakage of desiccated material. The ratio of cells:alumina:buffer is critical for the efficient extraction and solubilization of proteins and must be determined empirically. Alumina and extraction buffer (see below) were added simultaneously to cell material which was then ground vigorously until a homogeneous paste formed.

Extraction of soluble proteins

For the extraction of soluble proteins, the mortar was chilled and the ice-cold extraction buffer (50 mM Tris-HCl, pH 7.8, 20 mM KCl, 10 mM $MgCl_2$; modified after Oliver and Bewley 1984c) was supplemented with 1 mM PMSF, 50 μg ml^{-1} DNAse and 50 μg ml^{-1} RNAse (Sigma Chemical Co.). After centrifugation at 12,000 × *g*, for 15 min at 4° C (SS 34 rotor, RC-5B centrifuge, DuPont Instruments), the clear supernatant was mixed thoroughly with an equal volume of 20% w/v trichloroacetic acid (TCA) and kept on ice for 75 min. The precipitate was collected by centrifugation and was washed twice in 5% w/v TCA. After a subsequent wash in ice-cold acetone:water (50:50 v/v) and then ether, the pellet was dried under a stream of air.

For 2-dimensional electrophoretic analysis, the proteins were solubilized in IEF lysis buffer (9.5 M urea, 2% Nonidet NP-40, 2% ampholines (0.8% pH 5–7, 0.8% pH 6–8, 0.4% pH 3–10), 5% v/v β-mercaptoethanol), for 30 min, at room temperature. Samples were subjected to brief sonication to ensure complete dispersal of particulate protein aggregates. The solution was then cleared by centrifugation. An aliquot of the solution (usually 2 to 5 μl) was mixed thoroughly with Biofluor cocktail (New England Nuclear, Boston, MA), and the level of radioactivity was measured in a Packard Tri-Carb model 2405 liquid scintillation counter. Measurements were corrected for the quenching due to urea. The remainder of the protein solution was either used immediately or stored at −70° C until needed.

For 1-dimensional SDS-PAG electrophoretic analysis, the protein pellet was solubilized at room temperature in "cracking" buffer [0.2 M Tris, (pH 8.8), 2% w/v sodium dodecyl sulfate, 1 M β-mercaptoethanol, 15% v/v glycerol, 0.01% w/v bromophenol blue].

Total protein

Total proteins were solubilized directly by the grinding of cell material in the presence of alumina and the appropriate buffer (see above). The slurry was then centrifuged at 12,000 × *g* for 15 min.

Two-dimensional gel electrophoresis

Two-dimensional electrophoresis of proteins was accomplished using the procedure described by O'Farrell (1975). First-dimension electrofocussing tube gels were 110 × 2.5 mm diameter with the following composition: 9 M urea, 4% w/v acrylamide, 2% v/v Nonidet NP-40 and 2% ampholines (2:2:1 of pH 5–7, 6–8, 3–10 respectively). The gels were prefocussed before the protein samples were applied to the basic end of the gel. In several cases the samples were mixed, prior to loading, with a small quantity of Sephadex (Pharmacia) in lytic buffer, to prevent any precipitated protein from clogging the basic end of the gel. The sample was then overlaid with a solution of 9 M urea and 1% v/v ampholyte mixture. Equivalent amounts of radioactive material from different samples were applied to IEF gels. Gels were focussed for 20 h at a constant voltage of 300 V and then for 1.5 h at 400 V. In later experiments this was modified to 300 V for 18 h then 800 V for 3 h. After focussing a flat membrane pH micro-electrode (model MI-404, Microelectrodes Inc., Londonderry, NH) was used to determine the pH gradient in one of the gels. The sample gels were then incubated in equilibration buffer (O'Farrell 1975) and either used immediately, or stored at −20° C until needed. Tube gels were applied to vertical slab gels and molecular sieving in the second dimension was accomplished through SDS-PAG electrophoresis (Laemmli 1970) in a Protean chamber (version 1 or 2: BioRad Labs, Richmond, CA). Gels were 1.5 mm in thickness with either a 10 or 12% w/v concentration of acrylamide in the monomer solution. Molecular weight markers were obtained from Sigma Chemical Co. (products SDS-7 and SDS-6H). For certain slab gels, acrylamide was substituted for bis-acrylamide, and the gel was bound to Gelbond PAG which was used according to the manufacturer's instructions (FMC Corporation, Rockland, ME). After electrophoresis, gels were either stained, processed for fluorography, or processed for Western blotting.

Staining of gels

Gels were stained with coomassie brilliant blue R, destained, photographed, destained exhaustively, silver-stained and photographed (Merril and Goldman 1984).

Fluorographic detection of labelled proteins

Gels were incubated overnight in methanol:acetic acid (40:10 v/v) and were then impregnated with Enhance solution (New England Nuclear, Boston, MA), dried in a vacuum oven and the polypeptides were detected with Kodak X-Omat AR (XAR-5) film after exposure at −70° C.

Western blotting

Proteins were transferred from polyacrylamide gels to nitrocellulose sheets using an electrophoretic technique which is described by Symington (1984). The detection of antigen-antibody complexes employed protein A-Horseradish Peroxidase conjugate which was used according to the manufacturer's recommendations (BioRad Labs, Richmond, CA).

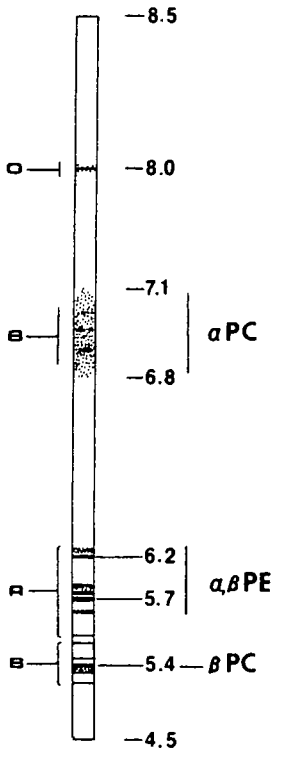

Fig. 1
Distribution of colored bands after iso-electric focussing of protein extract from control material. Numbers indicate pH values. *B* blue, *O* orange/brown, *R* red/pink; *PC* phycocyanin, *PE* phycoerythrin

Nomenclature

In view of the general paucity of information on peptide mapping in cyanobacteria, any system of nomenclature for describing the pattern of proteins detected in a 2-D gel is, by necessity, provisional. Consequently, the approach of Neel et al. (1984) has been followed. Briefly, the 2-D gels have been subdivided into a series of constellations each of which contains a recognizable "landmark spot(s)" (or streaks). Each constellation has been designated a letter, and major spots within a constellation have been numbered from left to right and top to bottom e.g. A-005 etc.

Results

Iso-electric focussing of proteins from cells in exponential growth gave a distinctive pattern of colored bands which was clearly visible (14 cm gels; 18 h at 400 V, then 3 h at 800 V) without staining (Fig. 1). A similar pattern of colored bands was observed when protein extracts from material which had been desiccated for 5 years was iso-electric focussed. The orange band (O) at pH ~ 8.0 corresponded to a polypeptide(s) which was detected as a diffuse area with an apparent molecular mass of 14 K (Fig. 2b). The colored subunits of the major phycobiliproteins were visible, prior to staining, in gels after molecular sieving in the second dimension (Fig. 2a, b) and also after Western transfer to nitrocellulose sheets. In the latter respect, these colored polypeptides served as useful orientation markers during analysis with antibody probes. Individual phycobiliprotein subunits were identified by their molecular mass, color and apparent iso-electric point. The protein index shown in Fig. 2a was the result of staining with coomassie brilliant blue R and was reproducible in numerous trials. Silver stain revealed a significant number of additional polypeptide spots (Fig. 2b), among which was a conspicuous acidic polypeptide of around 50 k. The most obvious proteins comprised four constellations which were designated B, C, D and E (Fig. 2b). In the gel which is represented by Fig. 2a, the β subunit of allophycocyanin is obscured by the intense staining of the β subunit of phycocyanin. Immunostaining with antibody directed against the nifH product (component II; Fe protein) of *Rhodospirillum rubrum* nitrogenase gave a single band in 1-dimensional Western blots, and a single spot on 2-dimensional Western blots. The reaction involved a single polypeptide with a molecular mass of 33.8 K and an isoelectric point between pH 4.5 to 5.5. The omission of agarose when IEF gels were applied to slab gels permitted the high resolution of low molecular mass proteins (note cluster below allophycocyanin).

The most conspicuous constellations which were detected with either coomassie blue or silver stain (Fig. 2a, b) were also detected after fluorographic analysis of cell extracts in which the proteins had been labelled in vivo with ^{35}S (Fig. 3a). Differences were, however, apparent. For example, protein A-001 was not detected in gels after coomassie or silver staining and, although phycobiliproteins represent the largest proportion of soluble protein in cells (Fig. 2b), the level of incorporation of ^{35}S in biliproteins was comparatively low in comparison to other less abundant polypeptides. For this reason, the ampholyte composition of IEF gels was adjusted when labelled proteins were to be analyzed, to permit the greatest resolution of the most heavily labelled proteins. The four constellations A, B, C and D, which were detected clearly by fluorography, included 17 of the most extensively labelled and/or most abundant proteins.

Chloramphenicol-sensitive protein synthesis was shown to persist for more than 90 min after the immobilization and rapid drying of cells at −99.5 MPa (Potts 1985). Figure 3b shows a fluorograph of the protein index of cells which had been labelled in exponential growth (as in Fig. 3a) and then immobilized, dried rapidly and incubated at −99.5 MPa for 30 min. Four conspicuous proteins (circled in Fig. 3a) diminished considerably as did the majority of the less conspicuous proteins in the size range 18 to 97 K. Of the most obvious proteins present in the control, the high molecular weight protein A-001(209 K) and the six proteins of constellation C showed no change when cells were dried for this time, while the acidic protein D-001 decreased slightly. No *de novo* proteins were detected during this 30 min period of drying – a period when measurements indicated that the cells continued to incorporate ^{35}S into protein at an elevated rate (Potts 1985). Immunoanalysis with nitrogenase Fe protein antibody detected a single cross-reactive polypeptide in extracts from control cells, cells which had been dried rapidly over 30 min, cells desiccated for 3 h at −99.5 MPa, and cells dried similarly for 3 h and then subjected to rewetting.

An increase in the amount of streaking on fluorographs accompanied the decrease in the intensity of a large number of different proteins during 24 h of desiccation (Figs. 3b, 4a, b). In a second series of experiments, $Na_2{}^{35}SO_4$ was added to cell suspensions immediately prior to the immobilization and drying of cells to permit resolution of those proteins which were made during the time of drying. In this case, proteins were labelled extensively after 2 h of drying and the index was qualitatively identical to that shown in Fig. 3b. After 24 h of drying, a similar decrease in the intensity of extant spots, as described above (Fig. 4a, b), was observed (data not shown).

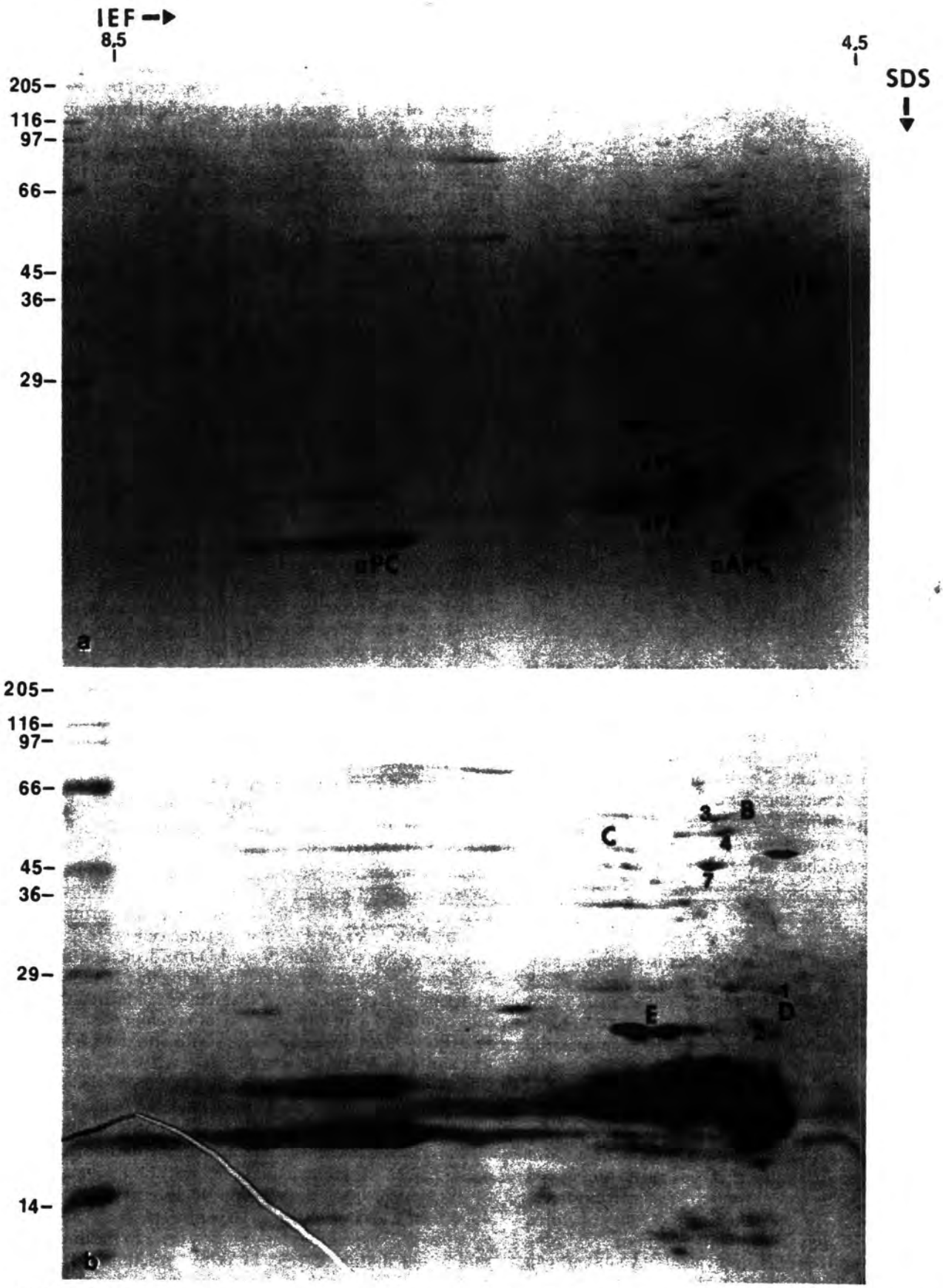

Fig. 2a, b. Protein index of *Nostoc commune* in exponential growth in liquid culture. **a** Gel stained with coomassie blue **b** same gel stained with coomassie blue then silver stain. *B, C, D,* and *E* are protein constellations (numbers refer to individual polypeptides); *APC* allophycocyanin, *PC* phycocyanin, *PE* phycoerythrin; *nifH* nitrogenase Fe protein. Protein markers (M_r = 14 to 205 K) were ran simultaneously with IEF gels

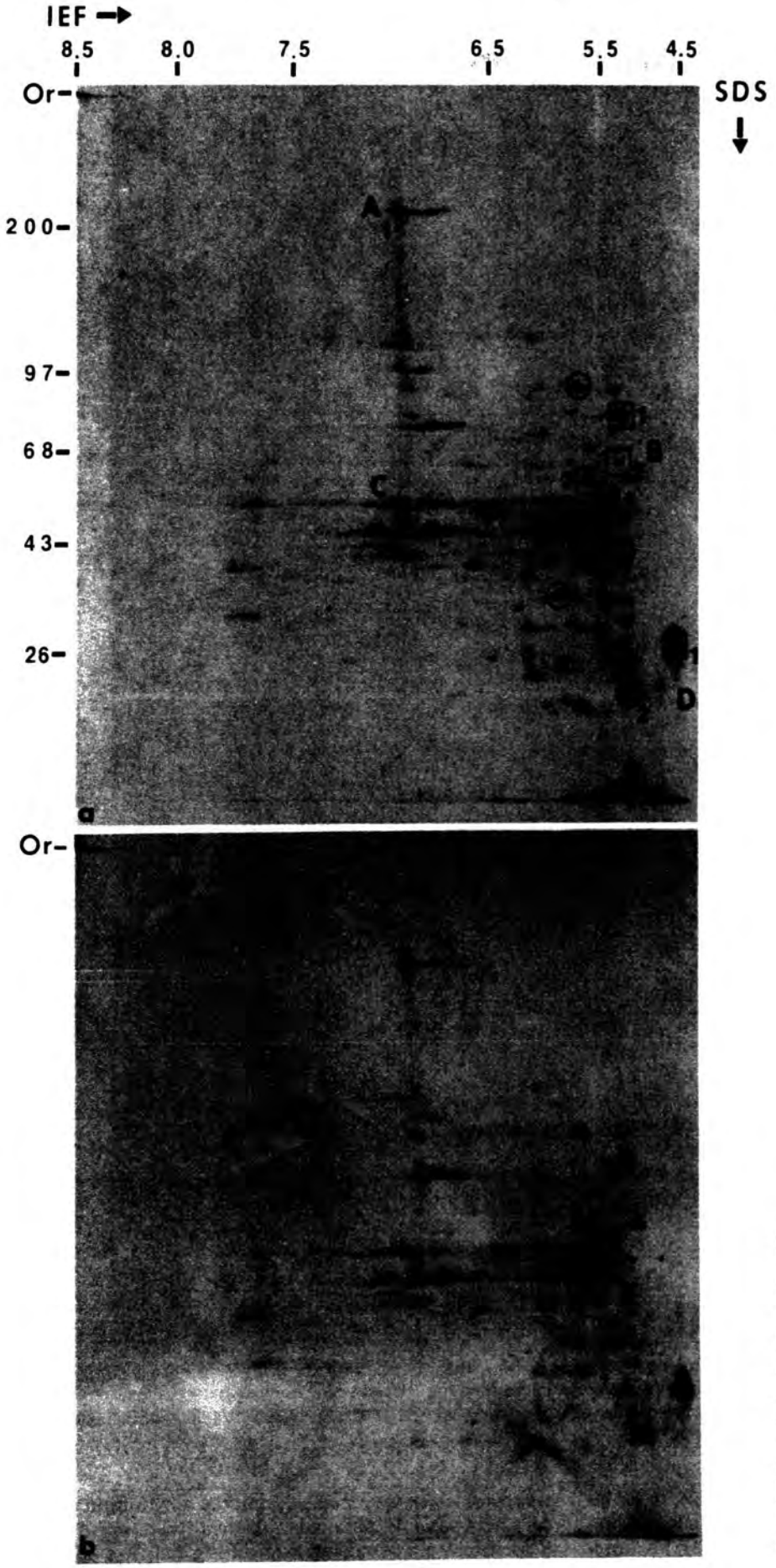

Fig. 3a, b
Fluorographic analysis of labelled proteins. **a** Protein index of cells in exponential growth in liquid culture. Letters designate protein constellations. Spots enclosed by a circle are examples of those which show a conspicuous change in intensity upon drying. Spots enclosed by squares show no or little change in intensity after immobilization of cells and rapid drying (see 3b). The sample size was 35 µl (63.084 cpm). **b** Protein index of immobilized cells exposed to −99.5 MPa for 30 min. The sample size was 27.4 µl (63.084 cpm)

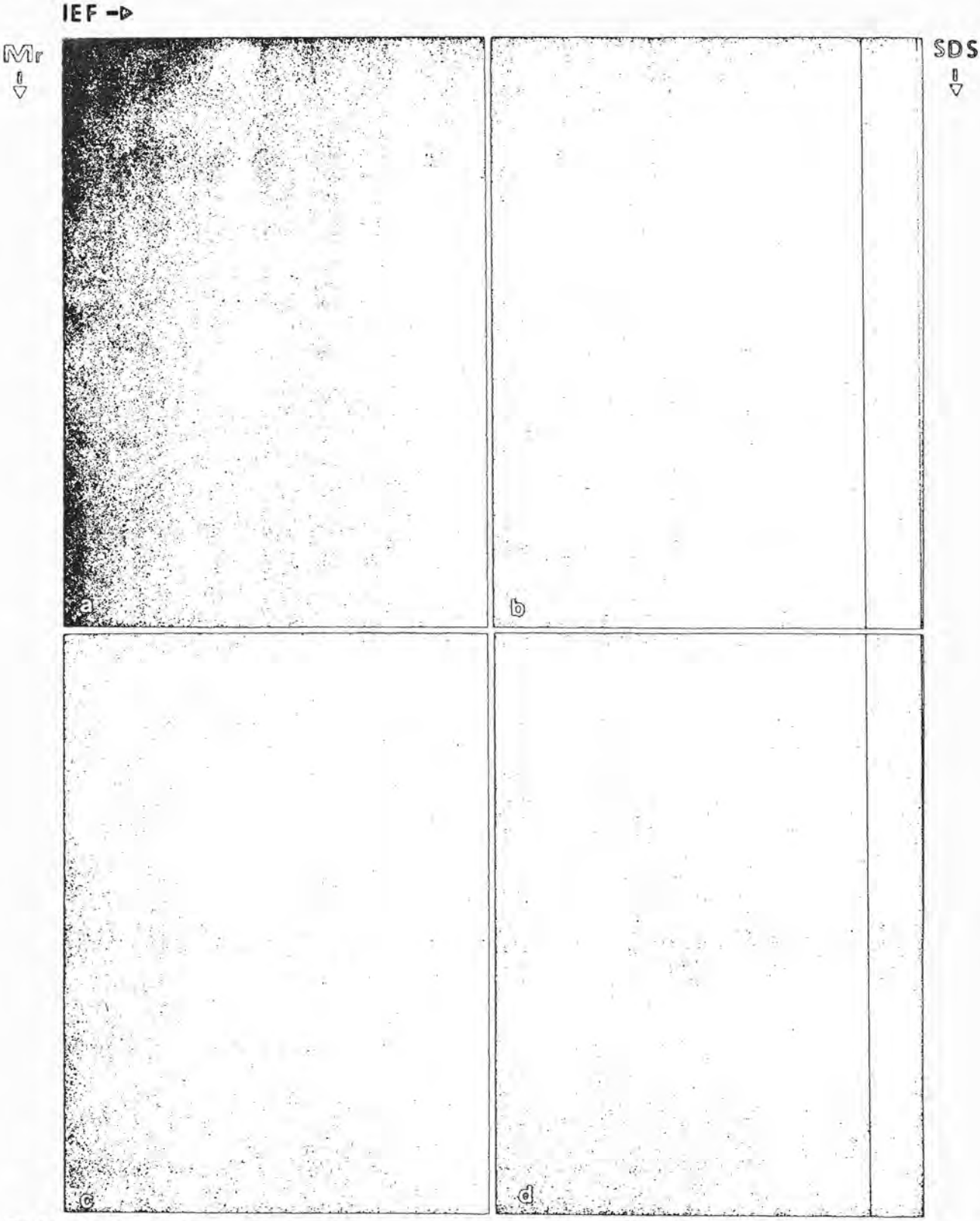

Fig. 4a–d. Changes in the protein index of cells in response to drying and rewetting. Equal amounts of radioactivity (20,000 cpm) were loaded on each gel. Letters refer to protein constellations. **a** Some conditions as in Fig. 3b except the drying time was 2 h at −99.5 MPa, in the light. **b** As for **a**; the drying time was 24 h. **c** Same material as **a**; the desiccated cells were rehydrated for 60 min, in the light, before proteins were extracted. **d** As for **c**; the rehydration time was 3 h

The "net" weight of desiccated, immobilized cells increased rapidly upon rewetting and after 10 min it represented more than 80% of the wet weight (Fig. 5). Measurements of the level of incorporation of ^{35}S in protein (protein turnover) which were made during the rehydration of these cells were variable, although a distinct trend was apparent. This involved a rapid increase during the first 60 min of rehydration, followed by a gradual decrease which con-

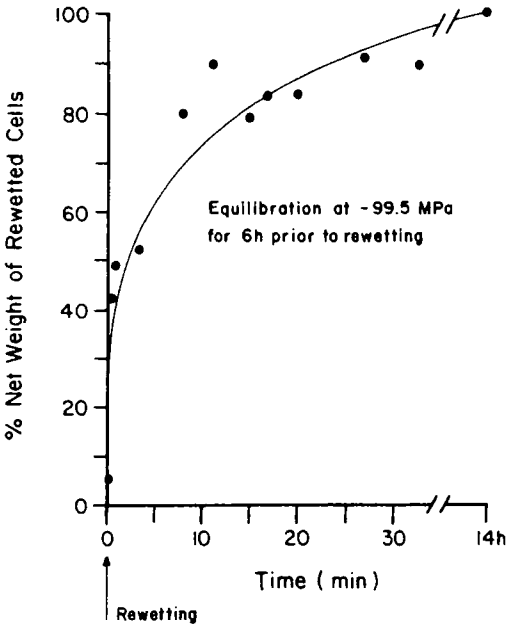

Fig. 5. Water uptake by immobilized, desiccated cells. The wet weight of cells before drying and immobilization was 2.74 g and the support was a hydrophobic nylon mesh. Rehydration was in the light, with distilled H_2O, at 32°C

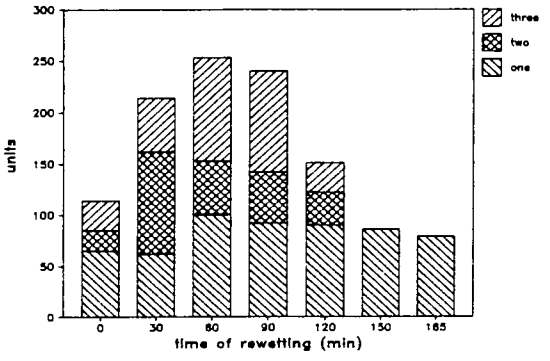

Fig. 6. Level of incorporation of ^{35}S in protein upon rehydration of desiccated cells. Cells were grown in the presence of $Na_2{}^{35}SO_4$ before immobilization at −99.5 MPa for 24 h. The material was rewetted with distilled H_2O, at 32°C, in the light and incorporation was measured with a technique described by Mans and Novelli (1961). The data, represented in a stacked-bar graph, were obtained from three separate experiments. For each experiment, the mean ($n = 3$) level of incorporation at each time point is expressed as a percentage of the maximum level of incorporation during the time of rewetting. Units = cumulative % of maximum incorporation

tinued for at least 2.5 h (Fig. 6). This trend was reflected in the protein indexes which were analyzed at different points during 3 h of rewetting (Fig. 4c, d). The result shown in Fig. 4d is the most extreme of those obtained for this time of rewetting. In retrials, the number of proteins detected was variable, although the trend was apparent i.e. an increase in the intensity of extant proteins (see C-001, C-002, C-004, C-006 and C-007), followed by a significant loss of labelled proteins upon prolonged rewetting. For the preparation of the four fluorographs which are illustrated in Fig. 4a, b, c and d, identical amounts of radioactivity were applied to IEF gels. Protein A-001 serves as an "internal standard" as the radioactivity in this polypeptide spot was found to vary only slightly during the different treatments.

A very low level of incorporation (over a time course of 3 h) was measured in desiccated cells which, instead of being labelled prior to immobilization, were rewetted in the presence of $Na_2{}^{35}SO_4$ (data not shown).

Discussion

The rapid drying and immobilization of *Nostoc* cells, and the rewetting of desiccated material lead, respectively, to marked downshifts and upshifts in cellular activity (Potts and Bowman 1985; Potts 1985; Potts and Morrison 1986). In other systems, such metabolic shifts are often accompanied by a change in the level of expression of genes which are often under the control of a common regulator (Ingraham et al. 1983; Hoch and Setlow 1985; Neidhardt et al. 1982). A typical heat-shock response has already been demonstrated for *Synechococcus* PCC 6301 (Borbély et al. 1985). The immobilization and rapid drying of *Nostoc* cells was accompanied, in the short-term, by only slight changes in the protein index, despite the detection of elevated rates of chloramphenicol-sensitive protein synthesis. Changes which take place during the prolongd drying of cells, especially in the light, are a consequence of protein degradation and not the result of specific changes in gene expression, although they are likely to have important consequences when the cells are rewetted. No new class of proteins were detected upon the rewetting of dried *Nostoc*, – the polypeptide spots which increased in intensity upon rehydration were either already present in the dried material, or they had been detected previously in control extracts.

In these respects, the data contrast with those obtained from studies directed at the effects of osmotic water stress on gene expression, although it must be noted that the effective water potentials which are produced through the addition of NaCl etc. to growth media are considerably higher than those which can be achieved in matric systems (Potts and Friedmann 1981; Potts et al. 1984). With cells of *Escherichia coli*, a decrease in the osmotic water potential (Ψ_o) of the growth medium leads to induction of the *hop* regulon and consequently an elevated rate of synthesis of more than three major proteins (Clark and Parker 1984). The differential synthesis of two major outer membrane proteins ompC and ompF is also influenced by Ψ_o. More specifically, it has been proposed that a 95 K polypeptide, the *kdpD* product, interacts with the promoter of the *kdp* operon in response to a rise in turgor pressure (see Epstein 1983). It is not known if similar controls operate in cyanobacteria in response to a change in Ψ_o, although a recent study does present evidence for a primary active, energy-dependent K^+ uptake system in both *Anabaena variabilis* and *Synechocystis* PCC 6714 that responds directly to changes in cell turgor pressure (Reed and Stewart 1985).

The response of *Nostoc commune* to water stress more closely resembles that shown by the gametophyte of a desiccation-tolerant moss, *Tortulu ruralis* (Oliver and Bewley 1984a, b, c; Bewley and Oliver 1983). Moss proteins in vivo were found to be very stable during desiccation and rehydration with no selective loss as a consequence of water stress. Upon rehydration of desiccated cells, there was an increase in synthesis of a number of proteins (termed rehydration proteins), that in control cells were only just detectable. A role for the rehydration proteins as protective

agents against desiccation was considered unlikely; protein synthesis declined rapidly during water loss and there was considered to be no time for the moss to synthesize new proteins during drying (in the moss system rapid drying is achieved within 15–60 min). In addition, rehydration occurred within minutes, prior to the synthesis of significant amounts of rehydration proteins (Oliver and Bewley 1984c). A more likely role for these proteins was thought to be in repair, either as enzymes or structural proteins. Under the conditions imposed in this investigation the time required for immobilized cells of *Nostoc* to achieve their air-dry weight at −99.5 MPa (90 min), was virtually the same as for the moss gametophyte (Oliver and Bewley 1984a). However, immobilized cells of *Nostoc* continued protein synthesis during this time at a significant rate. During the first 30 min of drying, the level of incorporation of ^{35}S in protein (cpm μg total protein^{-1}) increased by some 30% in relation to the level in cells prior to immobilization. Therefore, when Fig. 3a and Fig. 3b are compared, it is clear that, for the 30 min that followed immobilization, the cells continued to synthesize a collection of proteins which can be considered as insensitive to rapid-drying (e.g. A-001, constellation B), while a number, such as D-002, are sensitive to rapid drying. During longer periods of drying, the different sensitivities of certain of these proteins becomes more apparent. For example, C-006 was still conspicuous after 24 h of drying while proteins which were detected with an equivalent intensity prior to drying (C-005, C-006), as well as the majority of the proteins which were labelled only faintly (Fig. 1a), all decreased significantly.

The events that take place upon rewetting are likely to be complex. After 1 h of rewetting, clear differences were observed in the sequence of reappearance of labelled proteins. After 3 h of rewetting, the most conspicuous proteins detected were those seen in control cells e.g. constellation B. Incorporation experiments demonstrated that there was a rapid initial incorporation of ^{35}S into protein, followed by a decline during a further 2 h of incubation. During outgrowth and germination of the spores of *Bacillus megaterium*, approximately 20% of the proteins present in the dormant spore are degraded to free amino acids during the first 20 min of germination (Szulmajster 1982). These amino acids are then used for new rounds of protein synthesis. The potential role of proteases during the rewetting of dried *N. commune* is under investigation.

The identities of the proteins which show differential sensitivities to water stress remain unknown. Nitrogen fixation is one process which diminishes rapidly when cells are subjected to a drying period of less than 30 min at −99.5 MPa (Potts et al. 1984; Potts and Bowman 1985). Although the *nifH* product is present in cells which have been subjected to rapid drying, desiccation and subsequent rewetting, it is not known at present if the *nif* proteins are inactive, whether *nif* mRNA is present in desiccated cells and available during rewetting, or whether another component of the nitrogen fixation system is subject to regulation by water stress.

In summary, the immobilization and rapid drying of axenic cultures of laboratory-grown *N. commune*, and the rewetting of dried cell material, do not induce the synthesis of "heat-shock" proteins or any distinct class of proteins which could be considered to represent a "water-stress" regulon *per se*. However, the orderly and sequential restoration of cellular functions upon the addition of water to desiccated cells (e.g. Potts and Bowman 1985; Scherer et al. 1984) does suggest a strict control over gene expression during rehydration. Further analysis of this system is likely to yield important information on the role of water in the control of gene expression.

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Variation in Phospholipid Ester-Linked Fatty Acids and Carotenoids of Desiccated *Nostoc commune* (Cyanobacteria) from Different Geographic Locations

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Profiles of phospholipid fatty acids and carotenoids in desiccated *Nostoc commune* (cyanobacteria) collected from China, Federal Republic of Germany, and Antarctica and in axenic cultures of the desiccation-tolerant strains *N. commune* UTEX 584 and *Hydrocoleum* strain GOE1 were analyzed. The phospholipid fatty acid contents of the three samples of desiccated *Nostoc* species were all similar, and the dominant compounds were 16:1ω7c, 16:0, 18:2ω6, 18:3ω3, and 18:1ω7c. In comparison with the field materials, *N. commune* UTEX 584 had a much higher ratio of 18:2ω6 to 18:3ω3 (5.36) and a significantly lower ratio of 18:1ω7c to 18:1ω9c (1.86). Compound 18:3 was present in large amounts in the samples of desiccated *Nostoc* species which had been subject, in situ, to repeated cycles of drying and rewetting, but represented only a small fraction of the total fatty acids of the strains grown in liquid culture. This finding is in contrast to the data obtained from studies on the effects of drought and water stress on higher plants. Field materials of *Nostoc* species contained, in contrast to the axenic strains, significant amounts of apocarotenoids and a P384 pigment which, upon reduction with $NaBH_4$, yielded a mixture of a chlorophyll derivative and a compound with an absorption maximum of 451 nm. A clear distinction can be made between the carotenoid contents of the axenic cultures and the desiccated field materials. In the former, β-carotene and echinenone predominate; in the latter, canthaxanthin and the β-γ series of carotenoids are found.

Species of the genus *Nostoc* are among the most widespread of all nitrogen-fixing cyanobacteria. Communities of *Nostoc commune*, in particular, are prominent in those terrestrial limestone environments of tropical, polar, and temperate regions which are subject to extremes of water availability (25). An Aldabra Atoll (Indian Ocean), for example, macroscopic colonies persist in a desiccated state for approximately 6 months of the year (25). Throughout the monsoon season, however, the colonies resume and then cease cellular activities repeatedly as intermittent rains lead to cycles of rewetting and drying.

Both field and laboratory studies confirm that *N. commune* cells have a marked capacity to withstand long periods of desiccation and extremes of water stress (18, 22, 25). Upon rewetting, desiccated cells of field materials resume respiration first, then photosynthesis, and finally nitrogen fixation (22). A lag of 4 to 5 h was noted before the steady-state intracellular ATP pool was reached upon rewetting of field materials (21). With axenic laboratory-grown cultures of *N. commune* UTEX 584 this lag was of greater duration (19). Recent studies with *N. commune* UTEX 584 have shown that water stress induces marked changes in the protein index, the amounts of polysomes, and levels of chloramphenicol-sensitive protein synthesis (1, 17; M. Potts, Arch. Microbiol., in press), although there is, at present, no evidence for a water-stress regulon.

These preliminary studies suggest that the drying and rewetting of *Nostoc* cells can be used effectively to study the effects of water stress, an important environmental variable, on gene expression. However, if the molecular basis for desiccation tolerance is to be understood fully, it must be determined how *Nostoc* cells maintain the key components of their transcriptional and translational apparatus in a functionally intact state during prolonged periods of desiccation.

A previous investigation showed that the purified cytoplasmic membrane of *N. commune* UTEX 584 was enriched in carotenoids and contained significant amounts of a single fatty acid, compound 20:3ω3 (13). The latter was present at a level of 56.8% of the total membrane fatty acids; a unique feature which sets the membrane apart from all other cyanobacterial membranes which have been characterized to date (9, 12, 14, 15).

To assess further those features of *Nostoc* cells which may play a role in desiccation tolerance, we analyzed the phospholipid fatty acids (PLFA) and carotenoids of desiccated colonies from diverse geographic locations. The data were compared with those we obtained after the analysis of laboratory-grown cells of *N. commune* UTEX 584 and a desiccation-tolerant marine cyanobacterium.

MATERIALS AND METHODS

Microorganisms. Desiccated colonies of *N. commune* were collected from field sites in Hunan Province, China, and Reichenau (Konstanz), Federal Republic of Germany, and were provided kindly by T.-W. Chen and S. Scherer, respectively. The third sample was collected from an area of coastal lowland adjacent to the Ross Ice Shelf (Antarctica). For discussion purposes these three samples are referred to as *Nostoc* strain HUN, *Nostoc* strain REICH, and *Nostoc* strain ANT, respectively. At the time these samples were analyzed (see below), they had been stored in a desiccated state in the dark for either 18 months (*Nostoc* strain HUN, *Nostoc* strain REICH) or 7 years (*Nostoc* strain ANT).

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Laboratory cultures. For comparative purposes, analyses were performed on liquid cultures of *N. commune* UTEX 584 and a filamentous marine cyanobacterium which also expresses a marked tolerance to desiccation. Cultures of *N. commune* UTEX 584 were grown in liquid culture as described previously (18). The marine strain was isolated from stromatolitic crusts which were collected from an intertidal region of the Gulf of Elat, Israel, in 1978 (16). Cells were grown in an artificial seawater medium (ASN III) which lacked a source of combined nitrogen, at 32°C, under a photon flux density of 5 μmol of photons m^{-2} s^{-1}. This cyanobacterium has been assigned, provisionally, to the genus *Hydrocoleum* with the strain designation GOE1.

Lipid analysis. All glassware was rinsed with 6 N hydrochloric acid, distilled water, and chloroform-methanol (1:1, vol/vol). Between 100 and 200 mg of desiccated or lyophilized cells was subjected to a modified Bligh-Dyer extraction as described by Guckert et al. (8). The lipids were separated into three general classes by silicic acid chromatography (6, 8). The PLFA from the methanol fraction were methylated by milk alkaline methanolysis. The methods to quantify the fatty acid methyl esters (FAMES) are described in detail by Guckert et al. (8). The PLFA data are reported as the means of two analyses.

GC-MS. Gas chromatography-mass spectrometry (GC-MS) analyses were performed on a Hewlett-Packard 5996A system fitted with a direct capillary inlet. A cross-linked methyl silicone capillary column (8) was used. Samples were injected in the splitless mode at 100°C with a 30-s venting time, a 1-min isothermal interval followed by an increase in oven temperature of 10°C min^{-1} for 5 min, and then a rate of increase of 3°C min^{-1} until 280°C was reached. This was followed by an isothermal period of 10 min. This program was modified for the dimethyldisulfide (DMDS) adducts: the rate of temperature increase was changed from 3 to 1°C min^{-1} in the range of 250 to 280°C before the isothermal period for separation of *cis-trans* isomers. Helium was the carrier gas. The MS parameters were: electron multiplier voltage between 1,500 and 1,600 V, transfer line 280°C, source and analyzer 250°C, autotune file DFTPP normalized, optics tuned at *m/z* 502, electron impact energy equal to 70 eV. MS data were acquired and processed with a Hewlett-Packard RTE-6/VM data system.

After tentative identification by GC analysis, the PLFA were subjected to GC-MS analysis. Characteristic fragmentation patterns along with retention times were used to confirm the individual PLFA compounds.

Double-bond positions of compounds 16:1ω9c, 16:1ω7c, 16:1ω5c, 18:1ω9c, and 18:1ω7c were confirmed through the formation of their DMDS adducts as described by Dunkelblum et al. (4). Samples were dissolved in hexane, and then 100 μl of DMDS (gold label; Aldrich Chemical Co., Inc., Milwaukee, Wis.) and 1 to 2 drops of an iodine solution (6.0% [wt/vol] in diethyl ether) were added, and the samples were incubated at 50°C for 48 h. After cooling, additional hexane was added, and the iodine was removed by shaking with 5% (wt/vol) aqueous $Na_2S_2O_3$. The organic layer was removed, and the aqueous portion was reextracted with hexane-chloroform (4:1, vol/vol). The organic phases were evaporated under a stream of nitrogen in preparation for GC-MS analysis. The GC-MS analysis of the DMDS adducts indicated those major ions which arose from the fragmentation of the two CH_3S groups located at the site of unsaturation (Table 1).

Fatty acid nomenclature. Fatty acids are designated with respect to the total number of carbon atoms:number of double bonds followed by the position of the double bond from the omega (ω; aliphatic) end of the molecule. The suffixes c and t indicate *cis* and *trans* geometry, respectively. The prefixes i and a refer to iso and anteiso branching, respectively. Methyl branching is indicated by the prefix br. Cyclopropane fatty acids are designated with the prefix cy.

Extraction and analysis of carotenoids and chlorophylls. Before extraction, the desiccated or lyophilized sample was prewetted for 1 h on ice. After centrifugation (to remove excess water), the pellet was suspended in 10 ml of ice-cold methanol, and the solution was sonicated in five bursts of 30-s duration, with 30-s intermissions, on ice. Chloroform (10 ml) was added, and after 1 h of extraction the samples were centrifuged at 4°C. The supernatant was filtered through a Whatman 2V filter, and the pellet was reextracted with methanol and chloroform until it was colorless. A 0.5 volume of distilled water was added to the pooled supernatants, and the phases were allowed to partition at 4°C overnight. The chloroform fraction was filtered through a Whatman 2V filter, and then it was evaporated to dryness under a stream of nitrogen. The residue was dissolved in chloroform before its passage through a C18-SepPak cartridge and filtration in a centrifugal filter system (13). The pigments were analyzed by reverse-phase high-pressure liquid chromatography (HPLC).

Extracts were transferred to Whatman K6 silica gel thin-layer chromatography (TLC) plates, and chromatography was performed in a solvent mixture of petroleum ether-acetone-chloroform (3:1:1, vol/vol/vol). Individual bands were identified tentatively by their R_f values and their wavelength spectra which were recorded with a Hewlett-Packard 8450A diode array spectrophotometer. The area of gel within individual bands was scraped from plates and suspended in chloroform in the centrifugal filter system. After centrifugation, the wavelength spectrum was recorded in acetone, chloroform, ethyl acetate, hexane, and petroleum ether. The presence of allylic hydroxy groups was confirmed by treatment with HCl-chloroform; conjugated keto groups were detected after treatment with $NaBH_4$-ethanol.

RESULTS

The PLFA profiles of the three samples of desiccated *N. commune* were similar (Table 2). The dominant fatty acids present were compounds 16:1ω7c (10.12 to 18.29%), 16:0 (23.08 to 32.03%), 18:2ω6 (4.48 to 15.60%), 18:3ω3 (11.95 to 28.22%), and 18:1ω7c (10.38 to 20.63%). Iso-, anteiso-, methyl-, and cyclopropane-branched fatty acids were either not detected or represented a small percentage of the total FAMEs. The ratios of the amounts of 18:1ω7c to 18:1ω9c were similar for *Nostoc* strain HUN and *Nostoc* strain REICH (2.63 and 2.46, respectively) and significantly higher

TABLE 1. Characteristic ion fragments of derivatized products formed by reaction of monounsaturated FAMES with DMDS

FAME	M^+	Delta[a]	Delta-32	Omega[b]
il5:1ω11	ND[c]	ND	ND	215
16:1ω9c	ND	ND	157	173
16:1ω7c	362	217	185	145
16:1ω5c	ND	245	213	117
18:1ω9c	390	217	185	173
18:1ω7c	390	245	213	145

[a] Delta indicates double-bond position from carboxylic end of molecule.
[b] Omega indicates double-bond position from aliphatic end of molecule.
[c] ND, Not detected.

TABLE 2. PLFA profiles of field and laboratory cultures of cyanobacteria

Fatty acid[a]	% Composition[b]				
	Nostoc strain HUN	*Nostoc* strain REICH	*Nostoc* strain ANT	*N. commune* UTEX 584	*Hydrocoleum* strain GOE1
il5:1ω11	0.57	0.34	0.35	Tr[c]	Tr
il5:0	0.97	0.40	0.53	Tr	1.28
al5:0	0.47	0.42	0.97	0.75	Tr
il6:0	0.55	0.56	1.53	0.65	0.82
16:1ω9c	0.69	0.06	0.40	Tr	2.11
16:1ω7c	10.12	13.02	18.29	13.52	4.18
16:1ω5c	1.16	0.73	1.18	Tr	Tr
16:0	23.08	30.35	32.03	33.21	23.24
al7:0/17:1	1.28	0.56	0.80	2.65	0.76
cy17:0	Tr	Tr	Tr	Tr	0.37
17:0	0.44	0.14	0.16	0.43	0.35
br18:1	1.11	0.56	1.12	Tr	Tr
18:2ω6	15.60	9.34	4.48	17.80	13.59
18:3ω3	11.95	28.22	16.00	3.32	3.50
18:1ω9c	7.83	4.21	4.45	4.13	12.76
18:1ω7c	20.63	10.38	16.22	7.68	16.74
18:0	3.61	0.73	1.53	8.52	13.00
br19:1	Tr	Tr	Tr	Tr	7.43
cy19:0	Tr	Tr	Tr	7.33	7.68
Total 18:2ω6 + 3ω3	27.55	37.56	20.48	21.12	17.09
Ratio 18:2ω6/3ω3	1.31	0.33	0.28	5.36	3.88
Total FAME[d]	682.5	879.6	309.6	1,366.0	110.0

[a] Fatty acid identification based on GC retention data, GC-MS confirmation, and analysis of DMDS adducts of the monounsaturated components (Table 1) unless specified otherwise.
[b] Mean of two replicates expressed as a percentage of the total fatty acids.
[c] Tr, Trace amounts of fatty acids below selected cutoff of 0.05%.
[d] Total amount of FAME in picomoles per milligram (dry weight); mean of two replicates.

for *Nostoc* strain ANT (3.64). The total amounts of compounds 18:2ω6 plus 18:3ω3 were much less in Nostoc strain ANT than in the other two field *Nostoc* samples.

The PLFA profile of the laboratory-grown culture of *N. commune* UTEX 584 differed significantly from those of the field materials. Obvious differences, in comparison to the field samples, were the high ratio of compounds 18:2ω6 to 18:3ω3 (5.36), high concentrations of compounds cy19:0, 18:0, 17:0, and a17:1, and low concentrations of compounds 18:3ω3 and 18:1ω7c. The ratio of 18:1ω7c to 18:1ω9c was

TABLE 3. Characteristic wavelength spectra for total pigments of cyanobacteria (isolated by HPLC and TLC)[a]

EO index	Wavelength (nm) maxima				
	Nostoc strain HUN	*Nostoc* strain REICH	*Nostoc* strain ANT	*N. commune* UTEX 584	*Hydrocoleum* strain GOE1
0.24–0.29	385, 297, 414	384, 295	384, 262, 274, 297, 580		
0.47–0.51		475, 505, 446, 295 (67)			
0.53–0.55	478, 504, 446, 290 (66)	476, 505, 447, 295 (69)			
0.57–0.61					504, 474, 444, 274 (88)
0.58–0.63					505, 477, 444, 275 (64)
0.63–0.67	477	469, 292, 370	479, 262	449, 476, 425, 278 (42)	
0.70–0.73	482				
0.73–0.75					
0.75–0.76		426, 411, 658, 504 (1.0)			452, 476, 424, 276 (60)
0.76–0.78				424, 378, 660, 616 (1.6)	556, 484, 661, 451 (1.1)
0.77–0.80	427, 662, 614, 410 (1.2)	427, 661, 617, 410 (1.2)		424, 660, 376 (1.3)	
0.78–0.80				276, 451, 478, 422 (58)	426, 660, 614, 557 (1.3)
0.80–0.81			427, 662, 611, 577 (1.3)	425, 377, 663, 614 (1.4)	429, 663, 613, 485 (1.2)
0.82–0.84		428, 493, 660 (1.0)		426, 394, 660, 612 (1.6)	
0.82–0.85		462, 296		454, 281, 354	428, 661, 484, 614 (1.2)
0.83–0.84	461, 293			426, 408, 660, 613 (1.7)	457
0.94–0.96	405, 501, 662, 533 (3.7)	406, 664, 502, 532 (2.4)	406, 665, 501, 606 (2.3)		
0.95–0.97		407, 502, 532, 664 (2.4)	405, 533, 665, 505 (2.5)		406, 664, 502, 533 (2.2)
1.00	480, 451, 423	408, 505, 535, 665			410, 665, 534, 603 (2.2)
1.00–1.01		449, 479, 417 (40)		450, 476, 422, 276 (13)	452, 478, 424, 276 (29)

[a] Values in parentheses indicate the peak III/II ratio for carotenoids and soret/alpha peak ratio for chlorophylls. Elution order (EO) index is the retention time of the compound under the HPLC conditions employed divided by the retention time for β-carotene. Wavelength maxima are listed (left to right) for the highest to lowest peaks in the spectrum.

TABLE 4. Identity of pigments from cyanobacteria[a]

Order of elution	Compound
1	Tentative identity
2	Apo-beta-carotenal or -one
3	Myxol or 2-OH plectaniaxanthin
4	Plectaniaxanthin or saproxanthin
5	Saproxanthin or plectaniaxanthin
6	Zeaxanthin
7	Canthaxanthin/hydroxyechinenone
8	NI[b]
9	Crocoxanthin
10	Chlorophyll *a*
11	Chlorophyll *a*
12	Beta-cryptoxanthin
13	Chlorophyll *a*
14	Chlorophyll *a*
15	Echinenone
16	Chlorophyll *a*
17	NI[b]
18	Pheophytin *a*
19	Pheophytin *a*
20	Beta-carotene
21	Beta-carotene

[a] Pigments are listed in order with increasing time of retention on HPLC columns.
[b] NI, Not identified.

1.86, significantly lower than the range of values found for the field samples (2.46 to 3.64).

The phospholipid profile of laboratory-grown *Hydrocoleum* strain GOE1 most resembled that of *N. commune* UTEX 584, with a high ratio of 18:2ω6 to 18:3ω3 (3.88) and a low ratio of 18:1ω7c to 18:1ω9c (1.31). Of all the materials analyzed, this strain contained the lowest amounts of compounds 16:1ω7c and the highest amounts of compounds 18:1ω9c and 18:0. The concentrations of methyl-branched and cyclopropane fatty acids in *Hydrocoleum* strain GOE1 were high (7.43 and 8.05%, respectively).

The methods of HPLC and TLC described here made it possible to discriminate between, and identify, some 21 different pigments from the cyanobacterial samples (Tables 3 and 4). Apo-beta-carotenal (or -one), myxol- or 2-OH plectaniaxanthin, and saproxanthin (Table 4) were associated exclusively with the desiccated field materials and were present in high concentrations (Table 5). Pheophytin was also present in these field materials in high concentrations. The apocarotenoids were composed of a chromatophore of five conjugated double bonds, with at least one of the double bonds in a ring, and with either a conjugated aldehyde or a conjugated keto group. *Nostoc* strain HUN, *Nostoc* strain REICH, and *Hydrocoleum* strain GOE1 contained myxoxanthophyll-like pigments as deduced from the wavelength spectra, although the retention time on the HPLC columns did not correspond to that of pure myxoxanthophyll. *Nostoc* strain REICH was the only strain which contained a pigment which is identified either as myxoxanthophyll (lacking sugar) or 2-hydroxyplectaniaxanthin. *Nostoc* strain HUN, *Nostoc* strain REICH, and *Hydrocoleum* strain GOE1 contained a myxoxanthophyll-type carotenoid with two O atoms in the molecule identified as either plectaniaxanthin or saproxanthin. Plectaniaxanthin and saproxanthin were both present in *Hydrocoleum* strain GOE1. Ketocarotenoids were detected as canthaxanthin (*Nostoc* strain HUN, *Nostoc* strain ANT), hydroxyechinenone (*Nostoc* strain REICH), and echinenone (*Nostoc* strain HUN, *Nostoc* strain REICH, *N. commune* UTEX 584, and *Hydrocoleum* strain GOE1). Hydroxycarotenoids were present as zeaxanthin (*N. commune* UTEX 584), crocoxanthin (*Hydrocoleum* strain GOE1), and β-cryptoxanthin (*Nostoc* strain REICH, *N. commune* UTEX 584, *Hydrocoleum* strain GOE1).

DISCUSSION

The fatty acid profiles of cyanobacteria show great diversity (7). Based on a survey of laboratory-grown cultures of unicellular and filamentous strains, four metabolic groups were recognized by the degree of saturation of different major fatty acids (10). However, there does not appear to be a strict correlation between the presence of polyunsaturated fatty acids and the cellular organization in filamentous cyanobacteria (7). Like the higher-plant chloroplast, many cyanobacteria, particularly the filamentous forms, have fatty acid profiles with a high proportion of polyunsaturated C_{18} compounds, especially linolenic acid (18:3). Data suggest that the mechanism for the desaturation of C_{18} acids is different between cyanobacteria and the photosynthetic eucaryotes but that the mechanism for the desaturation of C_{16} acids seems to be similar between them (20).

Changes in the conditions used to grow laboratory cultures can lead to changes in the composition of lipid molecular species present in cells. In *Anabaena variabilis*, the composition of fatty acids is dependent upon temperature (20). The 18:1/16:1 and 18:2/16:1 species of monogalactosyldiacylglycerol are formed at 38°C, whereas the 18:2/16:0, 18:3/16:0, 18:3/16:1, and 18:3/16:2 species of monogalactosyldiacylglycerol are formed at 22°C. By limiting catalyst-mediated hydrogenation of fatty acids to cell-surface membranes, Vigh et al. (24) obtained direct evidence to support the hypothesis that the thermotrophic properties of lipids within cytoplasmic membranes, and not thylakoids, control chilling susceptibility of the unicellular cyanobacterium *Anacystis nidulans*. In contrast to these rather clear effects of temperature, the fatty acid compositions of *Anabaena* strain BCC 6310 and *Anabaenopsis* strain BCC 6720 were similar in cells grown photoautotrophically and in cells grown heterotrophically in the dark (10).

The phospholipid profiles of the desiccated *N. commune* colonies from different geographic locations are very similar, and the profile appears to change little upon prolonged storage of the cells in the desiccated state (7 years). Given the diverse environments from which the field materials were collected and the different times of collection, the similarities in the fatty acid profiles are striking. The profiles of desiccated *N. commune* are significantly different from those of the laboratory-grown cultures of *N. commune* UTEX 584 and desiccation-tolerant *Hydrocoleum* strain GOE1. In comparison with laboratory-grown cultures, the field materials, which have undergone multiple cycles of drying and rewetting, contain lower amounts of 18:0, equivalent amounts of 16:0, and elevated levels of 18:3ω3 with low ratios of 18:2ω6/3ω3. This trend is the opposite of that found when chloroplasts of *Gossypium hirsutum* L. cv. Reba were subjected to water stress (5). In this case, the galactolipid content, particularly digalactosyldiglyceride, decreased with decreasing water potential, and the percentage of linolenic acid (18:3), the major fatty acid of thylakoids, decreased, whereas that of linoleic (18:2) and oleic (18:1) acids increased. An accumulation of fatty acids having less than 16 carbon atoms was also observed (5). Rather different data were obtained in studies with the desiccation-tolerant and -intolerant mosses *Tortula ruralis* (Hedw.) Gaertn, Meyer

TABLE 5. Quantitative pigment composition of desiccated *N. commune* and laboratory-grown cultures of *Hydrocoleum* strain GOE1[a]

EO index[b]	Compound	Peak area/mg (dry wt)			
		Nostoc strain HUN	*Nostoc* strain REICH	*Nostoc* strain ANT	*Hydrocoleum* strain GOE1
0.26–0.29	Keto-		50,870	127,886	
0.49–0.51	Myxo-like		2,162		
0.57–0.58	Myxo-like				846
0.63–0.65	Canthaxanthin	3,931	12,145	3,897	
0.70–0.73	Chlorophyll *a*			503	
0.73–0.75	Chlorophyll *a*			4,645	
0.76–0.78	Chlorophyll *a*		1,956	9,277	2,185
0.77–0.80	Chlorophyll *a*	12,152	16,069	1,300	15,547
0.78–0.80	β-Cryptoxanthin	597			
0.80–0.81	Chlorophyll *a*		2,483		2,272
0.82–0.85	Echinenone	2,794	9,428	3,694	6,187
0.92–0.94	Pheophytin			1,279	
0.94–0.96	Pheophytin	8,743	18,459	22,225	10,228
1.00	β-Carotene	1,833	2,222	1,562	8,646

[a] Data for *N. commune* UTEX 584 are given in J. J. Olie and M. Potts, Appl. Environ. Microbiol., in press.
[b] EO, Elution order.

and Scherb and *Cratoneuron filicinium*, respectively (23). No changes in phospholipid composition occurred in either moss as a consequence of rapid drying, but after slow drying, there was a decline in some unsaturated fatty acids. Original levels were recovered upon rehydration of *T. ruralis* but not upon rewetting of *C. filicinum*. In addition, there was poor correlation between lipid peroxidation of fatty acids owing to desiccation and changes in the phospholipid fraction.

The total lipid composition of cyanobacterial cells may be representative of the thylakoid membrane fraction in view of the high mass of the thylakoids in comparison with the cytoplasmic and outer membranes (7). The similarities in the phospholipid profiles of the three desiccated materials suggest that thylakoid membranes undergo no gross changes upon prolonged desiccation. Furthermore, the differences noted in fatty acid compositions of field and laboratory cultures may reflect differences in composition of their thylakoids. As such, desiccation and water stress in field populations of *Nostoc* species may result in the differentiation of thylakoids with quite different properties from those of axenic cultures. We have shown already that the cytoplasmic membrane of *N. commune* UTEX 584 possesses features which may account, in part, for the resistance of the cells to the stresses of de- and rehydration (13) and that the membranes of desiccated *Nostoc* cells remain intact during prolonged periods of desiccation.

Studies have demonstrated relatively large amounts of carotenoids in the cytoplasmic and outer membranes of unicellular and filamentous cyanobacteria, and recently, a carotenoid-binding protein has been described (3, 9, 12, 14, 15). Apart from an intrinsic role in photosynthesis, carotenoids are thought to protect cyanobacteria and other bacteria (2) against photoxidative radiation (11). Radiation damage is likely to be of some consequence in field populations of nitrogen-fixing cyanobacteria, such as the desiccated colonies of *Nostoc* species. The similarity in the carotenoid contents of the field materials may therefore be of ecological significance. There are clear differences in the carotenoid compositions of laboratory-grown cultures and the desiccated field *Nostoc* samples. While beta-carotene is present in all samples, there is an evident shift from beta-carotene and echinenone, which predominate in the laboratory cultures, to canthaxanthin and the beta-gamma-carotene series in the desiccated cells. A major feature of the carotenoid content of desiccated *Nostoc* cells is the presence of high levels of apocarotenoids. The identity of these apocarotenoids proved difficult to establish. The P384 pigment which appeared through TLC analysis to be a chlorophyll derivative yielded, upon reduction with $NaBH_4$, a mixture of a chlorophyll derivative plus a compound with one absorption maximum at 451 nm. The absorption properties of this pigment(s) and its high concentration in desiccated cells suggest the potential for a role in protection against UV radiation.

The role of carotenoids in cyanobacterial membranes remains poorly understood. It is of interest that zeaxanthin in the cytoplasmic membrane of *Anacystis nidulans* undergoes a chilling-induced absorption increase around 390 nm, a sign of the phase change in the membrane, although it is not known whether this represents an alteration in the conformation or an aggregation of the pigment when the membrane lipids enter a phase-separated state (24). The absorption properties of carotenoids may be of some consequence in *Nostoc* membranes which have become "dehydrated."

Field populations of *N. commune* are pigmented, and the filaments are embedded in a thick dense mucilage. When dry the colonies appear black and are friable and brittle. When wet the colonies have the consistency of soft cartilage. These are quite different properties from those shown by dry immobilized cells of *N. commune* UTEX 584 and cells of this strain when grown in liquid culture. Such differences between the field populations and laboratory strains, as well as the differences in biochemical composition discussed above, suggest that a greater emphasis should be placed upon field materials with respect to the molecular analysis of desiccation tolerance.

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Stability of nucleic acids in immobilized and desiccated *Nostoc commune* UTEX584 (Cyanobacteria)

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1. SUMMARY

Two microscale methods were developed to isolate and analyze the [2-^{14}C]uracil-labeled nucleic acids of immobilized, dried cells of the desiccation-tolerant cyanobacterium *Nostoc commune* UTEX584. The incidence of single-strand breaks ('nicks') in DNA of light-desiccated, but not dark-desiccated cells, was demonstrated by the use of DNA modification enzymes.

2. INTRODUCTION

A capacity to tolerate desiccation, an important environmental extreme, is a feature common to many of the cyanobacteria [1–5], one of the largest sub-groups of Gram-negative prokaryotes [6]. This is particularly significant in view of the perceived utility of cyanobacteria as model systems for the study of nitrogen fixation, photosynthesis and cell differentiation [7,8]. Cells of *N. commune*, in particular, express a marked capacity for desiccation tolerance [9–12]. However, cyanobacterial cells, especially when desiccated, are notorious for their lack of susceptibility to a wide range of common lysis procedures [13,14]. Cells of *N. commune*, even in liquid culture, resist the effects of lysozyme, sodium dodecyl sulfate (SDS) and pronase, as well as other wall-specific degradative enzymes, including β-1,3-endoglucanase (lyticase [2]), cellulase and hemicellulase.

This communication describes two microscale methods for the recovery of unlabeled and labeled nucleic acids from desiccated *Nostoc*. We describe an apparent in vivo, light-dependent modification of DNA which occurs during the drying and prolonged storage of immobilized cells at a matric water potential (ψm) of -99.5 MPa (approx. 0.5 a_w).

3. MATERIALS AND METHODS

Liquid cultures of *N. commune* UTEX584 were grown in BG-11$_0$ medium [6] as described previously [12,15]. To label nucleic acids in vivo, we took advantage of a previous observation that uracil was incorporated into DNA more effi-

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ciently than either thymine or thymidine [16].

Aliquots (50 μl) of a cell suspension were transferred to 23-mm-diameter Whatman 3MM filter discs which were supported on steel pins [9]. To prevent non-specific binding of acid-soluble components the filters were first washed in an aqueous solution of uridine-3′-monophosphate and cytidine-2′,3′-monophosphate (each at 0.5 mg · mg^{-1}), and then dried. Immobilized cells were incubated either in the light, or in the dark, at a ψm of −99.5 MPa, at 32°C [17]. After different periods of desiccation, the nucleic acids were precipitated *directly* within the confines of these filters by the successive addition of two 50-μl aliquots of ice-cold trichloracetic acid (TCA; 10%, w/v). The filters were dried, then processed for RNA or DNA determination [16].

To allow quantitative recovery of nucleic acids from desiccated cell material aliquots of cell suspension were immobilized by placing them on the surface of a hydrophobic fine-mesh (pore size 0.04 mm^2) nylon support. The support was stretched over the mouth of a 12-cm-diameter grooved ring, cut from a section of PVC tubing, and was held tightly in place with a rubber band. Aliquots assumed the shape of regular spheres, in contact only with the nylon surface and the atmosphere, which was maintained at a constant water potential and temperature. More than 50 droplets, each with the same dimensions and shape, could be supported on a single support. Light-shielding of the cells was negligible, and attachment to the mesh was sufficiently tight to permit easy manipulation, but sufficiently loose to allow quantitative recovery of the material. After incubation under these conditions for 14 days, circular sections of the nylon with aggregates of immobilized cells were excised with a razor blade and were then ground in a chilled mortar at 0°C. A 250-μl aliquot of the extraction buffer (TE; 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) was added and grinding was continued with successive additions of TE buffer to a final volume of 1 ml. The suspension was then transferred to a 2-ml polyethylene screw-cap tube (Biospec Products), glass beads were added (25% v/v; 0.5 mm diameter), and the tube was agitated vigorously in a Mini Beadbeater (Biospec Products) for 50 s. The lysate was deproteinized with buffer-equilibrated phenol and chloroform-isoamyl alcohol (24:1, v/v), and nucleic acids were recovered and quantified [20].

4. RESULTS AND DISCUSSION

The level of acid-insoluble radioactivity bound to light-stored filters increased with the time of storage (Fig. 1). After 10 days, the level had increased by 45% above that measured directly after immobilization and drying of the cells. The bound radioactivity which was associated with dark-stored filters remained more or less constant over the 10 days of the experiment. Our previous work demonstrated that photobleaching occurs in immobilized light-stored cells, they lose chlorophyll, protein synthesis ceases after several hours, protein degradation is significant, and an intracellular ATP pool cannot be detected in them after such prolonged storage [9–11,14]. The apparent increase in bound DNA-incorporated radioactivity cannot be attributed to DNA synthe-

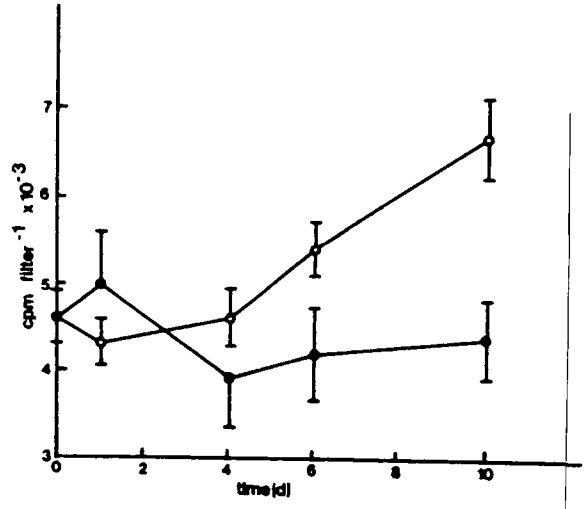

Fig. 1. Amount of radioactivity [^{14}C]uracil incorporated in DNA on filters with time of desiccation (corrected for background). ○, Filters stored in the light; ●, filters stored in the dark; $N = 3$. Prior to immobilization and desiccation, cells were suspended in 20 ml of BG-11_0 at a cell density of 70 μg · ml^{-1}, and incubated for 48 h in the presence of 100 μCi (3.7 MBq) of [2-^{14}C]uracil, specific activity 40–60 mCi (1480–2220 MBq) $mmol^{-1}$.

sis, and must represent more efficient binding during the extraction and washing process. Binding efficiency of DNA could be enhanced through the degradation of proteins, particularly those in association with membranes, which would decrease competition for binding sites within the upper confines of the filters. It is possible that the binding properties of the filter may also have been changed by the continual illumination. It is known that DNA can be bound covalently to supports made of nylon through the reaction of primary amine groups with thymidine residues in the presence of UV radiation (254 nm [18]). When the desiccated cells were rehydrated with distilled water, the amount of filter-bound radioactivity, associated with total nucleic acid, decreased (Fig. 2). The kinetics were similar for both light- and dark-stored cells and after 6 h of rewetting, the amount of radioactivity present on filters had decreased by almost half. The amount of radioactivity in DNA alone decreased by 20% within the first hour of rewetting and then remained stable during the next 5 h. Additional experiments determined that loss of immobilized material from filters, upon rewetting, was minimal (data not shown). A significant proportion of the decrease in bound radioactivity upon the rewetting of both light- and dark-stored cells reflects the breakdown of labeled RNA. A rapid turnover of pre-labeled protein also takes place in these cells upon rehydration [14]. Upon the rewetting of dried cells of the desiccation-tolerant moss, *Tortula ruralis*, a rapid turnover of RNA has also been measured [19]. A rapid turnover of macromolecules appears to be a feature of the recovery of cells from the air-dry state.

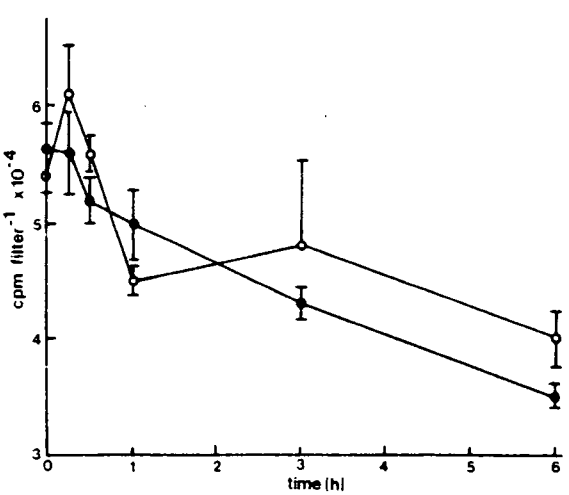

Fig. 2. Amount of ^{14}C incorporated in DNA and RNA on filters after 10 days of desiccation and then rewetting with distilled water, in the light. ○, Filters stored in the light; ●, filters stored in the dark; $N = 3$.

After treatment with RNAse, the susceptibility of DNA samples from light- and dark-stored cells to several DNA modification enzymes was tested as outlined in Fig. 3. In assays for DNA modification, a significant finding was the increased susceptibility of non-ligated DNA from light-stored cells to modification with DNA polymerase I (Fig. 4). The level of incorporation of [α-^{32}P]dCTP in light-stored DNA under these conditions was greater than when the sample was treated with DNA polymerase I and endonuclease I (nick translation [21]). For DNA samples from dark-stored cells, the opposite was true. The increased susceptibility of DNA from light-stored cells to treatment with DNA polymerase I suggests the presence of single-strand breaks ('nicks'), the level of incorporation was much less in assays with the Klenow fragment, which lacks 5′–3′ exonuclease activity. Ligated DNA showed a similar incorporation with DNA polymerase I to that of non-ligated DNA in incubations with Klenow fragment alone. Whether the greater incidence of nicks represents an increased susceptibility of the DNA to nicking due to light-dependent processes, e.g., photo-oxidation, or a reduced capacity to repair nicks during the drying process, remains to be investigated. These cells recover their activity upon rewetting [9–11], which suggests that a DNA re-

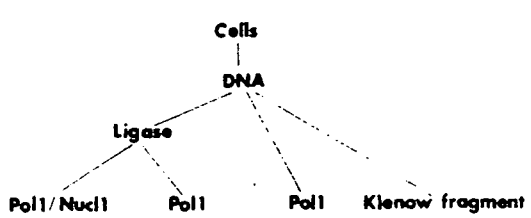

Fig. 3. Scheme for DNA modification assays. Pol I, DNA polymerase I; Nucl I, endonuclease I.

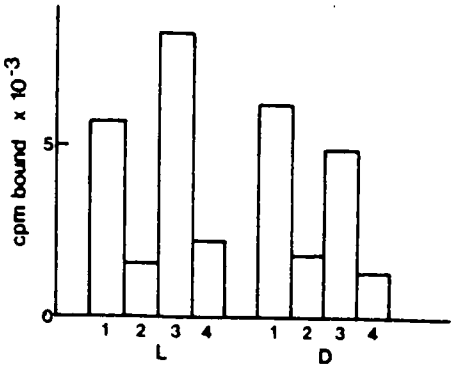

Fig. 4. Amount of ^{32}P incorporation in DNA after DNA modification assays. (1) Ligated DNA, treated with Pol I and Nucl I; (2) ligated DNA treated with Pol I; (3) unligated DNA treated with Pol I; (4) unligated DNA treated with Klenow fragment. L, DNA from cells desiccated in the light. D, DNA from cells desiccated in the dark. Ligation reactions were performed overnight at 16 °C. Enzymes were used according to the manufacturer's specification (Bethesda Research Labs/Life Technologies, Gaithersburg, MD). The level of incorporation was measured after the application of aliquots of the reaction mixture to DEAE filters (Whatman DE 81) and removal of unincorporated radioactivity.

rewetting [9–11], which suggests that a DNA repair system must be active at this time. Such light-stored cells undergo longer lags than dark-stored cells before the onset of nitrogenase activity can be detected [10]—a requirement for some DNA repair may account, in part, for these lags in recovery. This light-dependent modification of DNA may have effected the binding properties of the DNA (see above). During the extraction of nucleic acids from desiccated *Nostoc* cells attached to nylon supports, the Beadbeater proved essential. Yields were variable if manual grinding was the only method used to break the cells. Other methods of physical disintegration, such as treatment in the French pressure cell and X-Press, are not applicable at this scale. The yield obtained by the present method was 14 ng DNA · (mg wet wt)$^{-1}$. This is significantly less than yields which have been obtained from fresh material on a larger scale [22] but comparable with yields from micro-scale extractions of dried plant material such as those described by Rogers and Bendich [23]. The amounts of DNA which we are able to obtain from several portions of desiccated material on nylon supports are, clearly, sufficient for restriction enzyme analysis and Southern/Northern hybridizations.

These two methods should prove extremely useful for the study of gene expression in immobilized cells, particularly in light of recent reports that describe the superior characteristics of nylon membranes for quantitative in situ molecular hybridization [18], and the use of sensitive filter retention assays for DNA modification [24].

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In vitro translation of mRNA from *Nostoc commune* (Cyanobacteria)

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Abstract. RNA pools were extracted from cells of *Nostoc commune* UTEX 584 in exponential growth (liquid cultures) and from cells which had been immobilized and dried rapidly at −99.5 MPa. Levels of incorporation of ^{35}S-methionine, five- to sixfold higher than the endogenous level, were obtained after *in vitro* translation of the RNA preparations in a heterologous S30 cell-free system purified from *Escherichia coli* Q13. The levels of incorporation, obtained with a homologous *N. commune* UTEX 584 S30 system, were much lower. The requirement for magnesium in the heterologous system was 15–21 mM, translation of *N. commune* UTEX 584 RNA was inhibited when the RNA concentration was greater than 0.3 mg ml^{-1}, and translation was stimulated significantly by the presence of ammonium chloride. Few qualitative differences were observed between the pattern of proteins (SDS-PAGE) obtained after translation of the RNA pools from cells in exponential growth, and from those cells subjected to immobilization and rapid drying. The data suggest that short-term desiccation of *N. commune* UTEX 584 does not have a marked selective effect on the composition of the mRNA pool. In contrast, preparations of RNA from field materials of *Nostoc commune* HUN (desiccated for 5 years) were unable to drive high rates of translation in any of the systems tested and optimized for use in this study.

Key words: mRNA – Cyanobacteria – *In vitro* translation – Water-stress – Desiccationtolerance – *Nostoc commune*

Cyanobacteria encompass, within a single cell, a prokaryotic ultrastructure and a capacity for oxygenic photosynthesis as well as, in many strains, an ability to fix atmospheric nitrogen (Carr and Whitton 1982). Recent advances in the molecular biology of cyanobacteria include the analysis of the structure of *nif* genes and their organization (Golden et al. 1985), the cloning of the genes which code for photosystem II proteins (Lind et al. 1985), the development of conjugation and transformation systems (Buzby et al. 1985; Herrero and Wolk 1986) the construction of shuttle vectors and systems for genetic mapping (Gendel et al. 1983), and demonstration of heterospecific transformation (Stevens and Porter 1986). The control of gene expression in cyanobacteria remains, nevertheless, poorly understood, although the regulation is thought to be modulated largely in response to light, CO_2, and inorganic nutrients (Doolittle 1979).

Species of *Nostoc* are among the most widespread of all cyanobacteria and colonies of *N. commune* are especially prominent in limestone areas which are subject to extremes of water availability (Whitton et al. 1979). In these situations the cells resume and cease cellular activities repeatedly as intermittent rainfall leads to cycles of rewetting and drying. Our previous work with axenic cultures of *N. commune* UTEX 584 demonstrated that water stress (drying of cells, desiccation, and rewetting of cells) leads to changes in gene expression (Angeloni and Potts 1986; Potts 1985; Potts and Bowman 1985). In view of the fundamental significance of water in nucleic acid-protein interactions, we aim to gain an understanding of the molecular basis for desiccation-tolerance in *N. commune*. Central to this work is the need to assess the translational capacity of RNA pools from cells subjected to different degrees of water stress. There are few reports of the *in vitro* translation of cyanobacterial mRNA (Bazin 1970). A heterologous cell-free system from *Escherichia coli* PR7 was used by Gupta and Carr (1983) to translate polysomal RNA from *Nostoc* sp. MAC. Seven polypeptides co-migrated with the in vivo-labelled proteins upon gel electrophoresis of the translation products, and these had apparent molecular masses of 17–30 ($\times 10^{-3}$). Bacterial translation systems from one species are frequently incapable of initiating efficiently the translation of mRNA's of distantly related species (Stallcup et al. 1976). As discussed by several authors (Bryant et al. 1985; Doolittle 1979) one might expect problems in expressing cyanobacterial genes in *E. coli* if *E. coli* mRNA recognition specificity differs significantly from that in cyanobacteria. This study assesses the translation capacities of RNA preparations from cells of *N. commune* UTEX 584 in exponential growth, immobilized and dried cells, and from field materials of *N. commune* HUN desiccated for five years.

Materials and methods

Microorganisms. Nostoc commune UTEX 584 was grown in continuous axenic culture in a 1.8-l airlift fermenter (BRL/Life Technologies, Inc., Gaithersburg, MD, USA). The conditions of growth were 32°C, in BG-11$_0$ medium (Rippka et al. 1979), with a photon flux density of 10 µmol photons m^{-2}s^{-1}. Cells were harvested in the exponential phase of growth for the extraction of RNA (Angeloni and Potts 1986).

Offprint requests to: M. Potts

Desiccated colonies of *N. commune* were collected by Professor T.W. Chen in Hunan Province, China, in 1980, and were a kind gift of Dr. S. Scherer. For discussion purposes this material is referred to as *Nostoc commune* HUN.

A culture of *Escherichia coli* Q13, a derivative of *E. coli* K12, was obtained from the American Type Culture Collection, Maryland, USA (culture no. 29079). The strain is deficient in ribonuclease 1 and polynucleotide phosphorylase. Cells were grown in yeast-tryptone medium (Gupta and Carr 1983), at 37°C, with shaking. Cells were harvested in the exponential phase of growth ($OD_{550} = 2$).

Immobilization of cells. Axenic cultures of *N. commune* UTEX 584 were immobilized on hydrophobic nylon supports and dried at −99.5 MPa, at 32°C, for 24 h in the light as described previously (Potts 1985).

Preparation of E. coli S30 extracts. S30 extracts from *Escherichia coli* Q13 ($S30_{Eco}$) were prepared initially by using the method of Modolell (1971). Cells were disrupted by grinding them with alumina (type 305, Sigma). However, a modified form of the procedure of Bottomley (1982) gave more consistent results. In this case the cells were disrupted by passing them twice through a French pressure cell (Aminco, Silver Springs) at 69 MPa; the dialysis buffer was 10 mM Tris-HCl pH 7.8, 60 mM ammonium chloride, 10 mM magnesium acetate, 6 mM β-mercaptoethanol (freshly prepared and added separately immediately prior to use).

Preparation of N. commune UTEX 584 S-30 extracts. S30 extracts from *Nostoc commune* UTEX 584 ($S30_{Nos}$) were prepared as described above for *E. coli* using a method based upon the procedure of Bottomley (1982).

Wheat-germ system. Nuclease-treated wheat-germ extract and the components for in vitro translation were obtained from BRL/Life Technologies Inc. and were used according to the manufacturer's specifications.

Extraction and purification of RNA. All glassware was sterilized by autoclaving it prior to use. With the exception of the phenol extractions, all manipulations were carried out on ice with chilled buffer solutions.

Escherichia coli. Total cell RNA was isolated in the presence of guanidinium isothiocyanate and preheated phenol (60°C) as described by Maniatis et al. (1982). Prior to their use in in-vitro translation assays, samples of total cell RNA were treated with deoxyribonuclease I (RQ™DNase I, Promega Biotec, Madison, WI, USA).

Nostoc commune UTEX 584 – liquid cultures. Disruption of the cells in a French pressure cell gave high yields of undegraded RNA. Total cell RNA was isolated from freshly-harvested cells which contained ~ 4 μg chlorophyll *a* ml^{-1}. The washed cells were resuspended in two volumes of 50 mM Tris-HCl (pH 9) buffer and disrupted at 110−125 MPa in a ~ 36-ml capacity French pressure cell, at 4°C. The eluate was supplemented immediately with β-mercaptoethanol and sodium dodecyl sulfate (SDS) to give final concentrations of 6 mM and 1% w/v respectively. This suspension was extracted once with an equal volume of Tris-saturated phenol (pH 8.1) and shaken vigorously for 10−15 min at room temperature. The aqueous phase was removed, mixed with 1/10 volume of 4 M sodium chloride solution, and the total nucleic acids were precipitated by adding 2.5 volumes of ethanol, at −20°C, overnight. The precipitate was dissolved in sterile distilled water and treated with deoxyribonuclease I for 30 min on ice. The total cell RNA was extracted four times with two volumes of phenol:chloroform:isoamyl alcohol (24:24:1; v/v/v) and once with chloroform:isoamyl alcohol (24:1; v/v). The aqueous phase was mixed with 1/10 volume of 4 M sodium chloride solution and nucleic acids were precipitated with 2.5 volumes of ethanol, at −20°C, overnight. The pellet was washed with 70% v/v ethanol (containing 0.3 M sodium acetate pH 5.2), dried briefly, dissolved in an appropriate volume of sterile distilled water, and kept at −70°C until required.

A separate procedure was used to isolate total cell RNA enriched in tRNA. The material used was obtained from a distinct red-brown layer in a sucrose-density gradient which had been used to prepare polysomal RNA (Angeloni and Potts 1986). The fraction was extracted with a 0.86 volume of water-saturated phenol for 1 h, with shaking, at room temperature (Orozco 1982), and then centrifuged for 1 h, at 4°C, at 16,000 × *g*. The aqueous phase was extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1; v/v). The final aqueous phase was supplemented with 1/10 volume of 3 M sodium acetate (pH 7) and the nucleic acids were precipitated with 2.5 volumes of ethanol, at −20°C overnight. To deacylate aminoacyl-tRNAs, the pellet after the ethanol precipitation step was resuspended in 0.5 M Tris-HCl (pH 9; 1.2 mg ml^{-1} RNA final concentration) and incubated for 1 h, at 37°C. The tRNA was recovered by ethanol precipitation, dissolved in sterile distilled water, and stored at −70°C.

Nostoc commune UTEX 584 – immobilized cells. Essentially the same procedure was used as described above for the total cell RNA preparations with the following exceptions. The dried cells of *Nostoc commune* UTEX 584 were frozen in liquid nitrogen and ground to a powder in a precooled mortar. The extraction buffer contained chloramphenicol (50 μg ml^{-1}), the cell suspension was passed twice through a French pressure cell (110−125 MPa), and the detergent mixture was composed of 1% w/v NP40, 1% w/v Tween 20, 1% w/v Brij 35 and 30 mM magnesium chloride.

Nostoc commune (Hunan/China) – field materials. Dried field material of *Nostoc commune* Hun (0.2 g) was treated in the same manner as described for the dried *Nostoc commune* UTEX 584. The extraction buffer contained 50 mM Tris-HCl pH 9, 40 mM potassium chloride, 20 mM magnesium chloride, 50 μg ml^{-1} chloramphenicol, 50 μg ml^{-1} heparin, 120 mM β-mercaptoethanol. After disruption of the cells in the French pressure cell (a total of 2−3 passages) the cell suspension was treated with proteinase K (0.2 mg ml^{-1} final concentration) and four to five volumes of extraction buffer which contained 0.8%−2% w/v SDS or the NP40 detergent mixture (see previous section). After vigorous shaking for 10 min at room temperature, the RNA was extracted with phenol and collected by ethanol precipitation.

Conditions for in vitro translation of RNA. The composition of the system used for optimization of in vitro protein synthesis was based initially upon that described by Modolell

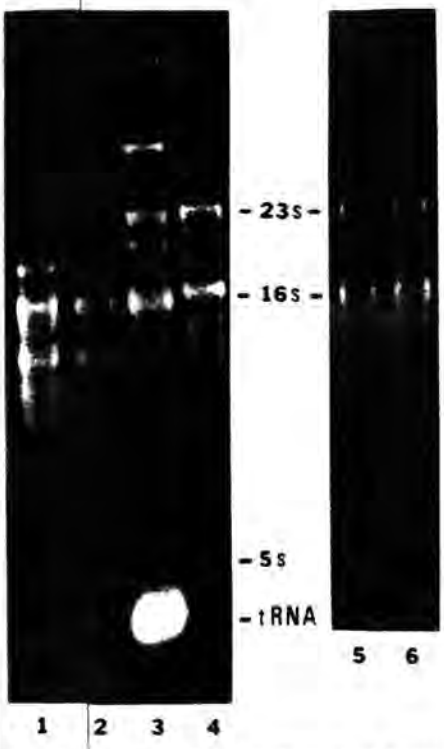

Fig. 1. Agarose gel electrophoresis of *Nostoc commune* UTEX 584 RNA. *Lanes 1, 2:* from cells in exponential growth (separate cultures); *lane 3:* from cells in exponential growth using "tRNA" procedure (see Materials and methods); *lane 4: Escherichia coli* rRNA markers; *lanes 5, 6:* cells in exponential growth, immobilized and dried rapidly at −99.5 MPa for 24 h (separate cultures)

(1971) and later on that described by Bottomley (1982). The translation assays had a final reaction volume of 25 µl and were incubated at 37 °C, for 30 min, unless stated otherwise. The incorporation of radioactivity into trichloroacetic acid-insoluble material was measured according to Mans and Novelli (1961). Aliquots of the reaction mixture were mixed with ice-cold acetone (100%) and the protein precipitates were analyzed by SDS-polyacrylamide gel electrophoresis (Potts 1985).

Electrophoresis and fluorography. Protein samples were denatured by heating them at 95 °C for 5 min in 2% w/v SDS, 1% v/v dithiothreitol (DTT), 30% w/v glycerol, 32 mM Tris-HCl (pH 6.8) and 0.005% w/v bromophenol blue. Samples were electrophoresed using the buffer system of Laemmli (1970) in polyacrylamide (15% w/v) slab gels. Gels were fixed, stained, impregnated with En^{3}hance (NEN DuPont, Boston, MA, USA) and exposed to Kodak-X AR-5 film, at −70 °C.

Agarose gel electrophoresis. Samples of total cell RNA were analyzed with 2% w v agarose gels in Tris-Borate buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA pH 8.0) according to the procedure described by Maniatis et al. (1982). Gels were stained in 0.5 µg ml^{-1} ethidium bromide, destained and photographed with type 55 Polaroid land film.

Chlorophyll a determination. Chlorophyll *a* concentrations were determined by the method of MacKinney (1941).

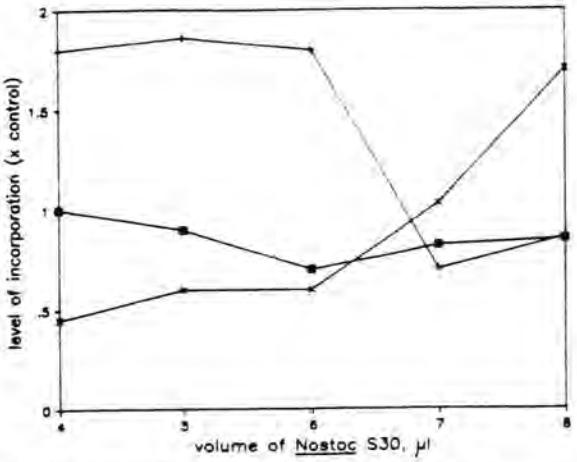

Fig. 2. Level of TCA precipitable ^{35}S-methionine incorporation in the homologous $S30_{Nos}$ system. Levels of incorporation were measured in relation to time of incubation at 37 °C and the volume of $S30_{Nos}$ in each reaction volume. Each data point represents the mean of three replicates and was calculated using the appropriate individual control value. + 30 min; ■ 60 min; × 90 min

Results

Preparation of Nostoc RNA

Preparations of RNA from cells grown in liquid culture contained significant amounts of 23 S, 16 S and 5 S rRNA, and lesser amounts of tRNA (Fig. 1). Incubation of these preparations with RNAse-free DNAse did not lead to any discernible change in the pattern of the bands observed in the gels (Fig. 1; lanes 1, 2). The identity of the 23 S and 16 S rRNA bands was confirmed through hybridization after Northern transfer with a biotinylated heterologous rRNA DNA probe from *Escherichia coli* (Angeloni and Potts, unpublished data). Preparations which were obtained through the use of the "tRNA" extraction technique were enriched significantly in tRNA (Fig. 1; lane 3) but also contained DNA, rRNA and RNA fractions which had the same mobility in agarose gels as the major RNA species detected in the "total" RNA preparations. In those preparations which were extracted from immobilized and desiccated cells, 23 S and 16 S rRNA's were detected after electrophoresis but streaking obscured the nascent pattern present in the gels (Fig. 1; lanes 5, 6). Preparations of RNA from desiccated or rehydrated colonies of field *Nostoc commune* HUN were resolved poorly under the same conditions of electrophoresis (data not shown).

Translation of Nostoc mRNA in the homologous cell-free system

In reaction mixtures with a total volume of 25 µl and containing RNA from cells in exponential growth, the level of incorporation of ^{35}S-methionine in de novo protein was influenced by the time of incubation at 37 °C and the volume of *N. commune* UTEX 584 S30 ($S30_{Nos}$) in the final reaction volume (Fig. 2). With an incubation time of 60 min, the level of incorporation varied little (range 0.7 − 1.0 × control) when different volumes of $S30_{Nos}$ in the range of 4 to 8 µl were used in the assays. The use of the same range of $S30_{Nos}$ volumes with either shorter or longer incubation times, resulted in marked differences in the level of incorporation. A time-dependent decrease in the level of incorporation

Fig. 3. SDS-PAGE of in vitro translation products. *E. coli* total RNA was translated in the $S30_{Eco}$-based system (*lane 2*) and in the $S30_{Nos}$-based system (*lane 4*). Controls (without the addition of RNA) are represented in *lanes 1* ($S30_{Eco}$) and *3* ($S30_{Nos}$). For the homologous *E. coli* RNA translation assays (corresponding to lane 2) only a fraction of the translation mixture was analyzed to prevent smearing on the fluorograph

occurred in assays which contained between 4 and 6 µl of $S30_{Nos}$ while larger volumes of $S30_{Nos}$ stimulated incorporation in long-term (90 min) assays.

Low levels of incorporation (0.75 to 1.2 × control) were also achieved with RNA samples from immobilized and dried cells (in the range 0.059 to 0.44 mg ml^{-1} of total RNA). A single polypeptide (mol. mass = 22×10^3) was detected after SDS-PAGE and fluorographic analysis of the reaction products from this assay. Aliquots of this same RNA preparation, when used in the same range of concentration, gave significantly higher levels of incorporation when the $S30_{Nos}$ was replaced with $S30_{Eco}$.

To assess further the limitations and versatility of the $S30_{Nos}$-based translation system, the system was primed with total RNA from *E. coli* Q13. Despite low levels of incorporation (slightly higher than background) a small number of polypeptides with a range in mol. mass of ~ 19 to ~ 22 ($\times 10^3$) were synthesized (Fig. 3; lane 4). Of these, the most conspicuous had a mol. mass of 22×10^3. This polypeptide was not detected when aliquots of the same *E. coli* RNA preparation were incubated in the $S30_{Eco}$-based translation system (Fig. 3).

Translation of Nostoc mRNA in a heterologous cell-free system

In the $S30_{Eco}$-based translation system, optimum incorporation was achieved with a concentration of 0.31 mg ml^{-1} *N. commune* UTEX 584 RNA (from cells in exponential growth) when used in a total reaction volume of 50 µl. The

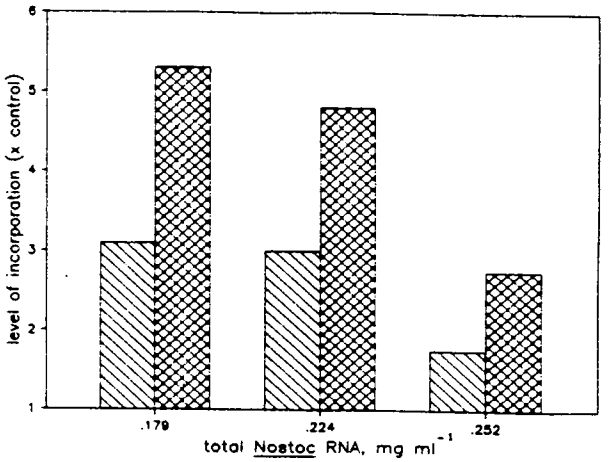

Fig. 4. Influence of incubation temperature on the level of ^{35}S-methionine incorporation in in-vitro translations of total *Nostoc* RNA. Assays were performed at 32 °C (▨) or 37 °C (■) ($S30_{Eco}$ system after Bottomley 1982) with different concentrations of total RNA from *N. commune* UTEX 584 in exponential growth

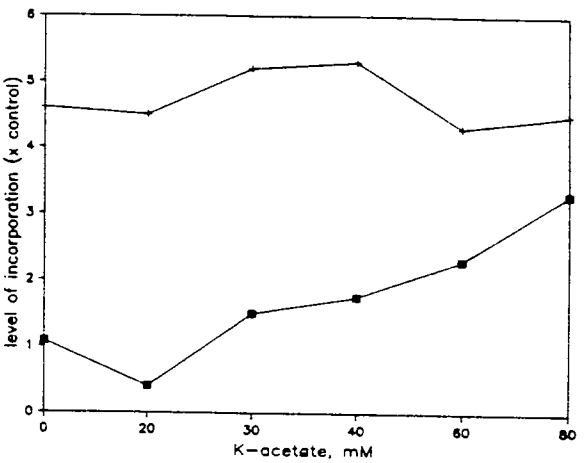

Fig. 5. Stimulation of ^{35}S-methionine incorporation in in vitro translation products in the presence or absence of ammonium chloride. *N. commune* UTEX 584 total RNA from cells in exponential growth was used in the $S30_{Eco}$ system with different concentrations of potassium acetate. + plus NH_4Cl; ■ minus NH_4Cl

level of incorporation achieved with this concentration of total RNA represented approximately 20% of that which resulted when an equivalent concentration of *E. coli* total RNA was used in this translation system. The optimum temperature for *in vitro* translation of *N. commune* UTEX 584 RNA was at 37 °C (Fig. 4). The levels of incorporation measured after incubation at 32 °C, the optimum growth temperature of *N. commune* UTEX 584 (Potts 1985), were significantly lower (Fig. 4).

During fluorographic analysis of the reaction products from $S30_{Eco}$ translation reactions three dense bands, which corresponded to polypeptides with molecular masses of ~ 66 × 10, were always observed (Figs. 3, 6). These bands were most dense in the gel lanes of the control ($S30_{Eco}$ minus RNA) despite preincubation of the $S30_{Eco}$ extract at 37 °C.

The in vitro translation of *N. commune* UTEX 584 RNA was stimulated significantly by ammonium chloride (Fig. 5). Optimum incorporation was achieved with an ammonium chloride concentration of 62 mM. In the absence of ammonium chloride the level of incorporation was significantly

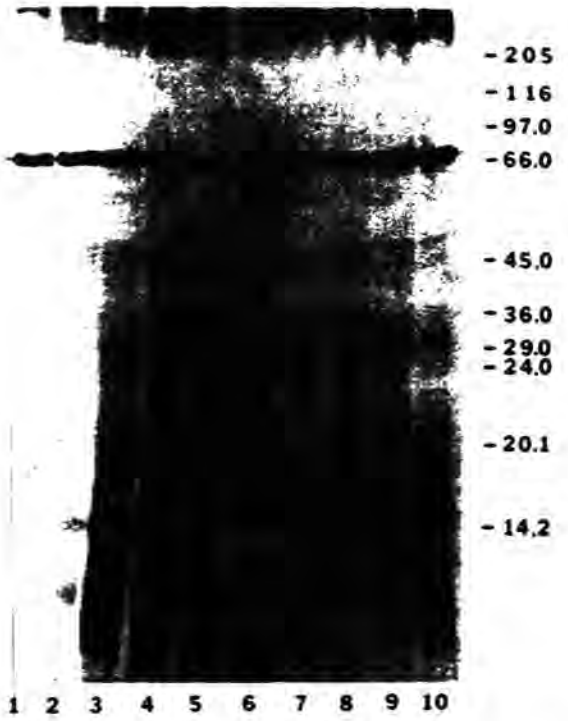

Fig. 6. SDS-PAGE of in vitro translation products from reactions with different RNA preparations. Samples were analyzed in duplicate to assess pipetting errors. *Lanes 1, 2:* control, no added RNA; *lanes 3, 4:* tRNA preparation (see Materials and methods), cells in exponential growth; *lanes 5, 6:* total RNA from cells dried for 24 h, 6.32 µg and 9.48 µg of RNA respectively; *lanes 7, 8:* total RNA (preparation 1) from cells in exponential growth; *lanes 9, 10:* total RNA (preparation 2) from cells in exponential growth

lower but the incorporation did increase in a linear fashion with increasing concentration of potassium acetate. Also, in the presence of potassium acetate, the concentration of magnesium acetate required for optimum incorporation was 15 – 21 mM. Concentrations of Mg^{2+} outside of this range decreased incorporation significantly. Under conditions where concentrations of Mg^{2+}, K^+ and ammonium chloride had been adjusted to permit optimum incorporation, low concentrations of calcium acetate (0.4 mM) had no effect on the rate of incorporation whereas high concentrations (5 – 7 mM) were inhibitory and decreased the final level of incorporation by more than 60%.

A slight stimulation in incorporation was observed when spermidine (0.4 mM) was added to those reaction mixtures which contained low concentrations of Mg^{2+} (15 mM magnesium acetate). At higher concentrations of magnesium acetate (21 mM), spermidine had no effect. Examination of autoradiograms suggested that the increased levels of incorporation achieved with spermidine were associated with the synthesis of low molecular mass polypeptides.

Translation capacities of the different RNA preparations

With five different samples of RNA prepared from *N. commune* UTEX 584 cells in exponential growth, and after immobilization and drying of cells for 24 h, equivalent levels of incorporation (five- to sixfold higher than the control) were achieved in the heterologous system. Electrophoretic and fluorographic analysis of the reaction products from the different translation reactions indicated virtually identical banding patterns with the most obvious polypeptides with mol. masses in the range ~ 8 to ~ 36 ($\times 10^3$; Fig. 6). The most singular qualitative difference between the different samples was the presence of a polypeptide (mol. mass = 14×10^3) in the reaction products of incubations where the RNA was prepared from cells in exponential growth and which contained a high concentration of tRNA (see Fig. 1; lane 3).

Direct extraction of RNA from dry colonies of *N. commune* HUN (desiccated for 5 years) yielded preparations which, in comparison to the RNA samples from *N. commune* UTEX 584, supported very low levels of *in vitro* translation. Preparations of RNA from rewetted materials of *N. commune* HUN gave significantly higher levels of incorporation, although the levels represented only 30% of those obtained with RNA from cells of *N. commune* UTEX 584 which had been immobilized and dried for 24 h under laboratory conditions. There was a clear positive correlation between the alleviation of inhibition and the time the cells of *N. commune* HUN had been rewetted prior to the extraction of RNA. In this regard, the time of rewetting was found to influence significantly the ultimate purity of the RNA preparation. When cells were dry the RNA preparations (after exhaustive purification steps) had $OD_{260\,280}$ values of 1.4 to 1.5. After 120 min of rewetting the cells, the RNA preparations obtained had $OD_{260/280}$ values of 1.9. In an attempt to achieve more efficient translation of the *N. commune* HUN RNA preparations, a heterologous wheat germ cell-free translation system (optimized for use with globin mRNA) was used. Preparations of RNA from both desiccated and rewetted cells were not translated and all inhibited the endogenous activity of the system (data not shown). The RNA preparation from laboratory grown cultures of *N. commune* UTEX 584 also failed to stimulate in vitro translation although no significant inhibitory effect on endogenous activity was observed.

Discussion

There are comparatively few reports of the RNA-directed synthesis of eubacterial (or cyanobacterial) proteins in *Escherichia coli* (Iname et al. 1985; Zubay 1973), presumably due to the difficulty in isolating intact messenger RNA's from bacterial cells and the fact that the mRNA's are translated immediately upon their synthesis. In the present study, the most efficient translation of *Nostoc commune* UTEX 584 RNA was obtained with a heterologous cell-free system based on an *Escherichia coli* S30 extract. Under optimum conditions, levels of incorporation obtained with *N. commune* UTEX 584 total RNA were five- to sixfold higher than the control. These levels of incorporation are high in comparison to the two- to fivefold level of stimulation obtained routinely for chloroplast mRNA's in wheat-germ systems (Reisfeld and Edelman 1982), and the threefold stimulation in incorporation obtained with polysomal RNA from *Nostoc* sp. strain MAC which was used in a S100 system purified from *E. coli* PR7 (Gupta and Carr 1983). In this latter study the translation system was composed of an *E. coli* S100 (purified over DEAE cellulose), supplemented with initiation factors, ribosomes and tRNA from the same strain of *E. coli*. Preparations of mRNA purified from *Nostoc* sp. MAC polysomal RNA gave incorporation levels tenfold higher than background with this system but they

did not drive in vitro translation when a crude S30 extract from *E. coli* PR7 was used. We made no attempt to isolate pure mRNA from *N. commune* UTEX 584 for translation purposes in view of the comparatively low yields of polysomal RNA which we obtained routinely from desiccated cells and cells in liquid culture (Angeloni and Potts 1986). Although there is some evidence for the existence of polyadenylated mRNA in cyanobacteria (Ownby et al. 1982), we did not attempt to enrich our RNA samples in polyA$^+$ mRNA through oligo-dT chromatography. However, several of the buffers used in the different extraction procedures described here were selected because they were expected to enrich for polyA$^+$ mRNA. The use of these buffers did, in fact, result in higher yields of total RNA.

The use of *N. commune* UTEX 584 total RNA in concentrations higher than ~ 16 µg per total reaction (0.3 mg ml^{-1}; optimum) led to a significant decrease in peptide synthesis in the heterologous system. Such an inhibitory effect could, as has been suggested, be due to the increased amounts of non-messenger components such as rRNA (Doolittle et al. 1979; Gupta and Carr 1983). Although the requirement for magnesium is lower for *E. coli* translation systems than for coupled transcription-translation systems (Bottomley 1982) – around 8 – 10 mM – our results suggest an optimum for *N. commune* UTEX 584 of 15–21 mM. This may reflect the requirement of a higher magnesium concentration for proper initiation of translation in cyanobacteria (see Laughrea et al. 1984; Tai and Davis 1979), or a lack of the correct initiation factors (Modolell 1971).

The homologous *N. commune* UTEX 584 S30 cell-free sytem proved to be unsuitable for the efficient in vitro translation of *N. commune* RNA. However, a single discrete band, corresponding to a polypeptide of mol. mass of 22×10^3 was, nevertheless, detected. It is significant that the same system, when primed with *E. coli* total RNA, gave rise to a prominent band on gels, of the same mol. mass (see Fig. 3). Whether these are one and the same, or different, polypeptides remains to be determined. In experiments where the conditions for the use of $S30_{Nos}$ were optimized, the final level of incorporation achieved appeared to be influenced by two antagonistic processes. Upon short-term incubation with $S30_{Nos}$ volumes of 4 – 6 µl (15 – 25% of the reaction volume) there is net incorporation in the system for a period of 30 min at the expense of factors which are exhausted during the translation reaction. Prolonged incubation of these reactions leads to proteolysis and, as the factors required for translation have become limiting, this leads to a net low level of incorporation. Larger volumes of $S30_{Nos}$ (> 6 µl) inhibit incorporation during short-term incubation (0 – 30 min). In the study of Gupta and Carr (1983) the time course of amino acid incorporation in vitro reached a plateau after 30 min. The components responsible for the inhibition remain unidentified at present but they appear to be inactivated or removed upon further incubation as, after approximately 60 min, translation can resume at the expense of translation factors still present in the reaction, leading to net incorporation. It is significant that the levels of incorporation achieved either in short-term incubations with small volumes of $S30_{Nos}$, or in long-term incubations with high volumes of $S30_{Nos}$, are quantitatively the same. The levels of incorporation in these experiments were such that it was not possible after examining autoradiograms to assess whether the translation products of 30 min reactions with low $S30_{Nos}$ volumes were qualitatively similar to the translation products of 90 min reactions which contained high $S30_{Nos}$ volumes. The proteases which are presumed to be responsible for the marked time-dependent decrease in incorporation seen in these experiments may originate either de novo through in vitro translation of protease transcripts in RNA preparations or, more likely, they may be endogenous in the $S30_{Nos}$ extract. A rapid and marked turnover of protein occurs when desiccated cells of *N. commune* UTEX 584 are rewetted (Potts 1985, 1986); this suggests that cell-free extracts would have the potential for high protease activity.

In the study by Gupta and Carr (1983) no increase in the level of incorporation in assays was detected when the *E. coli* S100 was replaced by a *Nostoc* sp. S100. Although optimum conditions for in vitro translation may differ markedly according to the strain of cyanobacterium under investigation, the apparent inability to stimulate high rates of in vitro translation of *Nostoc* spp. RNA in either a *N. commune* UTEX 584 S30 system (this study) or with a *Nostoc* S100 system (*Nostoc* MAC; Gupta and Carr 1983) under a wide range of experimental conditions, implies that there are basic limitations in the use of a homologous cyanobacterial in vitro translation system. Beside the importance of initiation factor(s) for the function of a stable complex of mRNA with ribosomes and those inhibitory proteins which specifically reduce initiation, in vitro studies with phage RNA's have shown that the conformation of the mRNA plays an important role in the differential translation of phage genes (Zubay 1973). Ultrastructural studies of *Nostoc commune* UTEX 584 cells show a high proportion of thylakoid membranes, making it likely that the mRNA has a quite different conformation within the living cell than when isolated as a pure fraction.

Model calculations suggest a roughly exponential dependence of mRNA functional half-life on the rate of ribosome initiation (King et al. 1986). If this is an important means of control, then differing codon usage might drastically affect the protein yield from some mRNA's. As such, it was expected that an availability of *N. commune* UTEX 584 tRNA could limit the in vitro translation of the RNA samples and, indeed, a marked accumulation of a polypeptide (mol. mass 14×10^3) was seen in assays which were enriched in tRNA. While this may reflect that the accuracy of translation is dependent upon the nature of the tRNA's present, only this single polypeptide resulted from the addition of a quite significant fraction of tRNA, and the overall quantitative incorporation was the same as in assays deficient in this tRNA fraction.

Few qualitative differences were observed between the one-dimensional pattern of proteins from in vitro translations of RNA pools from dried and immobilized cells of *N. commune* UTEX 584 and from cells of the same strain grown in liquid culture. Furthermore, no novel proteins appear to be synthesized when cells of this strain are subjected to water stress despite the fact that the protein synthesis machinery is maintained intact during rapid drying and short-term desiccation (Angeloni and Potts 1986). These data are similar to those obtained in studies with the desiccation-tolerant moss *Tortula ruralis* [Hedw.] Gaertn., Meyer and Scherb (Oliver and Bewley 1984) where it was suggested that rapid drying and short-term desiccation does not have any marked selective effect on the mRNA pool. In contrast, the RNA preparations from the desiccated field material of *N. commune* HUN were unable to drive high

rates of in vitro translation in the systems optimized for use in this study. While rewetting of the dried cells led to a marked improvement in the stimulation in incorporation by the RNA preparations, we do not know at present whether this represents de novo mRNA synthesis, or alleviation of some restraint upon translation such as a more efficient purification of the RNA from rehydrated cells. It is important to clarify this problem as field communities of *N. commune* HUN accumulate significant amounts of a discrete class of soluble proteins when they are subjected to repeated cycles of rewetting and drying (D. Silcutt, K. Jäger and M. Potts, unpublished data).

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Distinct fractions of genomic DNA from cyanobacterium *Nostoc commune* that differ in the degree of methylation *

(DNA-binding carbohydrates; buoyant density; methylcytosine; methyladenine; desiccation tolerance)

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Nostoc commune is a nitrogen-fixing cyanobacterium with a cosmopolitan distribution and a marked tolerance of desiccation (Whitton et al., 1979). We have been investigating the effects of water stress on gene expression in this cyanobacterium (Angeloni and Potts, 1986; Potts, 1985; 1986; Stulp and Potts, 1987). Little is known about the role of DNA methylation in the biology of cyanobacteria though several restriction-modification systems have been detected in species of *Nostoc* (Tandeau de Marsac and Houmard, 1987).

(a) Nuclease sensitivities and methylation status of the DNA fractions

Preparations of mRNA (Jäger and Potts, 1988) and genomic DNA were obtained from desiccated and rehydrated colonies of *Nostoc commune*. Each preparation of DNA was found to contain two distinct fractions of different buoyant density. The DNAs from each of the fractions, separated by CsCl density ultracentrifugation, were hydrolysed completely by DNAse I but showed different susceptibilities to a wide range of restriction endonucleases (Table I; Fig. 1). In particular, incubation of the two fractions of DNA with either *Dpn*I or *Mbo*I indicated that the DNA of lower buoyant density (fraction II) was highly methylated at adenines in the GATC sequences. The DNA of higher buoyant density (fraction I) was unmethylated at GATC sites (Fig. 1b). Fraction I was also digested extensively by both *Hpa*II and *Msp*I, and generated fragments less than 500 bp in size (Fig. 1b). The same two enzymes yielded partial digests of DNA from fraction II, and generated fragments in the size range 400 bp to 3 kb (Fig. 1c). The degrees of hydrolysis with either *Hpa*II or *Msp*I were equivalent to one another, with either DNA fraction, and where bands could be discerned after agarose gel electrophoresis of the digestion products, the patterns of the bands were very similar (Fig. 1c; lanes 4 and 6).

These data suggest that a significant proportion of cytosine residues at CCGG sites in fraction II DNA is methylated, and that methylation occurs at the internal and 5′-cytosine residues of these sequences with approximately equal frequency. No such methylation was seen in fraction-I DNA.

These digestions were also carried out with DNA purified from clonal axenic cultures of *N. commune*

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Abbreviations: A_{260}, absorbance at 260 nm; bp, base pair(s); kb, 1000 bp; mA, N^6-methyladenine; mC, 5-methylcytosine; T_m, melting temperature for DNA; TEM, transmission electron microscopy.

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TABLE I

Sensitivities of DNA fractions I and II (from *Nostoc commune*), and DNA from *Nostoc commune* UTEX584 to different restriction endonucleases

Enzyme[a]	Specificity[b]	Sensitivities and mean fragment size (kb)[c]					
		I		II		UTEX584	
*Ava*II	GG▽A▽CC● (T)	+	~1.2	-	>15	p	~2
*Bam*HI	GG○A●TCC	-	>15	-	>15	p	>6
*Bgl*II	▽A○GA●TCT	-	>15	-	>15	p	>6
*Eco*RI	G●A●TTC●	-	>10	+	~6.0	+	~4
*Hin*dIII	●A○AGCTT●	-	>15	p	>10	+	~3
*Kpn*I	GGTACC○○○	-	>15	-	>14	p	>6
*Pst*I	●CTGCA▽G●	+	~2	-	>15	p	~3
*Sal*I	GTCGA▽C●	+	~2	-	>15	p	~4
*Sau*3AI	G○ATC●	+	0.4	+	0.5	+	0.5
*Dpn*I	G○ATC●	-		+	~1	+	<1
*Mbo*I	G●ATC○	p	0.4	-		-	
*Hpa*II	○CCGG	+	0.5	p	~2	p	~2
*Msp*I	●CCGG	+	0.5	p	~2	p	~2

[a] The enzymes are described in Kessler et al. (1985).

[b] ●, mA or mC residues prevent cleavage; ○, mA or mC residues do not prevent cleavage; ▽, effect of mA or mC unknown.

[c] The average sizes of fragments generated by incubation with each of the enzymes are indicated in kb.

+, sensitive (main chromosomal band digested); -, insensitive (main chromosomal band undigested and no, or only traces of, smearing in gel lanes); p, partial hydrolysis (main chromosomal band visible but considerable smearing in gel lanes). Fig. 1 shows the extent of hydrolysis of the different DNA fractions by *Dpn*I, *Mbo*I, *Hpa*II and *Msp*I.

UTEX584 in exponential growth. A T_m value of 85.3°C was measured for the genomic DNA of *N. commune* UTEX584 (with a derived mol% G + C content of 39.03%) using the method of thermal denaturation. The DNA of *N. commune* UTEX584 aggregated as a single band during CsCl density ultracentrifugation with an apparent buoyant density equivalent to that of fraction II from the desiccated and rehydrated materials of *N. commune*. The DNA of *Nostoc commune* UTEX584 was highly methylated, contained both methyladenine and methylcytosine (Fig. 1a), and was hydrolysed only partially by a number of restriction endonucleases (Table I).

(b) Purification of DNA

The DNA from field materials of *N. commune* required extensive purification beyond that routinely required for laboratory-grown strains of cyanobacteria (Kallas et al., 1983; 1985; Mazur et al., 1980). Total DNA preparations, especially those from desiccated colonies, were brown-red in the initial stages of purification. Despite the extensive purification steps employed (see legend to Fig. 1), fraction I DNA remained bound to unidentified carbohydrate(s). In some cases fraction I also retained a brown-red color, albeit light, after purification from CsCl gradients. In contrast, fraction II DNA, purified from a range of desiccated and rehydrated materials, was not associated with carbohydrate or protein, as indicated by spot tests, electrophoretic analysis, and ratios of $A_{260/280}$ and $A_{235/260}$.

(c) Thermal denaturation and G + C content

Preliminary data from measurements of thermal denaturation suggest that DNAs from fractions I and II have different mol% G + C contents. At this time it has not been possible to achieve separation of

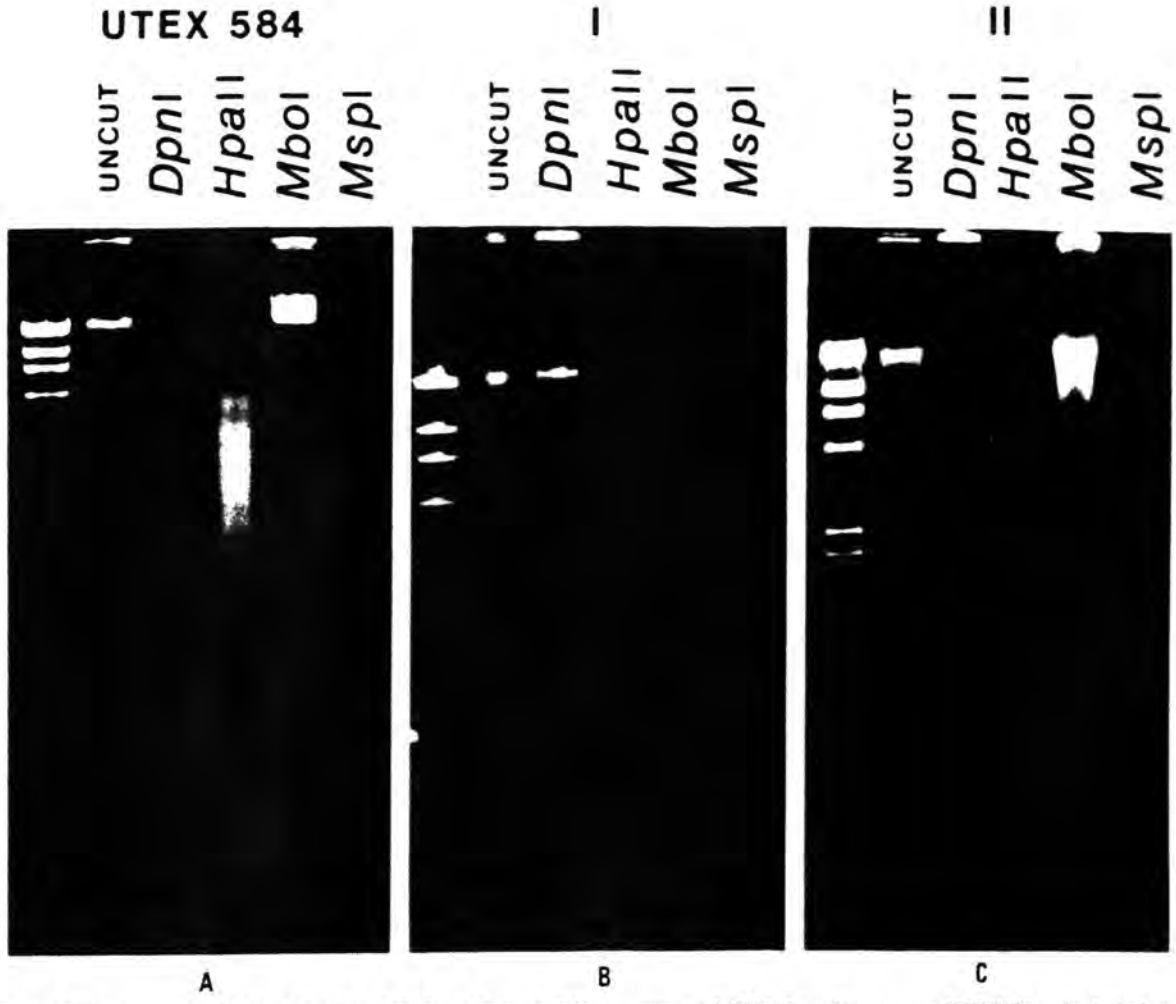

Fig. 1. Sensitivities of DNA fractions I and II (from desiccated *N. commune* and DNA from *N. commune* UTEX584 to hydrolysis by *Dpn*I, *Hpa*II, *Mbo*I and *Msp*I. Electrophoresis was performed in 0.7% w/v agarose minigels at 25 mA constant current in TBE buffer (0.089 M Tris · borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0). Phage λ DNA/*Hin*dIII markers were loaded in the first well of each of the gels. Digestions with the different restriction enzymes were performed at 37°C, for 60 min, according to the specifications of the manufacturer (Life Technologies Inc./BRL, Gaithersburg, MD, U.S.A.). DNA was obtained from desiccated *N. commune* using the following steps: rehydration of colonies for 30 min; grinding in liquid nitrogen; resuspension in 50 mM Tris · HCl, pH 8.5, containing 50 mM EDTA (TE) and 1 M NaCl, and shaking for 1 h (room temperature); centrifugation, resuspension in TE containing 0.2% w/v sarkosyl, incubation at 4°C overnight; centrifugation, resuspension of pellet in fresh TE/sarkosyl, freeze-thaw six times under liquid nitrogen; addition of lysozyme (10 mg/ml), incubation at 37°C for 2 h, addition of proteinase K (0.2 mg/ml) and sodium dodecyl sulfate (2% w/v); incubation at 37°C (1 h), then at 4°C (1 h), 65°C (30 min), 45°C (30 min); dilution of suspension with TE; exhaustive deproteinization; precipitation with ethanol and spooling of DNA; CsCl gradient ultracentrifugation (twice).

the carbohydrate from the DNA of fraction I nor measure the T_m of this DNA. However, total preparation of DNA from *N. commune* (containing both fractions I and II) had an apparent mol% G + C content of 51.95% (T_m = 90.6°C). The melting curve did not appear to be biphasic and no rapidly renaturing fraction was detected. Rapidly renaturing fractions, of undetermined identity, occur in DNA preparations from a wide range of cyanobacteria and, in some strains, may account for up to 24% of the total cell DNA (Herdman et al., 1979). These may represent repetitive sequences, or extrachromosomal elements such as phages and plasmids (Herdman, 1982). Of the latter, megaplasmids as large as 1000 kb have been described for several cyanobacteria (Rebière et al., 1986), and it has been suggested that undermethylation would specifically influence extrachromosomal elements in cyanobacteria (Padhy et al., 1988). However, if fraction I represents DNA of a megaplasmid(s), then the

and with a sequence complexity comparable to that of the genome.

(d) Conclusions

In summary, preparations of genomic DNA from colonies of *N. commune* consist of two major fractions differing in buoyant density. Fraction I is not detectably methylated, and even after extensive purification remains intimately associated with carbohydrate(s). Fraction II is highly methylated, is free of carbohydrate, and has the same buoyant density as the methylated genomic DNA purified from axenic cultures of *N. commune* UTEX584.

Large differences in the buoyant density of DNA preparations cannot be accounted for by their methylation status. As such, the finding of two discrete fractions of DNA from materials of *N. commune* could be accounted for by one or more of the following: (a) the presence of two different microorganisms with genomic DNAs of different buoyant densities; (b) a difference in mol% G + C content between two fractions of DNA from a single microorganism; (c) specific binding by carbohydrate(s) to fraction I; or (d) the presence of epichromosomal DNA, such as a plasmid. Colonies of *N. commune* collected from the field are not axenic, but detailed examination of thin sections of desiccated and rehydrated colonies, using both light microscopy and TEM, failed to locate any organism within the colonies other than the filamentous cyanobacterium *N. commune*. Surface contaminants, while present, represent an insignificant proportion of the biomass as judged from culture studies and microscopic examination, and they cannot account for the yield of DNA found in fraction I of DNA preparations. The procedure for the isolation of DNA from the colonies involved scrupulous washings of the colonies (including washes with 50% v/v ethanol), incubation of materials in the presence of 1 M NaCl and detergent solutions for extended periods of time (sometimes days), and then centrifugation (and in some cases sonication steps) before final recovery of the cells and cell disruption prior to the recovery of DNA. As a consequence, we consider that fractions I and II both derive from *N. commune*.

Colonies of *N. commune* contain large amounts of sheath material within which the filaments are DNA would appear to be maintained in the cell in an unusually high copy number (Rebière et al., 1986) embedded. Non-specific attachment of carbohydrate to DNA is likely to occur during purification of DNA. However, our data suggest a specific association of carbohydrate(s) to the less-methylated DNA of fraction I. The identity of fraction I DNA, and the means of association with the carbohydrate(s), are now under investigation.

ACKNOWLEDGEMENTS

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Quick Screening of Plasmid Deletion Clones Carrying Inserts of Desired Sizes for DNA Sequencing

WEN-QIN XIE and MALCOLM POTTS

Plasmids pWQX001 and pWQX005, constructed from pGEM-4 with an insert of 6.5 kb, were unidirectionally digested with exonuclease III and exonuclease VII. The DNA digests were ligated and used to transform competent cells of Escherichia coli *DH5-alpha. The size of the deletion plasmid carried by each transformant was estimated through agarose gel electrophoresis of crude lysates without any purification of the plasmid DNA. Colonies carrying plasmid DNAs with different deletions of the insert were grown and their DNAs were purified through a miniprocedure. The size of each purified plasmid DNA was determined accurately after linearization of the plasmid with an appropriate restriction endonuclease. The remainder of the DNA preparation was sufficiently pure to be sequenced using Sanger's dideoxynucleotide chain termination method. An easy, quick procedure is described for the preliminary selection of templates for DNA sequencing after construction of deletion clones of recombinant plasmid DNA using exonuclease III and exonuclease VII. This procedure permits a rapid screening of large numbers of colonies and selection of those carrying plasmid DNAs with inserts of the desired sizes for sequencing. This procedure does not require purification of the deletion plasmid DNA.*

The use of exonuclease III and exonuclease VII makes it possible to construct overlapping deletion clones from a DNA fragment for subsequent DNA sequencing [1]. Selection of those deletion clones with inserts of the desired sizes is, however, tedious work, especially when the original DNA fragment to be sequenced is large. Size analysis after purification of plasmid DNA from each deletion clone is also labor intensive. Therefore, a rapid and efficient procedure for the screening of deletion clones is needed. We have developed a technique, based upon a procedure described by Deininger and Smith [2], to screen large numbers of deletion clones rapidly in preparation for DNA sequencing. This procedure does not require any plasmid purification and permits one person to screen easily more than 300 colonies a day.

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Materials and Methods

Restriction endonucleases and DNA modification enzymes were purchased from Life Technologies Inc./BRL (Gaithersburg, MD) and were used as suggested by the manufacturer. All chemical reagents were of analytical grade.

Deletion Clones.

A recombinant phage carrying a 6.5-kb *Eco*RI–*Eco*RI fragment was isolated from a lambda gt10 genomic library of *Nostoc commune* UTEX 584 (Cyanobacteria) DNA. In preparation for sequencing, the insert was subcloned, in both orientations, at the unique *Eco*RI site of pGEM-4 (Promega Biotech, Madison, WI) using standard procedures [3], to construct pWQX001 and pWQX005, respectively. Deletion clones of the insert were constructed in both orientations by essentially following the procedure of Messing [1], using *Escherichia coli* strain DH5-alpha ($r_k^- m_k^+$) as the host cells. Four micrograms of each subcloned plasmid DNA were double digested with restriction endonucleases *sph*I and *Bam*HI. The digested DNA was precipitated with 2.5 volumes of 95% (v/v) ethanol and 10/1 volume of 3 M sodium acetate, and was redissolved in 100 µl of exonuclease III buffer (50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM DTT). Exonuclease III (3 µl, 65 units/µl) was added to the DNA solution, and the solution was mixed immediately and then incubated at 37°C. During the incubation aliquots of the reaction solution (3 µl) were removed every 30 seconds and transferred to ice-chilled 0.5-ml Eppendorf tubes, each containing 2 µl of 10× exonuclease VII buffer (1× = 50 mM KPO_4, pH7.0, 8 mM EDTA, and 1 mM DTT) to terminate the digestion of exonuclease III. Each of the Eppendorf tubes contained six aliquots (a pool) that had been removed consecutively from the reaction mixture. Thus six pools of exonuclease III digested DNA were generated. Each pool of DNA was then treated with 1 µl of exonuclease VII (1 U/µl) at 37°C for 1 hour, and the enzymes were

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inactivated by heating the solutions at 70°C for 5 minutes afterward. To each of the tubes, 1.5 μl of 0.2 M $MgCl_2$, 4 μl of 2 mM solution of dATP, dGTP, dCTP, and dTTP, and two units of Klenow fragment were added, and the mixtures were incubated at room temperature for 30 minutes to achieve DNA fragments with blunt ends. The DNA was ligated with T4 DNA ligase using a standard procedure [3]. Finally, the DNA was used to transform competent cells of *E. coli* DH5-alpha ($r_k^- m_k^+$) [4], and the transformants were grown on SOB–agar plates (SOB = 2% w/v Bacto tryptone, 0.5% w/v Bacto yeast extract, 10 mM NaCl, 0.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$), with each plate supplemented with 100 μg/ml ampicillin (AMP).

Competent Cells

Competent cells of DH5-alpha were prepared as described by Hanahan [4].

Preliminary Screening of Deletion Clones

Using sterile toothpicks, transformants derived from each separate pool of the treated DNA samples were transferred one by one from master plates and then streaked on SOB–agar (AMP, 100 μg/ml) plates. The plates were incubated at 37°C overnight. At this stage, the plates can be kept at 4°C for up to 3 weeks, or they may be used directly for further analysis. About 5–10% of the cell mass from each streak was removed using a sterile toothpick and transferred to a 0.5-ml Eppendorf tube by mixing vigorously. Each tube contained 10 μl of protoplasting buffer (20 mM Tris-HCl pH 7.5, 5 mM EDTA, 50 mM NaCl, 20% w/v sucrose, 100 μg/ml RNase A, and 50 μg/ml lysozyme), and the buffer became turbid immediately upon mixing. The suspensions were incubated at room temperature for a further 20–30 minutes and were then analyzed by agarose gel electrophoresis.

Electrophoresis

An agarose gel (0.7% w/v) containing 0.5 μg/ml ethidium bromide and 0.05% w/v sodium dodecyl sulfate (SDS) was used for electrophoretic analysis of the samples. The protoplast suspensions (each 10 μl in volume) and DNA molecular size markers were loaded into the gel slots after preloading of each well with 5 μl of lysis buffer (2.5 mM EDTA, 2% w/v SDS, 5% w/v sucrose, and 0.04% w/v bromophenol blue). The electrophoresis was performed with either a TBE (89 mM Tris-borate, 89 mM boric acid, and 2 mM EDTA) or TAE (40 mM Tris-acetate and 2 mM EDTA) buffer system [3]. In our laboratory, we routinely use a Life Technologies Inc./BRL large horizontal gel apparatus and prepare the gel with two combs (total of 40 wells) for the screening.

Minipreparation of Plasmid DNA

Colonies carrying plasmids with processive deletions of the insert were grown in 5 ml of SOB (AMP, 100 μg/ml) media for 12 hours, and the plasmid DNAs were purified from a 1.5-ml aliquot of each 5-ml cell suspension using a miniprocedure [5]. After purification the DNA was dissolved in 25 μl of distilled water. One microliter of each purified DNA sample was linearized with an appropriate restriction enzyme, and the sample was again analyzed by agarose gel electrophoresis to determine the size of the plasmid DNA more accurately. The remainder of each DNA sample was sufficient for one sequencing reaction.

Sequencing

The selected DNA templates (each 20 μl in volume) were sequenced using Sanger's dideoxynucleotide chain termination method [6]. The quality of DNA purified through the miniprocedure was sufficient to permit DNA sequencing with Klenow fragment.

Results and Discussion

Unpurified preparations of plasmid DNA, with inserts of different sizes, were resolved easily on an agarose gel (Figure 1). The number of colonies processed for each screening was determined solely by the number of wells available per gel. Agar, carried over during the picking of colonies, as well as incubation of the samples at room temperature for more than 40 minutes, both can contribute to the samples becoming too viscous to load in the wells of the agarose gel. Preparations of DNA from a bacterial colony carrying the vector plasmid only, and DNA from a colony carrying the undeleted recombinant plasmid, can be used as markers during electrophoresis. Because the DNA preparations were analyzed without purification, all the plasmid DNAs were supercoiled

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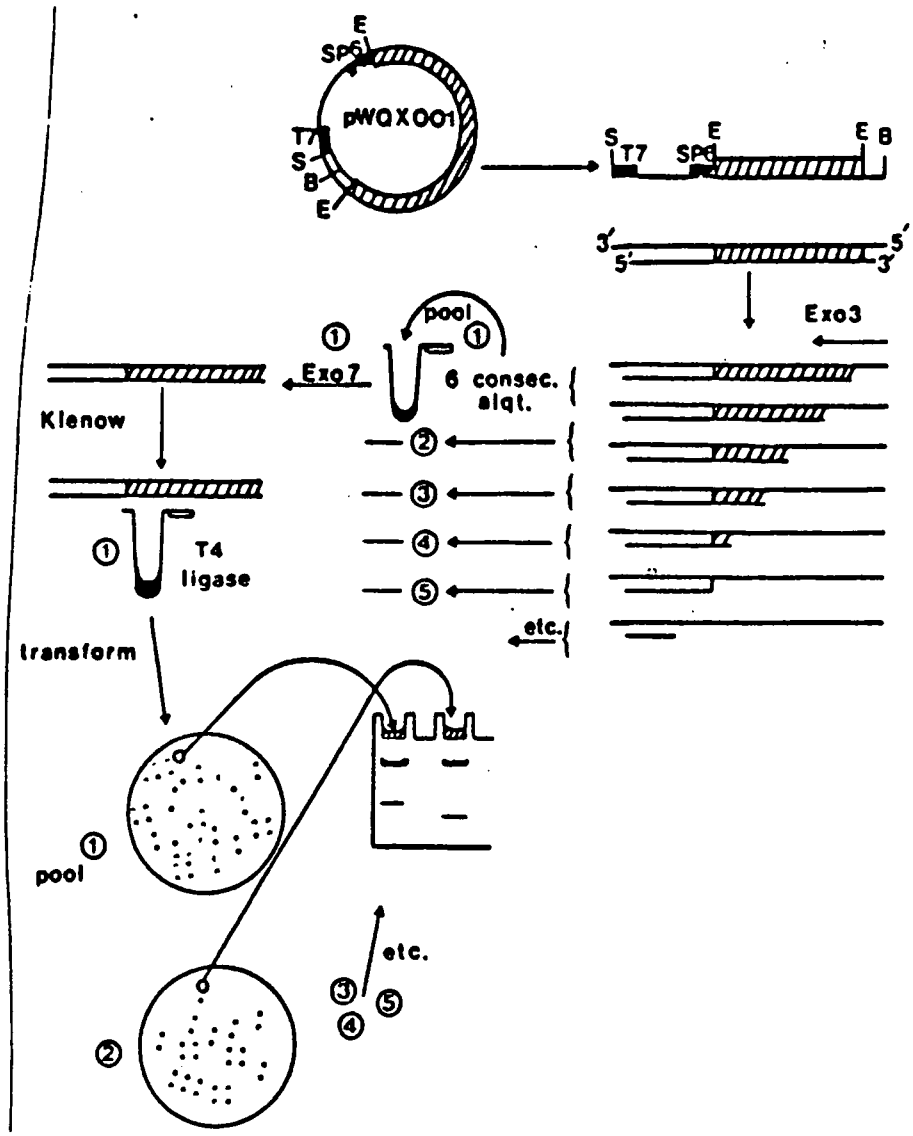

Figure 1. Outline of procedure for rapid screening of deletion clones. B, *Bam*HI; E, *Eco*RI; S, *Sph*I; Exo3, exonuclease III; Exo7, exonuclease VII; Klenow, the large (Klenow) fragment of DNA polymerase I; cross-hatching represents the cloned 6.5-kb *Eco*RI-*Eco*RI fragment of *Nostoc commune* DNA in the pGEM-4 vector, positions of the SP6 and T7 promoters in the recombinant parent plasmid (pWQX001) are indicated. Numbers refer to the different pools of aliquots containing different sizes of digested DNAs. The complete series of steps for pool 1 is indicated; in practice, each pool would be subjected to the same series of steps.

and ran faster than linear molecules of equivalent size. In contrast, plasmid DNA would exist in two forms, supercoiled and open circle, if the DNA was purified. The actual sizes of the plasmid DNAs were larger than those estimated using linear DNA size markers. About 300 deletion clones, derived from the deleted DNA of the original 6.5-kb insert, were checked through this procedure. A total of about 72 clones carrying a full range of deletions of the insert (in both orientations) were selected, and plasmid DNAs were purified from them through minipreparation for further study. Among the 72 clones, about 52 selected plasmid DNAs were sequenced using Sanger's dideoxynucleotide chain termination method [6].

Using this procedure, unpurified plasmid DNAs, which differ in size by about 3.5%, can be resolved. The smaller the vector DNA is, the better the resolution that can be obtained. The apparent size of a plasmid estimated through this procedure, when linear DNA size markers are

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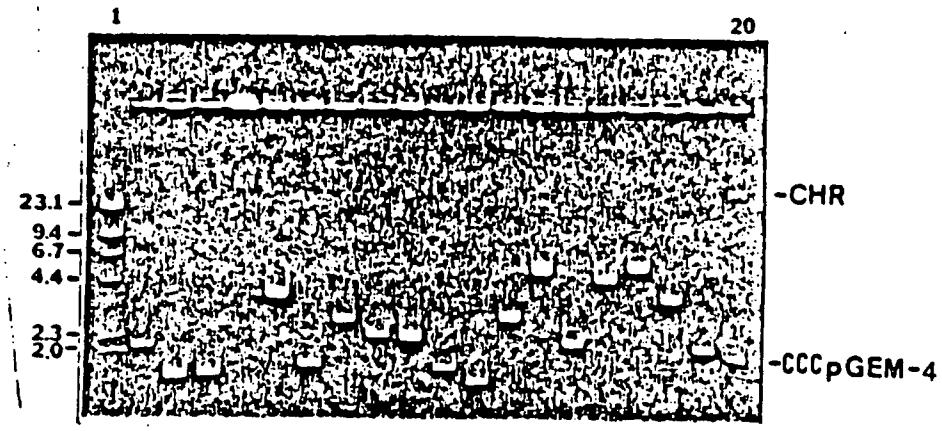

used, is smaller than its actual size. The latter can be determined after the plasmid DNA has been purified and linearized.

Figure 2. Agarose gel electrophoresis of unpurified plasmid DNA from deletion clones. Lane 1, *Hin*dIII–lambda DNA markers (kb); lanes 2–19, covalently closed circular (ccc) plasmid DNA from deletion clones; lane 20, ccc pGEM-4 DNA (2.85 kb); CHR, chromosomal DNA.

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Cyanobacterial RNA Polymerase Genes *rpoC1* and *rpoC2* Correspond to *rpoC* of *Escherichia coli*

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The DNA-dependent RNA polymerase (ribonucleoside triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) of cyanobacteria contains a unique core component, γ, which is absent from the RNA polymerases of other eubacteria (G. J. Schneider, N. E. Tumer, C. Richaud, G. Borbely, and R. Haselkorn, J. Biol. Chem. 262:14633–14639, 1987). We present the complete nucleotide sequence of *rpoC1*, the gene encoding the γ subunit, from the heterocystous cyanobacterium *Nostoc commune* UTEX 584. The derived amino acid sequence of γ (621 residues) corresponds with the amino-terminal portion of the β′ polypeptide of *Escherichia coli* RNA polymerase. A second gene in *N. commune* UTEX 584, *rpoC2*, encodes a protein which shows correspondence with the carboxy-terminal portion of the *E. coli* β′ subunit. The *rpoBC1C2* genes of *N. commune* UTEX 584 are present in single copies and are arranged in the order *rpoBC1C2*, and the coding regions are separated by short AT-rich spacer regions which have the potential to form very stable secondary structures. Our data indicate the occurrence of divergent evolution of structure in the eubacterial DNA-dependent RNA polymerase.

The transcription of genes is directed through the activity of DNA-dependent RNA polymerase (ribonucleoside triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6). In eubacteria, a single form of the core RNA polymerase, together with ancilliary sigma factors, is responsible for the synthesis of virtually all cellular RNAs (5). The RNA polymerase of *Escherichia coli* consists of at least four different subunits, β, β′, α, and σ, and is present in two main enzyme forms, core ($\beta\beta'\alpha_2$) and holoenzyme (core plus σ; 4). The two genes encoding the β (*rpoB*) and β′ (*rpoC*) subunits of this RNA polymerase are adjacent to one another and are cotranscribed from the major promoter PL10 (5). The basic ($\beta\beta'\alpha_2$) design has been found in the RNA polymerases purified from representatives of gram-positive and gram-negative eubacteria (17, 38). Recently, however, an additional core component, γ, has been described for the RNA polymerase ($\beta\gamma\beta'\alpha_2\sigma$) of the cyanobacterium *Anabaena* sp. strain PCC 7120 (32). The γ subunit is serologically unrelated to the other subunits of the cyanobacterial RNA polymerase, but anti-γ serum cross-reacts with both *E. coli* β′ subunit protein and subunit A of the RNA polymerase from *Sulfolobus acidocaldarius*, an archaebacterium (31). The γ subunit has since been detected in the RNA polymerases of 15 out of 15 taxonomically diverse cyanobacteria, including two *Nostoc* species (31).

Three different nuclear RNA polymerases are found in eucaryotes, each one responsible for the transcription of a different class of genes (17). Comparison of the amino acid sequences of the largest subunit, A, of RNA polymerases II and III from *Saccharomyces cerevisiae* and the β′ subunit of the *E. coli* RNA polymerase revealed six regions (I to VI) of marked conservation (1).

The RNA polymerases of archaebacteria appear to be more closely related to those of eucaryotes (6, 38). The subunit compositions of the enzymes from representatives of the halophilic or methanogenic and sulfur-dependent thermophilic branches of the archaebacteria differ. It is unclear whether archaebacteria, like eucaryotes, possess multiple species of RNA polymerase (6, 38).

The chloroplast DNA of higher and lower plants contains regions with sequence similarity to *rpoA*, *rpoB*, and *rpoC* of *E. coli* (11, 23, 24). In the present study, we show that in the cyanobacterium *Nostoc commune* UTEX 584 two separate and linked genes correspond to different portions of the single *rpoC* gene of *E. coli*. One of these genes, for which the complete sequence is presented, encodes the γ subunit.

MATERIALS AND METHODS

Microorganisms and growth conditions. *N. commune* UTEX 584 and *Anabaena variabilis* PCC 7118 were grown as described previously (25) in liquid BG-11_0 medium (28) and BG-11 medium, respectively.

Recombinant DNA analyses. Routine methods were used for the manipulation of DNA (8, 18). Restriction endonucleases were obtained from BRL/Life Technologies Inc. and were used according to the specifications of the manufacturer. Plasmid and bacteriophage DNAs were purified as described previously (18, 33).

Synthesis of biotinylated *rpo* DNA probes. The plasmids pPD489 and pPD490 were generous gifts of P. Dennis, University of British Columbia, Vancouver, British Columbia, Canada. These two plasmids are derivatives of pBR322. pPD489 carries an *Eco*RI fragment (2.6 kilobase pairs [kb]) from within *E. coli rpoC*, and pPD490 carries an *Eco*RI fragment (2.8 kb) from within *E. coli rpoB* (5). The *Eco*RI fragments were purified, and nick translation (27) was used to label the DNA fragments with biotin-11-dUTP. The biotin-11-dUTP, DNA polymerase I, and DNase I were obtained from BRL/Life Technologies Inc. Southern blotting (34) was used both to determine the sizes of those fragments of *N. commune* UTEX 584 genomic DNA which hybridized to these probes and to optimize the conditions for filter hybridization. Procedures for Southern transfer and hybridization were as described by Mason and Williams (19).

Screening of the λgt10 library. A recombinant library of genomic DNA from *N. commune* UTEX 584 was constructed with the phage insertion vector λgt10 (imm^{434} *h527*) and propagated in *E. coli* C600 (*hfl*) by standard methods (8).

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The library was constructed with *Eco*RI restriction fragments of *N. commune* UTEX 584 genomic DNA in the size range of 3 to 7 kb. Fragments of DNA of the appropriate size were isolated by sucrose density gradient centrifugation (15). Plaques of recombinant phages were transferred from agar plates (with soft overlays) to nitrocellulose filters (diameter, 82 mm; 19), and the library was screened (13) with the biotinylated *rpoC* DNA probe. Biotinylated DNA-DNA hybrids (positive plaques) were visualized through the use of a colorimetric assay which used streptavidin-alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate, and Nitro Blue Tetrazolium (BRL/Life Technologies Inc.). Positive plaques were isolated and subjected to two rounds of plaque purification. The cloned *Eco*RI fragments of *N. commune* UTEX 584 DNA were isolated from the purified phage DNA and subcloned, in both orientations, in pGEM-4 (Promega Biotec).

Deletion cloning and DNA sequence analysis. Processive digestion of the cloned DNA with exonuclease III and exonuclease VII was used to generate overlapping deletion fragments (35, 36). The linear fragments were then treated with the Klenow fragment of DNA polymerase I and blunt end ligated (with T4 ligase) for 5 h at room temperature, and the deletion plasmids were used to transform (9) *E. coli* DH5-α (r_K^- m_K^+; BRL/Life Technologies Inc). Plasmid preparations from the different transformants were used in DNA sequencing reactions with [α-^{35}S-thio]dATP (>400 Ci mmol^{-1}; Dupont, NEN Research Products) and the Klenow fragment of DNA polymerase I, following the dideoxy-chain termination method of Sanger et al. (29).

DNA sequence analysis. DNA sequence data were manipulated with the IBI-Pustell software of International Biotechnologies and PCGENE (IntelliGenetics).

In vitro transcription-translation of *N. commune* UTEX 584 *rpo* genes. The expression of *N. commune* UTEX 584 *rpo* genes was studied in a procaryotic cell-free coupled transcription-translation system obtained from Dupont, NEN. DNA templates for use in the in vitro system were purified after two rounds of cesium chloride ultracentrifugation and dialysis (18). Proteins were synthesized in the presence of carrier-free L-[^{35}S]methionine (1,115 Ci mmol^{-1}). Conditions for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the determination of incorporation of [^{35}S]methionine in protein were as described previously (14), with the exception that gels were prepared for autoradiography without the use of fluorographic enhancing agents.

In vivo transcription-translation of *rpo* genes. The 6.5-kb *Eco*RI DNA fragment of *N. commune* UTEX 584 DNA was subcloned in the pUC18 expression vector (BRL/Life Technologies Inc.) and a recombinant plasmid (pX1) was used to transform *E. coli* JM109. Expression from the *lac* promoter was induced with IPTG (isopropyl-β-D-thiogalactopyranoside). Gene products were analyzed through Western blotting (immunoblotting) and immunolabeling as described previously (26), with the exception that the buffer used in electroblotting was supplemented with 0.01% (wt/vol) sodium dodecyl sulfate, and the transfer time was 2 h (24 V/A).

Antibodies. Antiserum raised against the core ($\beta\gamma\beta'\alpha_2$) of the RNA polymerase of *Anabaena* strain PCC 7120 was a gift from G. J. Schneider and R. Haselkorn.

RESULTS

Isolation of *N. commune* UTEX 584 *rpo* genes. From Southern analyses, it was determined that the *E. coli rpo* probes hybridized with a single 6.5-kb *Eco*RI fragment of *N. commune* UTEX 584 genomic DNA. Subsequently, a recombinant phage, isolated from one positive-hybridizing plaque after screening of the gene library, was found to contain a DNA insert of 6.5 kb which gave strong hybridization signals under stringent conditions with both of the *E. coli rpo* probes used. Through the use of a set of deletion clones generated from one strand of the insert, it was determined that the *E. coli rpoB* probe hybridized only with those deletion fragments larger than 4.5 kb. The *rpoC* probe hybridized with all deletion fragments larger than 1.5 kb. It was assumed, therefore, that the 6.5-kb fragment contained sequences corresponding to both *E. coli rpo* genes. A nick-translated biotinylated probe generated from the purified 6.5-kb *N. commune* UTEX 584 DNA fragment hybridized, under stringent conditions, with a single 6.5-kb *Eco*RI fragment of *A. variabilis* PCC 7118 DNA (data not presented).

Organization of *N. commune* UTEX 584 *rpo* sequences. Sequence analysis was completed for both strands of the 6.5-kb DNA fragment by using 52 overlapping deletion clones. Three potential coding regions of 2,217, 1,866, and 2,151 base pairs (bp), separated by relatively short AT-rich sequences, were detected. The first, and incomplete, coding region showed extensive sequence similarity (data not presented) with portions of both the *rpoB* gene of *E. coli* and open reading frame 1070 isolated from tobacco chloroplast DNA (22). The second, and complete, region, of 1,866 bp (designated *rpoC1*; Fig. 1), represented an open reading frame of 622 codons which showed sequence similarity with the first 1,800 bp of *E. coli rpoC* (Fig. 2). The third region, of 2,151 bp (a portion of *rpoC2*; Fig. 1), showed sequence similarity with the remainder of *E. coli rpoC* (Fig. 2). For further discussion purposes, the fragment of DNA carrying the incomplete portions of *rpoB* and *rpoC2* and the complete sequence of *rpoC1* is referred to as *rpoBC1C2*. The amino acid sequences derived from *rpoC1* and *rpoC2* indicated that regions homologous to conserved domains within the β′ subunit of *E. coli* RNA polymerase were distributed between the two *N. commune* UTEX 584 gene products (Fig. 2, 3, and 4).

Intergenic sequences of the three *rpo* coding regions. The two nucleotide sequences separating the three *rpo* coding regions in *N. commune* UTEX 584 were AT rich and contained regions of extensive sequence similarity (Fig. 1). Several distinct components were recognized in the intergenic sequences of the *N. commune* UTEX 584 *rpo* genes through visual alignment. These components (TTAG repeat, TTAATT, and CAAAC sequences) were spaced at equivalent distances upstream from the presumed translational initiation codons of *rpoC1* and *rpoC2*. Potential ribosome-binding sequences (GGA and AGGA) were located 15 and 10 bases upstream from the translational initiation codons of *rpoC1* and *rpoC2*, respectively. The sequences from both intergenic regions have the potential to form very stable secondary structures (Fig. 5A and 5B).

Subunit composition of *N. commune* UTEX 584 core RNA polymerase. The β′, β, γ, and α subunits of *N. commune* UTEX 584 RNA polymerase, with M_rs of >180,000, approximately 159,000, 72,000, and 43,000, respectively, were identified through cross-reaction with *Anabaena* strain PCC 7120 core antiserum (data not presented).

Expression of *N. commune* UTEX 584 *rpoBC1C2* sequences in cell-free system and in *E. coli*. All of the ^{35}S-labeled products from the expression of *rpoBC1C2* in the cell-free system were precipitated with the *Anabaena* strain PCC 7120 core-specific antiserum, and immunoanalysis detected

```
                10        20        30        40        50        60
          Trm   |         |         |         |         |         |
    1 TAAGGAGTGCTGAGTGCTAAATGCTAAATGCTGAGTATTAAAAAACTCAGTAATTAGCATTTAGC

   66 ACTTAGAAATCTCTTAATTCAAACCTTAAAACTAAAATATACTCAAAACTCGGAACTCAGCACTT
          Met
  131 AAGTATGAGACCTGCCCAAACTAATCAGTTTGACTACGTAAAAATCGGCTTGGCTTCCCCTGAAC
  196 GTATTCGTCAGTGGGGCGAGAGAACATTGCCCAATGGTCAAGTAGTCGGTGAAGTTACCAAGCCA
  261 GAGACAATTAACTACCGAACTCTCAAGCCAGAGATGGATGGCTTATTTTGTGAGCGGATCTTTGG
  326 TCCGAAAGATTGGGAATGCCATTGTGGCAAGTATAAAGAGTCCGTCATAGAGGTATTGTCTGTGA
  391 GCGCTGTGGTGTTAGAAGTCACCGAGTCACGGGTGCGTCGTCACCGTATGGGCTATATCAAACTC
  456 GCCGCACCAGTAGCTCACGTTGGTTACTCAAAGGCATTCCTAGCTATATTCCATTCTGTTGGATA
  521 TGCCCTACGCGATGTCGAGCAGATTGTCTATTTCAACTCTTATGTTGTCCTGAGTCCTGGTAATG
  586 CTGAAACTTTAACTTACAAACAACTACTCAGTGAAGATCAGTGGTTGGAAATAGAAGACCAAATT
  651 TATAGCGAAGATTCTCTGCTGCAAGGCGTAGAGGTAGGTATTGGCGCTGAAGCGCTATTGCGTTT
  716 GCTTGCCGATATCAATTTGGAACAAGAAGCGGAAAGCCTACGGGAAGAAATTGGCAACGCCAAGG
  781 GACAAAAGCGGGCCAAGCTAATTAAGCGACTGCGGGTAATTGACAACTTCATCGCAACTGGTTCC
  846 AAACCAGAGTGGATGGTAATGGCAGTTATTCCTGTGATTCCCCCTGACTTGCGCCCAATGGTACA
  911 GCTAGATGGCGGACGGTTTGCCACCAGCGATTTAAATGATTTATATCGACGGGTAATTAACCGCA
  976 ACAATCGTTTGGCACGCTTGCAAGAAATTTTAGCACCAGAAATTATTGTGCGGAACGAAAAGCGG
 1041 ATGCTGCAAGAAGCAGTGGATGCTTTGATTGACAATGGTCGTCGGGGACGCACTGTAGTAGGGGC
 1106 AAATAACCGACCACTGAAATCTTTGTCCGACATTATTGAGGGTAAGCAAGGACGTTTCCGACAAA
 1171 ACTTGTTAGGTAAACGGGTTGACTACTCTGGACGTTCGGTTATTGTAGTCGGGCCAAAGCTGAAA
 1236 ATTCACCAGTGCGGTTTACCTAGAGAAATGGCAATTGAGCTATTTCAACCATTTGTAATTAACCG
 1301 CCTGATTCGTAGCGGTATGGTAATACATCAAGCTGCGCCAATGATATCGCGTAATGACCCCAGTG
 1366 TTTGGGATGTGCTGGAAGAAGTGATTGAAGGACACCCAGTGATGCTAAACAGGGCGCCAACATTG
 1431 CACCGTTTGGGTATTCAGTCTTTTGAACCGATTTTGGTAGAAGGTAGAGCGATTCAACTGCATCC
 1496 TCTGGTGTGTCCAGCGTTTAACGCCGACTTTGACGGGGACCAAATGGCGGTACACGTCCCTCTTT
 1561 CTTTAGAAAGTCAGGCTGAAGCGCGATTGTTGATGTTGGCTTCTAACAATATTTTGTCACCAGCC
 1626 ACGGGTAAACCGATCATCACGCCTAGCCAAGATATGGTGTTGGGAGCCTATTATTTAACAGCAGA
 1691 AAATCCCGGTGCGACAAAAGGTGCAGGAAAGTACTTTTCTTCGCTAGAGGATGTAATCATGGCTT
 1756 TCCAGCAAGAACAAATTGACTTGCACGCCTATATTTACGTGCGGTTTGACGGTGAAATAGAATCA
 1821 GACCAACCGGATACGGAACCCGTGAAAGTGACGGAAAACGAGGATGGTACCCGTACATTACTCTA
 1886 TAAGTTCCGTCGAGTCAGACAAGACGCTAAAGGCAATGTACTTTCCCAGTATATATACACAACTC
 1951 CAGGTCGCGTTATTTACAATAATGCTATTCAGGAAGCACTAGCAAGCTAAAAGTAAGGAGTGAAA
                                                    Trm
 2016 AGTTAGGAGTTAGGAGTTATTAATTACAAACTCCTAACTCCTAACTCCTAACTATTAATTTAGGA

                Met
 2081 CTAATGACTAATGACTAACGAAAAAATGATTTTTCGCAATCGGGTGGTTGATAAAGGTCAACTGA
 2146 GAAATTTAATTTCTTGGGCCTTTACCTATTATGGTACGGCGCGAACCGCAGTAATGGCAGACAAA
 2211 CTGAAAGATTTGGGATTTCGCTACGCTACCAAAGCAGGGGTTTCCATCAGTGTAGATGACTTGAT
 2276 GGTGCCACCAACTAAGCGATTGCTCTTAGAAGCAGCCGAAGAAGAAATTCGGGCTACTGAAACCC
 2341 GTTACCAACGAGGAGAAATCACAGAAGTAGAACGCTTCCAAAAGGTAATCGATACCTGGAACGGT
 2406 ACTAGTGAAGCTTTGAAAGATGAAGTAGTCGTTCACTTCAAAAAGACTAATCCCCTAAGCTCCGT
 2471 GTATATGATGGCATTTTCCGGTGCACGGGGTAACATCTCTCAAGTGCGGCAATTGGTGGGGATGC
 2536 GGGGACTGATGGCAGATCCTCAAGGGGAAATTATCGATTTGCCCATCAAAACCAATTTCCGTGAA
 2601 GGATTAACTGTAACGGAATACATTATTTCGTCTTACGGTGCCAGAAAAGGATTGGTGGTACAGCC
 2666 TTCACGGACGGCTGACTCTGGTTATCTCACCCGCCGATTGGTGGATGTATCCCAGGTGTATTATT
 2731 CGGGATTTGACTGCGGCACGCCAGAGCTTAGCATTCGACCAATGACAGAAGGGGCCAAAACCTTG
 2796 ATTCCTCTAGCAACTCGCTTGATGGGACGGGTAATTGGCGAAGATGTGCTGCATCCGGTAACAAA
 2861 AGAAGTGATTGCAGCACGCAATTCCCCAATTTCTGAGGATTTGGCGAAGAAAATTGAAAAATCTG
 2926 GGGTGGGCGAAGTTGTGGTGCGATCGCCACTAACTTGTGAAGCTGCACGTTCTGTCTGTCAGCAC
 2991 TGCTACGGCTGGAGTTTGGCACACGCAAGCATGGTAGATTTGGGTGAAGCTGTGGGGATTATTGC
 3056 CGCCCAAAGTATCGGCGAACCTGGTACCCAGCTAACCATGCGGACATTCCACACAGGTGGGGTGT
 3121 TTACTGGAGAAGTGGCGCAACAAGTGCGTTCCAAAATCGATGGTACTGTCAAGCTTCCTCGCAAA
 3186 CTGAAGACCAGAACATATCGTACTCGCCACGGGGAAGATGCCCTCTATGTTGAGGCTAATGGCAT
 3251 CATGCTTTTGGAGCCAACAAAAGTAGGTGATGTTACCCCAGAAAACCAAGAGGTTCATCTTACCC
 3316 AAGGTTCAACACTATATGTATTTGATGGAAATAAGGTAAACAAGGTCCAGTTGTTAGCAGAGGTT
 3381 GCCCTTGGTGGACGTACAACTCGGACTAATACAGAAAAAGCAGTTAAAGACGTCGCTTCTGACTT
 3446 AGCAGGGCAAGTGCAATTTGCCGAAGTTGTTCCAGAACAAAAAACTGACCGTCAAGGCAACACTA
 3511 CAACCACACGCCGCACGCGTGGCTTGATTTGGATTTTGTCTGGGGAAGTTTACAACTTGCCGCCA
 3576 GGGGCCGAATTGGTGGTGAAAAATGGTGATGCGATCGCTTCAAATGGAGTTTTAGCAGAAACCAA
 3641 GTTAGCCAGTTTGCACGGCCGTGTGGTGCGCTTGCCAGAAGCTACCCCAGGTAAGAGTACCAGGG
 3706 AAATTGAGATTATCACCGCTTCTGTAGTCTTAGACCAGGCAACGGTGACAGTTCAAAGTTCTCAA
 3771 GGACGTAATAACTACTTAGTTTCTACTGGCAACAACCAAGTATTTAACCTCCGGGCTACACCAGG
 3836 CACAAAAGTGCAAAATGGTCAAGTAGTAGCTGAGTTAATTGATGACCGCTATCGCACAACCACTG
 3901 GTGGATTCCTGAAATTTGCTGGTGTAGAAGTCCAGAAAAAAGGCAAAGCCAAGCTGGGTTATGAA
 3966 GTCGTGCAGGGCGGTACCTGTTGTGGATCTCCTGAAGAAAGCCACGAAGTTAATAAAGATATCTC
 4031 CTTGCTGTTGGTGGAAGACGGCCAGTTTGTGGAAGCTGGCACCGAAGTCGTGAAAGATATCTTCT
 4096 GCCAAAACAGTGGTGTGGTAGAAGTGACCCAGAAAAACGACATCCTCCGGGAAGTCGTGGTGAAG
 4161 CCAGGGGAACTGCTGATCGTGGACGATCCAGAATCAGTCATCGGGCGAGATAACACCTTCATCCA
 4226 ACCAGGTGAGGAATTC
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FIG. 1. Nucleic acid sequence of *rpoC1* and a portion of *rpoC2*. The sequence presented begins with the translational termination codon (Trm) of *rpoB*. Translational initiation codons for *rpoC1* and *rpoC2* are indicated (Met). In the sequences between the coding regions of *rpoB*, *rpoC1*, and *rpoC2*, light underlining indicates direct repeats and bold underlining indicates two regions of sequence similarity (see Fig. 5A and B). Putative Shine-Dalgarno sequences are indicated with overlining.

two strong bands, corresponding to polypeptides with M_rs of 71,000 and 94,000, when *rpoC1* and *rpoC2* were expressed from a *lac* promoter in *E. coli* JM109(pX1) (data not presented).

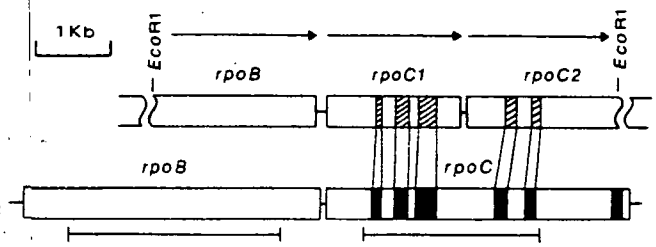

FIG. 2. Structural organization of *rpo* genes in *N. commune* UTEX 584 (upper) in comparison with *E. coli* (lower). The direction of transcription is indicated with arrows. The portions of the *E. coli* *rpo* genes used as probes are underlined. Regions of extensive sequence similarity (corresponding to segments I to VI [1]) are indicated by shading (*E. coli*) and hatching (*N. commune* UTEX 584).

DISCUSSION

Immunoanalysis has shown the cyanobacterial RNA polymerase to have a core structure of $\beta\gamma\beta'\alpha_2$ with what appears to be a single species of σ factor (31, 32). In the cyanobacterium *N. commune* UTEX 584, two genes, *rpoC1* and *rpoC2*, correspond to the single gene (*rpoC*) in *E. coli* which encodes the β′ subunit of RNA polymerase. The amino acid sequences derived from *rpoC1* and the region of *rpoC2* which was sequenced in this study correspond, respectively, with the amino-terminal and carboxy-terminal portions of the *E. coli* β′ subunit polypeptide. The γ subunit polypeptide contains 3 of the 6 highly conserved domains shared between the *E. coli* β′ subunit and subunit A of eucaryotic RNA polymerases (1). Our data provide evidence of divergent evolution of structure in the genes encoding the large subunits of eubacterial RNA polymerase.

Expression of *N. commune* UTEX 584 *rpoC1* and *rpoC2* in a cell-free system and in *E. coli* generated translation products which cross-reacted with *Anabaena* strain PCC 7120 core antiserum, and the cross-reactive polypeptide with an M_r of 71,000 comigrated with the γ protein of *N.*

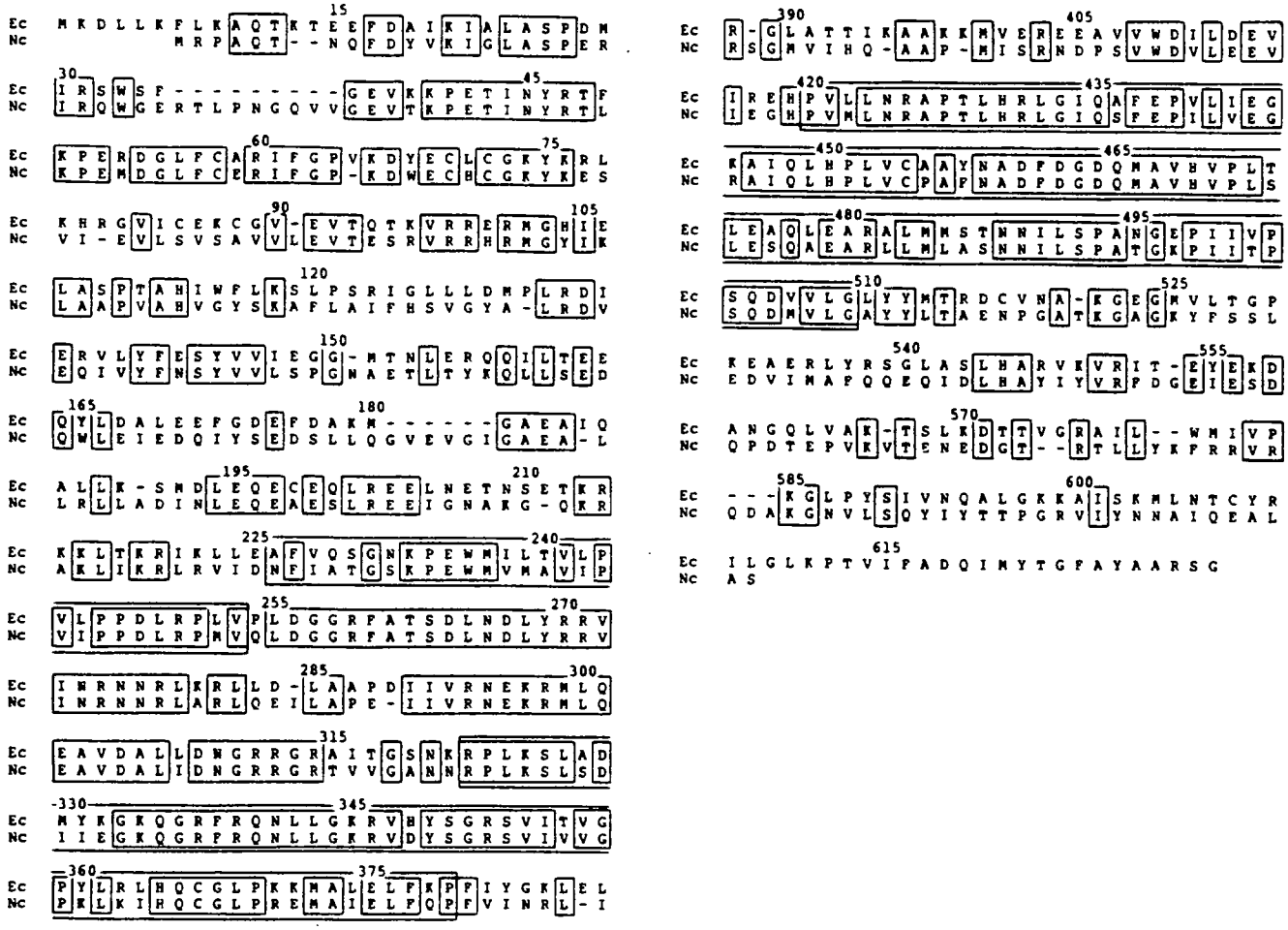

FIG. 3. Alignment of the derived amino acid sequence of γ from *N. commune* UTEX 584 (Nc) with the N-terminal portion of *E. coli* β′ (Ec). Dashed lines indicate a shifting of the sequences to permit the best fit. Numbering refers to the amino acid residues in the *E. coli* β′ subunit. Regions of sequence correspondence are enclosed in a single box. A double box around a region of sequence indicates the segments in the *E. coli* β′ subunit with extensive sequence similarity to component A of eucaryotes (1).

commune UTEX 584. In addition, the *rpoC1* gene from *Anabaena* strain PCC 7120 has been expressed from a T7 promoter of *E. coli*. It yields a protein with an M_r of 66,000 identified by γ-specific antibody in Western blot analysis; thus, it is clear that γ is the primary product of the *rpoC1* gene (K. Bergsland and R. Haselkorn, unpublished data). The *rpoC1* gene product of *N. commune* UTEX 584 has an M_r of 71,000, a value which agrees closely with the predicted M_r obtained from sequence analysis (M_r = 70,200). Both of these values are somewhat larger than the values for γ reported in a recent study of 15 taxonomically diverse strains of cyanobacteria (range in M_r, 65,000 to 68,500; 31) and are equivalent to the values reported (M_r = 72,000) for the γ subunits of two other cyanobacteria (10, 20).

The genes encoding the β, γ, and β′ subunits of *N. commune* UTEX 584 RNA polymerase are present in single copies, are transcribed in the same direction, and are arranged in the order *rpoB*, *rpoC1*, and *rpoC2*, respectively. As the γ subunit has been found in all those cyanobacteria tested to date (31), and as the 6.5-kb *Eco*RI DNA fragment harboring the *N. commune* UTEX 584 *rpoBC1C2* genes hybridized with a single 6.5-kb *Eco*RI fragment of *A. variabilis* PCC 7118 DNA, it seems likely that the arrangement of *N. commune* UTEX 584 *rpoBC1C2* genes described here will be found in other cyanobacteria. While the degree of sequence similarity between the intergenic regions of the *N. commune* UTEX 584 *rpo* genes is striking, the functional significance of these AT-rich sequences (with respect to transcription or translation), other than having the potential to encode very stable secondary structures, remains unclear. Short AT-rich spacer regions also separate *rpoC1* and *rpoC2* in the chloroplast genomes of *Marchantia polymorpha*, spinach, and tobacco (11, 23, 24).

Evidence for differences in the subunit compositions of archaebacterial RNA polymerases has been provided by immunochemical analyses (37–39). In halobacteria and the methanogenic bacteria, two subunits, B′ and B″, cross-react with a single subunit, B, present in the thermophile *Sulfolobus acidocaldarius* (37, 38). In addition, two subunits in archaebacterial RNA polymerases, A and C, are homologous with N-terminal and C-terminal portions of the β′ subunit of *E. coli*, respectively (37). Data from hybridization studies and DNA sequencing have shown that the order of the structural genes encoding A, B′, B″, and C in the methanogenic bacterium *Methanobacterium thermoautotrophicum* is B″, B′, A, and C (2, 30, 37). The division of β′ determinants between A and C in the archaebacterial enzymes is similar to the division of antigenic determinants between γ and β′ in *Anabaena* strain PCC 7120 (32), and the arrangement of the genes encoding archaebacterial A and C,

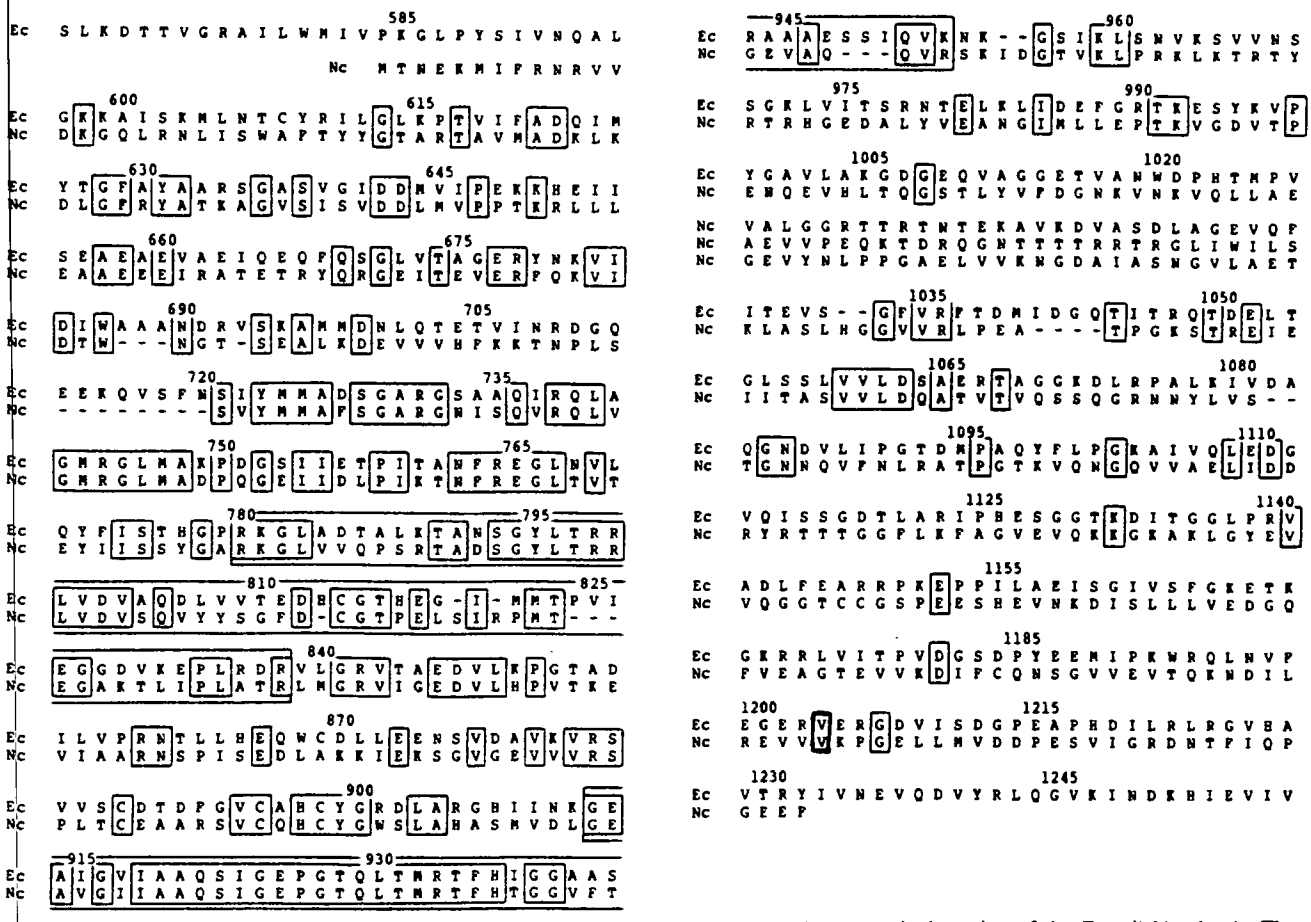

FIG. 4. Alignment of the derived amino acid sequence of RpoC2 (β′) with the carboxy-terminal portion of the *E. coli* β′ subunit. The conventions for numbering and labeling are as described in the legend to Fig. 3.

downstream of B (B″ + B′; 30), corresponds to the arrangement described here for *N. commune* UTEX 584. However, *Anabaena* strain PCC 7120 anti-γ subunit reacts with subunit A from *S. acidocaldarius* and *Anabaena* strain PCC 7120 anti-β + β′ reacts with both A and B of *S. acidocaldarius*, but neither of the *Anabaena* strain PCC 7120 sera react with the C subunit of *S. acidocaldarius* (31).

The form of structural organization of *rpo* genes described in this study has been conserved, with modifications, in the chloroplast genome. Sequences which correspond to *E. coli rpo* genes occur in the chloroplast genomes of higher and lower plants (11, 23, 24), and recent evidence suggests that these chloroplast *rpo* genes are expressed in vivo (16). Two separate chloroplast DNA sequences, *rpoC1* and *rpoC2*, correspond to *E. coli rpoC* but differ from it by the apparent insertion of an intron, an intergenic region (to create two genes), and a large section of nonhomologous coding sequence in *rpoC2* (11). RNA mapping data for spinach *rpo*

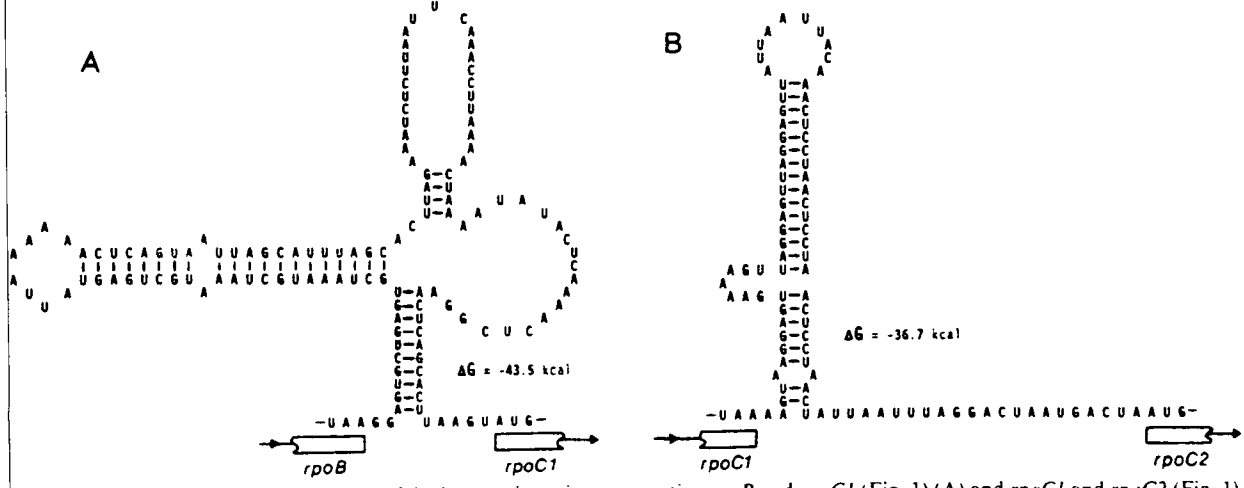

FIG. 5. Predicted secondary structures of the intergenic regions separating *rpoB* and *rpoC1* (Fig. 1) (A) and *rpoC1* and *rpoC2* (Fig. 1) (B).

sequences indicate that two isoforms of the B′ subunit may exist because of alternative splicing within the *rpoC1* sequence (11). The exact subunit composition of the spinach chloroplast RNA polymerase is uncertain.

The function(s) attributed to the large subunit of RNA polymerase has been ascertained mainly through detailed work with the RNA polymerase from *E. coli*. One conclusion from our study is that the function(s) which resides in the single β′ subunit of *E. coli* RNA polymerase (template binding; 21) may be divided between two different gene products in cyanobacteria. Such a division of function(s) could provide an additional level of control over the initiation of transcription in cyanobacteria. Although the cyanobacterial RNA polymerase shows promoter specificities similar to those of the *E. coli* enzyme, differences are apparent (32). Reconstitutions of subunits from both types of enzyme did not result in active hybrids, although these experiments were performed before the unique structure of the cyanobacterial enzyme became apparent (10). One question remains puzzling—what is the function(s) of the largest subunit of cyanobacterial RNA polymerase? Mainly because of its size, the largest subunit was considered to be the structural and functional equivalent of the *E. coli* β′ subunit (10, 20, 32). As shown here, the derived sequence of *N. commune* UTEX 584 *rpoC1* contained three segments (Fig. 2) which corresponded to three (I, II, and III) of the six highly conserved stretches of sequence observed in a comparison of the A subunit of eucaryotic and the β′ subunit of *E. coli* RNA polymerases (1). Furthermore, the derived sequence from *N. commune* UTEX 584 *rpoC2* contains two additional segments with extensive similarity to conserved regions IV and V of RNA polymerases (1; Fig. 4). The remainder of the derived sequence of RpoC2 and the carboxy-terminal region of the *E. coli* β′ polypeptide showed only minimal correspondence. A comparison of the sizes of the β′ subunits in RNA polymerases from diverse strains of cyanobacteria (10, 20, 31, 32) and that derived from our partial sequence analysis of *rpoC2* suggests that approximately 650 codons of *rpoC2*, with unknown function, remain to be analyzed.

The results from comprehensive immunochemical studies and data from DNA-sequencing studies provide convincing evidence for a common origin of the different RNA polymerases (1–3, 7, 12, 30, 37, 38). The structure of the original enzyme and the functions of its components can, however, only be speculated upon at present (37). Either the form of organization of *rpo* genes described here, which appears to have been conserved in the genome of chloroplasts, could have arisen through the splitting of a single ancestral gene in a fashion analogous to the splitting of component B into B′ and B″ in certain of the archaebacteria (37, 38) or, more likely, the genes may represent the vestiges of two separate primitive sequences, similar to those encoding A and C of the archaebacterial enzyme, respectively.

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Gene Cluster *rpoBC1C2* in Cyanobacteria Does Not Constitute an Operon

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The core enzyme of the cyanobacterial DNA-dependent RNA polymerase contains a unique component, γ, which is absent from the corresponding enzymes of other eubacteria. In the heterocystous cyanobacterium *Nostoc commune* the gene encoding γ, *rpoC1*, is immediately adjacent to, and downstream of, *rpoB*. The *rpoC1* gene, and a 3′ adjacent gene, *rpoC2*, correspond to the single *rpoC* gene found in *Escherichia coli* with respect to those domains conserved within their translational products. Northern analysis and primer extension assay show that in *N. commune*, *rpoC1* and *rpoC2* are transcribed separately from *rpoB*. The promoter of *rpoC1C2* can direct the expression of a promotorless *lacZ* gene in *E. coli*. As a consequence, cyanobacterial *rpo* gene expression is distinct from the mode of cotranscription described for the equivalent sequences found in other eubacteria, archaebacteria, and plant chloroplasts. Also in this paper, a simple protocol for RNA isolation, which should be applicable for RNA isolation from plant cells, is presented.
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The transcription of genes is directed through the activities of the multicomponent enzyme DNA-dependent RNA polymerase (ribonucleoside triphosphate:RNA nucleotidyl transferase, EC 2.7.7.6). In *Escherichia coli* the core enzyme consists of three different polypeptides, β, β′, and α, present in a stoichiometry of 1:1:2, respectively (1). Association of the core with the sigma polypeptide, of which several different forms have been identified, constitutes the promoter-specific, transcriptionally active holoenzyme $[\beta\beta'\alpha_2]\sigma$ (2). The genes encoding β and β′ in *E. coli*, *rpoB*, and *rpoC*, respectively, are adjacent to, and immediately downstream of four *rpl* genes encoding the ribosomal large-subunit proteins L11 and L1, L10 and L7/12 (3). *rplJ* (L10), *rplL* (L7/L12), *rpoB*, and *rpoC* constitute the *rif* operon, a single transcriptional unit, whose expression is subject to complex translational autoregulation (3).

Studies of the RNA polymerases from cyanobacteria provided evidence for an additional subunit, γ, in the core enzyme which cross-reacted with antiserum directed against the β′ subunit of the *E. coli* enzyme (4). In a recent study we provided the nucleotide sequence of *rpoC1*, the gene encoding the γ subunit of the enzyme from the heterocystous cyanobacterium *Nostoc commune* UTEX 584. In *Nostoc*, two adjacent genes *rpoC1* and *rpoC2* correspond, with respect to domains conserved in their protein products, to the single *rpoC* gene of *E. coli* (5). These data provide evidence for divergent evolution of the eubacterial RNA polymerase and demonstrate that the organization of cyanobacterial *rpo* genes has been retained, with some modifications, in the genomes of plant chloroplasts (6).

In this study we report that the *rpoC1* and *rpoC2* genes of *N. commune* are transcribed separately from *rpoB*. In this respect the form of transcription of *N. commune rpo* genes differs from that of the *rpoBC* gene in *E. coli*, differs from that of the *rpoBC1C2* genes of plant chloroplasts, and differs from that of the *rpoB(B′B′)AC* genes of archaebacteria, which share similarities with cyanobacteria in their *rpo* gene organization (7).

MATERIALS AND METHODS

rpo sequences. The construction and screening of a gene library of *N. commune* DNA, the isolation of *rpoB*, *rpoC1*, and *rpoC2*, and DNA sequence analysis of *rpoC1* and *rpoC2*, are described in a previous publication (5). The plasmids used in this study, and the organization of the three genes are shown in Fig. 1. The nucleotide sequences of *rpoC1* [1866 base pairs (bp)] and the 5′ portion of *rpoC2* (2151 bp) are deposited in Genbank under accession number M29747 (locus NOSRPOCB). A detailed structural analysis of *rpoB* and its 3′ flanking region is the subject of another study and will appear elsewhere.

Isolation of total RNA. Cells (5 g wet wt) of *Nostoc* were frozen under liquid nitrogen, ground to a powder, resuspended in 1.5 ml of a solution

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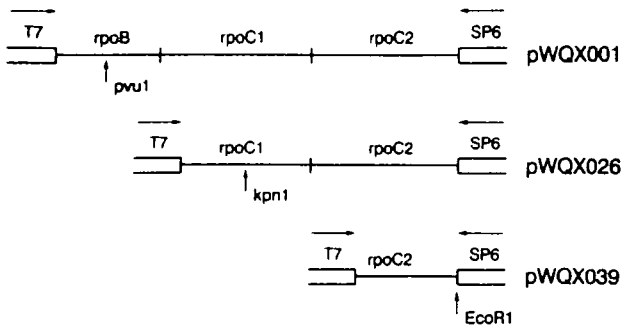

FIG. 1. Plasmids used in this study are shown here. Plasmid pWQX026 and pWQX039 are deletion derivatives of pWQX001 [Ref. (5)]. The insert of pWQX001 is cloned at the *Eco*RI site of vector pGEM4 in the orientation indicated. The arrows indicate the sites for restriction enzymes used to digest the plasmid DNA to provide appropriate sites for run-off transcription in ribo-probe synthesis.

of 1 M sodium acetate plus 1% SDS[3] at pH 5.2 (buffer A), and vortexed for three 2-min intervals. The tube was chilled on ice between each of these intervals. Five milliliter of cold buffered phenol and 200 μl of 2-mercaptoethanol were added and the suspension was mixed by further vortexing. Nine milliliters of Buffer A was added to dilute the solution and the mixture was incubated at 50°C for 10 min. Five milliliters of chloroform/isoamyl alcohol (24:1) was added, the two phases were separated by centrifugation, and the aqueous phase was subjected to further extractions with phenol/chloroform/isoamyl alcohol (25:24:1) until the interface was free of debris. The nucleic acids were precipitated with 2 vol of 95% ethanol (v/v) and were collected by centrifugation. The pellet was resuspended in 1.7 ml of diethylpyrocarbonate-treated water. The RNA was purified using lithium chloride as described (8) and trace amounts of DNA were removed with RQ DNAse (Promega Biotec).

The protocol for RNA isolation described in this report is a simple and efficient method for RNA purification, especially if the source has thick sheath material like *N. commune*. The quality of the RNA obtained is adequate for Northern analysis and reverse transcription assay.

Preparation of riboprobes. Three plasmids, pWQX039, pWQX026, and pWQX001, constructed during deletion and sequence analysis of the *rpoBC1C2* genes (9), were used as templates to synthesize single-stranded RNA probes specific for intragenic regions of *rpoC2*, *rpoC1*, and *rpoB*, respectively (Fig. 1). Plasmid DNA was digested with the appropriate restriction endonuclease (Fig. 1) to provide a runoff site for the termination of transcription. Single-stranded RNA probes were synthesized at 40°C using T7 RNA polymerase (Promega Biotec) in the presence of [α-^{32}S]UTP (1284 Ci/mmol, New England Nuclear).

Northern analysis. RNA samples were denatured and resolved in 1% w/v agarose gels in the presence of formaldehyde (8). The RNA was transferred to Nytran membranes (0.2-μm pore size, Schleicher and Schuell) through capillary action (8). The membranes were prehybridized at 50°C for 4 h in RNA hybridization buffer containing 55% deionized formamide (v/v), 5× SSC (20 SSC: 3 M NaCl and 0.3 M sodium citrate), 50 mM sodium phosphate (pH 6.5), 0.5% SDS, and 4.5× Denhardt's (50×: 1% each of polyvinyl pyrollidone, Ficol, and BSA) solution. The membranes were hybridized with ^{35}S-labeled probes overnight under identical conditions, then washed twice for 5 min at room temperature in 1× SSC plus 0.1% SDS, twice for 25 min at 60°C in 0.1× SSC plus 0.1% SDS, and rinsed for 10 min at room temperature in 1× SSC. Finally, the filters were exposed to X-ray film.

Primer-directed extension assay. A synthetic oligonucleotide (16-mer) primer, $^{5'}$GTTTGGCAGGTCTCA$^{3'}$, was synthesized using an Applied Biosystems 381A DNA synthesizer. The primer has the same sequence as the DNA template strand of *rpoC1* from position +17 to +2 with respect to the first base of the putative translational initiation codon [corresponding to bases 136 to 151 in Fig. 1 of Ref. (5)]. Ten micrograms of total RNA from *Nostoc* and 10 ng of the primer were used in the primer extension assay, which was performed as described by Alam *et al.* (10). At the same time, a DNA sequence ladder was generated using the same primer and pWQX001 (Fig. 1) as the template DNA.

Cloning of the rpoC1C2 promoter into a promoter reporter vector pCB267. Plasmid pCB267 is a promoter reporter vector. It contains two promoterless genes (*Pho*A and *lac*Z) and a multiple cloning site between the two genes (11). The promoter of *rpoC1C2* was subcloned into the vector by replacing the sequence between *Bam*H1 and *Hind*III sites, in front of the *lac*Z gene, using a standard procedure (8). The insert DNA containing the promoter was prepared as following: two oligo primers, 5'CCGGATCCTAAGGAGTGCTGAGTGCT3' (26 mer) and 5'CCAAGCTTACTTAAGTGCTGAGTTC3' (25 mer), were used to do the site-directed subcloning of the promoter into the reporter vector. The first oligo has the sequence from −99 to −82 (+1 being the first nucleotide transcribed) plus a site for restriction enzyme *Bam*H1 at its 5' site (underlined). The second primer corresponds to the sequence from +30 to +13 flanked by a site for restriction enzyme *Hind*III at its 5' site (underlined). These two oligos were used to amplify a DNA fragment containing the promoter sequence of *rpoC1C2* using plasmid pWQX001 (Fig. 1) as a template DNA through polymerase chain reaction technique (12). The resulting DNA fragment (144 bp) was then double digested with the restriction enzymes *Bam*H1 and *Hind*III. The recombinant DNA was used to transform competent DH5α cells using a standard protocol and the resulting cells were selected using a LB plate supplemented with Ampicillin (50 μg/ml) and X-Gal as described (8).

Assay for β-galactosidase activity. The β-galactosidase activity derived from the *lac*Z gene with or without the *rpoC1C2* promoter was assayed and determined using a substrate *O*-nitrophenyl-β-D-galactoside as described (11).

RESULTS AND DISCUSSION

In contrast to the RNA polymerases of a wide range of gram-positive and gram-negative eubacteria which are homogenous with respect to their subunit structure, the core enzyme from cyanobacteria contains a unique component, γ (4). The goals of this study were to understand how *rpoC1*, the gene encoding γ, is transcribed.

Transcription of rpoC1 and rpoC2 is separate from that of rpoB. Northern analysis using antisense riboprobes specific for intragenic regions of the three contiguous genes *rpoB*, *rpoC1*, and *rpoC2* detected two transcripts of 3.1 kb and 5.6 kb (Fig. 2). The *rpoB* probe hybridized only with the 3.1-kb transcript. Under routine conditions of gel electrophoresis this transcript comigrated with 23 S rRNA and the massive abundance of the 23 S RNA interfered with the resolution of the transcript. The *rpoB* probe gave stronger cross-hybridization signals with 23 S, 16 S, and 16 S precursor f1 rRNA than did either *rpoC1* or *rpoC2*, during trials using a range of different conditions for hybridization stringency. Both the *rpoC1* and *rpoC2* probes hybridized only with the single larger transcript of 5.6 kb (Fig. 2). Overexposure of autoradiograms of Northern analysis provide no evidence of any other transcript (data not shown). The transcriptional start site for *rpoC1C2* was analyzed using primer extension and found to be a T residue, 30 bases upstream from the A residue

[3] Abbreviations used: SDS, sodium dodecyl sulfate; SSC, standard saline citrate; BSA, bovine serum albumin

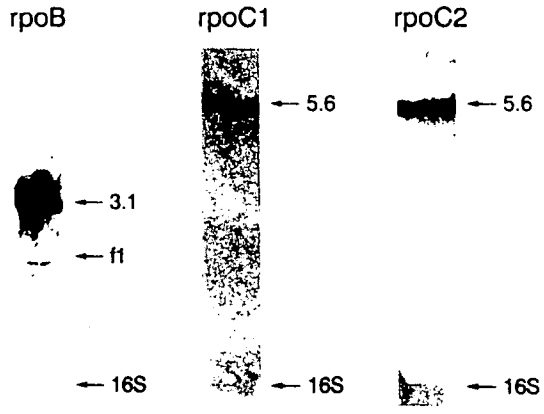

FIG. 2. Northern analysis of total *Nostoc commune* RNA (10 μg each) after being separated on a 1% agarose gel. The ribo-probe used in each panel is indicated. The conditions for hybridization are described in the Materials and Methods section.

of the AUG translational start codon of *rpoC1* [Fig. 3A; see Ref. (5)]. A weaker signal was detected at the T residue location at position −27. The transcriptional start site is located within a region of very stable secondary structure which may account for the background signals detected after overexposure of the autoradiogram used in the assay (Fig. 3A). These data suggest that the transcription of *rpoC1* and *rpoC2* is separate from the transcription of the upstream *rpoB* gene, i.e., the 3.1- and 5.6-kb transcripts arise from two separate promoters as opposed to being generated through post-transcriptional processing of a larger transcript.

Promoter activity of the 5′ upstream sequence of N. commune rpoC1C2. To further confirm this view, we tested the 5′ upstream sequence of *rpoC1C2* for a promoter activity by using a promotorless *lacZ* gene in a promoter reporter pCB267. There should be no *lacZ* gene activity unless a promoter sequence is subcloned into the vector with the right orientation (11). The 5′ upstream sequence from −99 to +30 of *rpoC1C2* (+1 being the first nucleotide transcribed) was subcloned into the promoter reporter vector pCB267 in front of *lacZ*. The recombinant DNA could direct the expressions of the *lacZ* gene and result in blue colonies on the X-Gal plate while the control plasmid pCB267 without the insert DNA was negative for the *lacZ* gene and generated white colonies only. The β-galactosidase derived from the *lacZ* gene in the recombinant construct or the vector has activity of 40 and *D* less than 1 unit [for unit definition see Ref. (11)]. Despite the structure difference between the RNA polymerase of *E. coli* and cyanobacteria (4, 5), the two different kinds of promoter do share enough similarity and the polymerase can recognize the heterologous promoter. However, the cyanobacterial promoter is a weak promoter for the RNA polymerase of *E. coli.* Therefore, we propose that the transcription of *rpoC1C2* is separated from that of *rpoB* in *N. commune.* The transcription start site of the *rpoB* gene is under investigation.

The separate transcription of *rpoB* and *rpoC1C2* genes in *Nostoc* is in contrast to the situation in *E. coli* where *rpoB* and *rpoC* are cotranscribed together with upstream *rpl* genes from the single promoter PL10 (3). In the genome of spinach chloroplasts, where there is an arrangement of *rpoB, rpoC1,* and *rpoC2* sequences similar to that found in *Nostoc,* the *rpoBC1C2* genes appear to constitute an operon (6). The corresponding *rpo* sequences present in representatives of both branches of the archaebacteria are also cotranscribed (13, 14).

Promoter-like sequences of rpoC1C2 occur in a region of secondary structure. The location of the transcriptional startsite of *rpoC1C2* makes it possible to identify potential regulatory sequences upstream of the genes (5). The sequence TTAAAA occurs between positions −14 and −9 relative to the transcriptional startsite of *rpoC1,* the sequence at −37 to −32 is TTAGAA. The sequences found at the −10 and −35 regions upstream of *rpoC1* do show some similarities with those considered for other cyanobacterial genes where the relevant data are available but it has proved difficult to define consensus sequence elements within promoter regions (15). We would rather emphasize that the putative −35 and −10 regions of *rpoC1* are juxtaposed around the base of the upper loop of the secondary structure shown in Fig. 3B [see (5)]. The transcriptional startsite is located within the right-hand loop (Fig. 3B, arrow). Such spatial arrangement could facilitate binding and recognition by RNA polymerase.

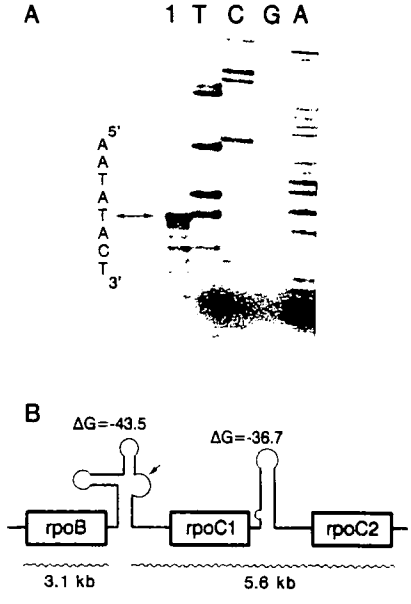

FIG. 3. Identification of transcriptional start site for *rpoC1C2* genes. (A) The start site for *rpoC1C2* genes was determined by primer extension and a sequence ladder was generated using the same primer as the one used for primer extension (see the Materials and Methods section). (B) A proposed transcriptional mode for *rpoB, rpoC1,* and *rpoC2* genes. The arrow indicates the transcriptional start site for *rpoC1C2* genes.

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Cell-water deficit regulates expression of *rpoC1C2* (RNA polymerase) at the level of mRNA in desiccation-tolerant *Nostoc commune* UTEX 584 (Cyanobacteria)

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Abstract

Immobilization and short-term air-drying of the cyanobacterium *Nostoc commune* strain UTEX 584 leads to a complete depletion of its cellular *rpoC1C2* mRNA pool. This mRNA is required for the synthesis of the γ and β' subunits of DNA-dependent RNA polymerase (RNA-P). In contrast, RNA-P remains stable in cells during long-term desiccation as judged from immunoblotting analyses of protein extracts using RNA-P core-specific antibodies. The data indicate that the extant RNA-P holoenzyme in air-dried cells drives the rapid de novo transcription of *rpoC1C2* that ensues in response to cell rehydration.

Keywords: Desiccation tolerance; Cyanobacteria; *Nostoc commune*; RNA polymerase; Gene expression

1. Introduction

The removal of cell-bound water through air-drying, and the addition of water to air-dried cells, exercise marked effects on the distribution and activities of bacterial communities. Yet desiccation, as a major stress parameter in nature, has continued to escape critical attention and the mechanisms that contribute to the desiccation tolerance of prokaryotic cells remain obscure [1].

Of those microorganisms that express a capacity to tolerate extremes of water deficit, many cyanobacteria – and one form in particular, *Nostoc commune* – have a marked tendency to do so [2]. *N. commune* becomes visually conspicuous in terrestrial limestone regions, especially those where there is a limited and variable availability of water [3]. Here, colonies of this cyanobacterium are subjected to intermittent wetting and often extended periods of desiccation. Air-dried cells of *N. commune* can maintain their viability despite decades of storage in the air-dried state [4]. A principal consideration in any appraisal of the resilience of these cells is the stabilities of their proteins. Do desiccated cells provide an environment that is conducive to the enhancement of protein stability, or do tolerant cells accumulate proteins that are inherently more stable than those found in sensitive cells? To begin to answer these questions we have commenced structural analyses of gene products synthesized by *N. commune* strain UTEX 584 (*Nostoc* 584). This strain has provided a conve-

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nient model to study the consequences of air-drying at the molecular level [4–11]. The present account describes our analysis of the turnover of DNA-dependent RNA polymerase (RNA-P) in cells of *Nostoc* 584 subjected to different cell-water deficits.

The stoichiometry of eubacterial RNA-P is $[\beta\beta\alpha_2]\sigma$ [12]. In contrast, the cyanobacterial enzyme contains an additional subunit, γ, and has the stoichiometry $[\beta\gamma\beta'\alpha_2]\sigma$ [13]. Cloning and sequence analysis of the genes from *Nostoc* 584 that encode the β (*rpoB*), γ (*rpoC1*) and β' (*rpoC2*) subunits of its RNA-P indicated that γ and β' correspond to the N-terminal and carboxy-terminal regions of eubacterial β', respectively [14]. Furthermore, *Nostoc* 584 *rpoC1* and *rpoC2* are transcribed separately from *rpoB* as a single, 5.6-kb dicistronic message (Fig. 1A; [15]).

RNA-P is the pivotal component of the transcription apparatus. If cells are to recover from desiccation, either their complement of RNA-P must remain intact or their pool of *rpo* mRNA transcripts must remain intact. Successful recovery with the former stipulation requires that cells retain intact DNA templates, ancillary transcription factors and a pool of ribonucleoside triphosphates. De novo translation with the latter stipulation requires that the cells retain active ribosomes, charged tRNAs and ancillary translation factors. To understand which of these sets of conditions may prevail we subjected cells to different water stresses, studied the turnover of *rpoC1C2* mRNAs, and monitored the fate of the subunits of RNA-P as intracellular markers for the holoenzyme.

2. Materials and methods

2.1. Growth and immobilization of cells

Cells of *Nostoc* 584 were grown as described [6] and were harvested in the mid-log phase of growth by centrifugation. The cell pellets were divided into portions of approximately 0.7 g (wet weight) which were then spread evenly, as pastes, over the surface of inert nylon meshes [9]. The cells were incubated in an atmosphere with a matric water potential (Ψ_m) of -99.5 MPa and were allowed to dry, under a continuous incident photon flux density of approximately 50 μmol photons m^{-2} s^{-1} at the surface of the culture vessels, for periods between 1 and 5 days. These conditions are comparable to those sometimes experienced by colonies of *N. commune* growing in situ. Water loss from colonies ceased after 5 days of drying at which time the colonies were judged to be desiccated. Desiccated cells were rehydrated when necessary through the addition of sterile $BG11_0$ medium [16].

2.2. Isolation and purification of mRNA

The method of Xie and Potts [15] was used to purify the intracellular mRNA pools of the air-dried cells as well as air-dried cells that had been rehydrated for 5 min, 10 min, 30 min, 60 min, 24 h or 4 days. RNA pools were extracted from *Nostoc* 584 cells and were transferred to nylon sheets as described following resolution in 1% (w/v) glyoxal agarose gels [15]. An 878-b riboprobe (antisense), complementary to bases 1241 to 2119 of *rpoC2*, was synthesized from a deletion plasmid [17] using T7 RNA polymerase in the presence of [^{35}S]UTP (1284 Ci mmol^{-1}, New England Nuclear). Nylon membranes were prehybridized in a plastic bag with RNA hybridization buffer (5 × SSC, pH 7.0, 50 mM $NaPO_4$, pH 6.5, 0.5% w/v SDS, 4.5 × Denhardt's solution and 55% w/v formamide) at 50°C, for 4–5 h. The prehybridization buffer was then discarded and the bag was refilled with fresh buffer containing the ^{35}S-labelled riboprobe. Hybridization was performed at 50°C, overnight, with gentle shaking. After hybridization, the membrane was rinsed in 1 × SSC/0.1% (w/v) SDS buffer for several minutes with one buffer change. The filter was then washed in 0.1 × SSC/0.1% (w/v) SDS buffer at 60°C, for 50 min, with two buffer changes. Finally, the membrane was rinsed with 1 × SSC/0.1% (w/v) SDS buffer and dried. RNA–RNA hybrids were visualised by autoradiography and the signals were quantified using scanning densitometry (Shimadzu). The data presented here are representative of those obtained in multiple trials.

2.3. Isolation of proteins and Western blotting

Replicate samples were used to obtain extracts of total cell proteins in Laemmli buffer [18] using the methods of Hill et al. [4]. Cells were first frozen in

liquid nitrogen, ground to a powder in a chilled mortar and then transferred to 15-ml tubes. 2 ml of Laemmli buffer [18] were added and the mixture was sonicated (Fisher sonic dismembranator with microprobe) for three consecutive periods of 30 s each, at a setting of 30. The efficiency of breakage of cells was monitored using light microscopy. Cell debris was removed by centrifugation at 35 000 rpm in a Beckman Ti50 rotor for 1 h, at 4°C. The proteins from equivalent amounts of cell lysate were resolved in 8% (w/v) polyacrylamide gels and were processed for SDS-PAGE and Western blotting as described [19].

3. Results

rpoC1C2 transcripts were not detected in RNA preparations from cells that had been immobilized and dried in air for 24 h – a period of time that does

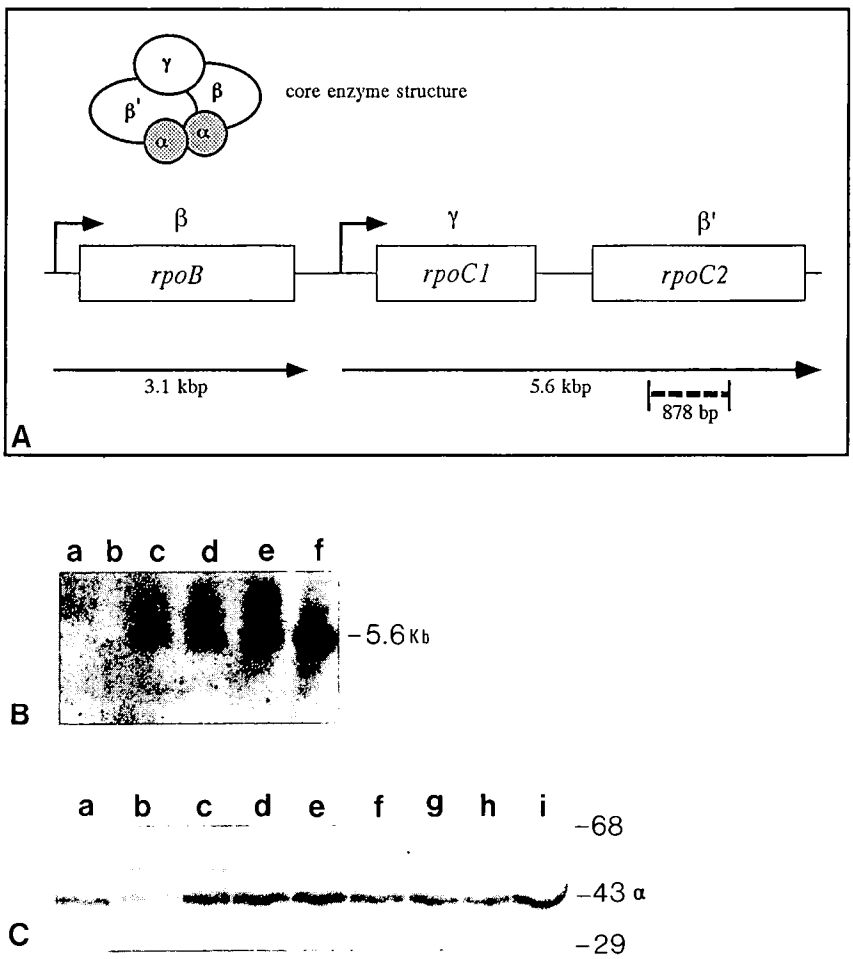

Fig. 1. (**A**) Organization of the *rpoB* and *rpoC1C2* operons and structure of the core enzyme of *Nostoc* UTEX 584 RNA polymerase. Straight arrows denote the direction of transcription and numbers indicate the sizes of the transcribed *rpo* DNA fragments. Bent arrows signify the positions of characterized promoters. The hatched line indicates the region of *rpoC2* that was used to generate a riboprobe. (**B**) *rpoC1C2* transcripts are degraded during air-drying of cells and then are rapidly synthesized upon cell rehydration. Autoradiograph of a Northern blot after probing equivalent amounts of RNA pools from cells that had been: a, 1 day dry; b, 5 min rewet; c, 10 min rewet; d, 30 min rewet; e, 60 min rewet; f, control (liquid culture). The position of the 5.6-kb *rpoC1C2* transcript is indicated (see **A**). (**C**) The α subunit, a marker for the core RNA-P, remains stable in cells subjected to different water deficits. Western blot of equivalent amounts of protein extracts from cells that had been: a, 1 day dry; b, 5 days dry; c, 10 min rewet; d, 30 min rewet; e, 60 min rewet; f, 5 h rewet; g, 24 h rewet; h, 4 days rewet; i, control (liquid culture). Numbers indicate and marked the positions and sizes of molecular mass markers.

not lead to desiccation (Fig. 1B). Resolution of greater amounts of RNA through the overloading of gels, as well as the implementation of increased exposure times during autoradiography, failed to detect any *rpoC1C2* transcripts in these preparations. In cells that had been dried for longer periods and which were desiccated, *rpoC1C2* mRNA was first detected in extracts from cells that had been rewetted for 10 min and an extractable pool of *rpoC1C2* mRNA, equivalent in size to approximately 70% of the pool present in control cells (in liquid culture), was present after 60 min of rewetting (Fig. 1B). We confirmed that the water status of the cells did not interfere with the efficiency of extraction of their RNA pools. The amounts and quality of rRNA present in control cells, and in cells that had been dried for different time intervals, were judged to be equivalent following spectrophotometric measurements, after analysis by agarose gel electrophoresis, and after probing Northern blots with an *Escherichia coli* rRNA probe ([20]; data not presented).

To assess the effects of water deficit on the protein complement of dried cells their protein extracts were probed with antibodies specific for the core of RNA-P. Antiserum raised against the core RNA-P of *Anabaena* sp. strain PCC 7120 gave a pronounced reaction against the α subunit of the *Nostoc* 584 RNA-P. These experiments demonstrated that there was no noticeable change in the relative amounts of the RNA-P α subunit in cells that were subjected to a range of different water deficits in comparison to the level of α in cells grown in liquid culture (Fig. 1C). The antiserum gave weaker reactions with the other components of the core enzyme as has been observed with the Rpo proteins of other strains of cyanobacteria and as is documented in Fig. 2 of ref. [21]. Nevertheless, protein extracts from air-dried cells, from rehydrated cells, and from cells grown in liquid culture (control), generated signals from the γ, β and β' subunits of the *Nostoc* 584 core RNA-P of equivalent intensity (data not shown).

4. Discussion

Cells of *Nostoc* 584 lose the capacity to maintain a pool of *rpoC1C2* mRNA following their immobilisation and short-term drying, air-dried cells lack any detectable *rpoC1C2* mRNA, and there is a conspicuous rise in the pool size of *rpoC1C2* mRNA upon rehydration of cells. It is possible that Northern analyses may have failed to identify very scarce transcripts. However, previous studies have shown that there is a loss of integrity of nucleic acids in desiccated cells, RNA preparations from cells subject to long-term desiccation are poorly resolved by agarose gel electrophoresis, and RNA preparations from such cells fail to drive in vitro translation [10,22]. These data are consistent with a mode of action where RNA-P remains stable, and functional, in desiccated cells and is responsible for the very rapid rise in the *rpoC1C2* pool following subsequent rehydration. Confirmation of this latter fact is the finding that the α subunit of RNA-P – a marker of the presence and the abundance of the core enzyme in cells -- as well as the other subunits of the core, were present in all of the *Nostoc* 584 extracts in approximately equivalent amounts (see Fig. 1C). Prokaryotic cells may contain up to around 10 000 copies of RNA-P [23] so it is conceivable that only a fraction of those copies present in desiccated cells remains functionally active and capable of initiation of transcription upon cell rehydration. These data indicate that *rpoC1C2* gene expression is regulated by water stress at the level of mRNA and the rise in the *rpoC1C2* mRNA pool size upon cell rehydration, i.e. de novo transcription, is at the expense of extant RNA-P holoenzyme. It is not possible to state, at this point, whether the regulation is at the level of mRNA stability or *rpoC1C2* transcription.

Irrespective of the quantities of transcriptionally active RNA-P in rehydrating cells there is a net requirement for intact DNA templates. The rapid rise in the *rpoC1C2* mRNA pool upon rehydration implies a priori that intact *rpoC1C2* DNA is available, but because our previous studies have shown that the genomic DNA of desiccated *Nostoc* 584 is subject to a substantial degree of light-dependent nicking the *rpoC1C2* transcription described here may, in fact, derive from DNA that has undergone rapid repair during cell rehydration [10]. The present data, and previous observations that show the intracellular RNA pools from desiccated *N. commune* cannot drive in vitro translation, show that the drying of a cell leads to a general depletion of its pool of mRNAs.

Desiccated cells of *N. commune* contain massive amounts of a highly stable (several decades) water stress protein (Wsp) that is implicated in the modification and/or synthesis of UV-A/B-absorbing pigments [4]. Enzymes of lipid biosynthesis remain functional in these cells as evidenced by an instantaneous onset of lipid biosynthesis upon rewetting [11]. At least one secreted enzyme, a protein tyrosine phosphatase, retains its phosphomonoesterase activity following drying and rewetting [24,25], and the Fe protein of nitrogenase remains structurally intact in cells following their storage for more than a decade in the air-dried state [5]. If protein stability is a key to the survival of desiccation-tolerant cells, then what is the basis for the stability? Those proteins from extremophiles that have been studied show no obvious primary sequence divergence from the corresponding proteins found in mesophiles [26]. *N. commune* places a considerable metabolic investment in its secreted UV-absorbing pigments, secreted Wsp proteins and a secreted extracellular glycan [27]. These components may contribute to, and form part of, an environment, a protective environment, that is conducive to the maintenance of protein stability.

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Rehydration induces rapid onset of lipid biosynthesis in desiccated *Nostoc commune* (Cyanobacteria)

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Water, which contained [1,3-^{3}H]glycerol, [^{35}S]sodium sulfate, or [^{32}P]sodium orthophosphate, was used to rehydrate air-dried cells of the desiccation-tolerant filamentous cyanobacterium *Nostoc commune*. The cells retained their capacities for the uptake and transport of all three compounds and, in response to rewetting, they mobilized the radiolabels into lipid precursors and initiated complex lipid biosynthesis. The onset of these events, measured in short-term, long-term and pulse-chase labeling experiments, was judged to be very rapid. The radiolabeled pool sizes of the major membrane species phosphatidylglycerol (PG) and sulfoquinovosyl diacylglycerol (SQDG) reached steady-state within several minutes, while those of the two abundant membrane glycolipids, mono- and di-glycosyldiacylglycerol (MGDG, DGDG), achieved uniform labeling within 2 h. The pattern of sulfolipid synthesis was generally more complex than the other lipid species. Analysis of the maturation of SQDG through differential labeling provided the only example of a lag in lipid maturation during the early stages (minutes) of cell rehydration. In this instance, the lag appeared to be associated specifically with the incorporation of $^{35}SO_3^-$ by the sulfoquinovose. During the initial 2 h of rewetting there was complete turnover of ^{3}H-label in the pools of the principal lipid precursors 1,2-*sn*-diacylglycerol and 1,3-diacylglycerol. In contrast, the accumulation of label by the major lipid of the heterocyst cell-wall, a non-saponifiable glycolipid, became detectable only after 24 h of rewetting. The present data are discussed in relation to the basis for desiccation tolerance in *N. Commune*.

Introduction

A limited number of organisms can overcome the obstacle of a fluctuating or a sustained cell water deficit and, significantly, some do so without resorting to the synthesis of spores. Vegetative cells of the heterocystous cyanobacterium *Nostoc commune* can withstand prolonged desiccation (years) and then recover their metabolic capabilities rapidly upon cell rehydration. The specific mechanisms which provide *N. commune* with the capacity to withstand these stresses are generally unclear, although it seems probable that they reflect a highly complex array of interactions at the structural, physiological and molecular levels. However, to emphasize that there is inherent order within the rehydrating *N. commune* cell, it has been shown for field materials, as well as for immobilised axenic cultures of *N. commune* strain UTEX 584 (*Nostoc* 584), that there is a reproducible, sequential recovery of metabolic capacities upon rewetting of cells beginning with respiration, then photosynthesis, and finally nitrogen fixation [1]. One, but not the only explanation for these recoveries is a step-wise, highly-ordered insertion of proteins (synthesized de novo), into membranes.

Studies of a wide range of strains suggest that cyanobacteria contain only one quantitatively important phospholipid, phosphatidylglycerol (PG), and three glycolipids (mono- and di-galactosyl diacylglycerol) and sulphoquinovosyl diacylglycerol (MGDG, DGDG and SQDG, respectively; see Ref. 2). A glycolipid, 1-(*O*-α-D)-glucopyranosyl-3,25-hexacosanediol [3], is the principal component of the heterocyst cell envelope in *Anabaena variabilis*, where that lipid is thought to be absent from the envelopes of vegetative cells. As a consequence of a unique pathway in glycolipid synthesis, cyanobacteria also contain small amounts of monoglucosyl diacylglycerol which, through epimeriza-

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tion, is the precursor of MGDG [4]. These principal lipid components accumulate in the intracellular (thylakoid) and cell envelope membrane systems of these microorganisms so that, in general, the lipid compositions of cell extracts reflect membrane composition. There is some compartmentalization of lipid biosynthesis. In *Anacystis nidulans* PCC 6301 the UDP-glucose:1,2-diacylglycerol glucosyltransferase of glucolipid biosynthesis was located in both the cytoplasmic and thylakoid membrane systems but not in the outer membrane [5]. In *Synechocystis* sp. PCC 6714 the triton-insoluble cell wall fraction contained PG, phosphatidylcholine (PC), sulfolipid and an unidentified polar lipid, but no MGDG or DGDG [6].

Lipid composition in cyanobacteria responds to changes in environmental conditions. With *Spirulina platensis* UTEX 1928, under nitrogen-starvation, cells increased total lipid content but underwent inhibition of synthesis of polar membrane lipids [7]. In addition, triacylglycerols were not formed in response to nitrogen limitation. Marked changes in lipid content in response to salt-stress also have been documented. Moderate halophiles and halotolerants adapt their membrane lipid composition by increasing the proportion of anionic lipids, often PG and/or glycolipids, which in the moderate halophilic eubacterium *Vibrio costicola*, a non-photosynthetic eubacterium, appears to be part of an osmoregulatory response to minimize membrane stress at high salinities [8]. Upon transfer of cells of *Synechococcus* sp. PCC 6311 from low salt to high salt, changes in membrane lipids were apparent within 1 h [9]. The amount of the bilayer destabilizing lipid MGDG decreased, while DGDG increased and, after 3 days, the ratio of MGDG : DGDG in cytoplasmic and thylakoid membranes of high-salt grown cells was about half of that of low salt grown cells.

An essential and recognizable feature of *N. commune* cells is that despite their being subjected to repeated water stresses (drying, desiccation, rewetting) they retain their characteristic ultrastructure and the integrity of their inner and outer cell membranes and their thylakoids [10,11]. This observation is significant because the transition from the desiccated state to the fully rehydrated state is expected to involve dramatic changes in protein conformation, membrane geometry, and protein-lipid interactions. The tendency for lipids to undergo peroxidation in response to desiccation makes cell membranes primary targets of water stress-induced damage. The consequences of such damage to lipids may be especially pronounced. For example, phospholipid cofactors, and the lipid activator-second messenger 1,2-*sn*-diacylglycerol, regulate protein kinase C, a critical component of the signal transduction pathways that cells utilize to recognize and respond to a variety of extracellular agents [12].

Previous studies on the lipids of *N. commune* reported fatty acid fingerprints and the composition of the cytoplasmic membrane in strain UTEX 584 [13,14]. In the present study we sought to determine the patterns of lipid biosynthesis and turnover following rehydration of desiccated *N. commune* and to determine how these compared to the recoveries of respiration, photosynthesis, nitrogen fixation and ATP synthesis observed in previous studies [1,15–19].

Materials and Methods

Microorganisms and growth conditions

Cells of an axenic isolate of *Nostoc commune* strain UTEX 584 (*Nostoc* UTEX 584) were grown as described previously [15] in BG or $BG11_0$ liquid media [20]. Field materials of *Nostoc commune* Vaucher were collected in the Hunan and Guangshi Provinces of China in 1981, and were air-dried prior to their storage in the dark [21]. The rehydration, washing and repeated subculturing of field material, on semi-solid media, induces the formation of numerous spherical colonies, of between 1 and 5 mm-diameter, in which the filaments of *N. commune* grow immobilised within a carbohydrate matrix of sheath material (Hill and Potts, unpublished data). These colonies and axenic cultures were air-dried on glass or plastic Petri dishes under a matric water potential of −95 MPa, in the light, at 32°C. Desiccated cells were rehydrated in liquid media supplemented with the appropriate radioactive lipid precursor and then allowed to rehydrate fully under matric conditions (see below).

Lipid extraction and separation

Lipids were extracted from cells after their rehydration for 48 h in $BG11_0$ medium, and from an equivalent weight of desiccated cells. Cells were dispersed into 18 vol. of chloroform/methanol (2:1, v/v) with a Polytron PT-20 homogenizer (Kinematica, Lucerne, Switzerland) operated at 75% of maximum speed, and the dispersion was stirred at room temperature for 30 to 60 min. Insoluble materials were recovered by centrifugation ($4000 \times g$, 15 min), and re-extracted, as above, with chloroform/methanol mixtures (once with 2:1, and twice each with 1:1 and 1:2 v/v mixtures). Combined extracts were evaporated to near dryness under nitrogen or in vacuo. Lipid-containing residues were dissolved in chloroform/methanol (2:1, v/v), and washed to remove nonlipid contaminants according to Folch et al. [22]. Amounts of lipids recovered from a given mass of cells were determined gravimetrically. Efficiency of lipid extraction was determined by hydrolysis of the residue recovered after chloroform/methanol extraction in aqueous 6 N HCl, for 16 h, at 110°C. Released fatty acids were extracted into hexane, and amounts were estimated by thin-layer chromatographic separation and densitometry.

Incorporation of lipid precursors

Du Pont/New England Nuclear supplied [1,3-^{3}H] glycerol, 40 Ci mmol^{-1}; [^{35}S]sodium sulfate, 1488 Ci mmol^{-1}; and [^{32}P]sodium orthophosphate, 8810 Ci mmol^{-1}.

For short-term labeling, desiccated cells (about 5 mg) were rehydrated in 100 μl of liquid medium which contained 1 to 10 μCi of a radiolabeled lipid precursor. After absorption of the solution, the colonies were allowed to rehydrate fully over time by transferring them to the surface of a solid moist agar plate (1.2% w/v agar in $BG11_0$). These conditions were used to match, as far as possible, the wetting conditions of desiccated colonies growing in situ. Incubation was continued at 32°C, under a photon flux density of 30 μmol photons m^{-2} s^{-1}. After different periods of rehydration (see figure legends) samples were removed from the plates and homogenized in chloroform-methanol for lipid extraction, as described. Radioactivity in aliquots of washed lipid extracts was determined in Betamax scintillation fluid (ICN, Irvine, CA), using a Beckmann LS3500 liquid scintillation counter.

Long-term labeling studies were as described above, with the sampling times given in the figure legends. For pulse-chase experiments, cell material was rehydrated for 1 h in medium containing the radiolabeled lipid precursor, and then cells were rinsed with medium and incubated in $BG11_0$ agar which contained a 5000-fold excess of unlabeled lipid precursor. One sample was subjected to lipid extraction immediately after the pulse period (1 h), and other samples were removed as described (figure legends).

Lipid separation and detection

Lipids were separated on Merck high-performance thin-layer chromatography (TLC) plates. Neutral lipids were resolved by development of plates in hexane/diethyl ether/acetic acid (45:5:1, v/v). Polar lipids were resolved by development of plates in chloroform/methanol/water (35:11:1.5, v/v), or in methyl acetate/*n*-propanol/chloroform/methanol/0.25% aqueous KCl (25:25:28:10:7, v/v) [23]. Reference lipids used as standards on TLC plates were 1,2-*sn*- and 1,3-diacylglycerols (1,2 DG and 1,3 DG), cholesterol, oleic acid, triolein, methyl oleate, cholesterol oleate, choline-, ethanolamine-, serine-, inositol-, and glycerolphosphoglycerides, phosphatidic acid, mono- and di-galactosyl diacylglycerols. These lipids were from Matreya, Pleasant Gap, PA, or from Sigma (St. Louis, MO). For detection of separated constituents, plates were sprayed with 50% aqueous sulfuric acid, or were dipped into 3% w/v cupric acetate in 8% phosphoric acid [24], and heated in an oven at 180°C. Selective spray reagents, used to aid in lipid identifications were: antimony trichloride, and the Liebermann-Burchard reagents for sterols, orcinol (for glycolipids), ninhydrin for amino-containing lipids, Dragendorf reagent for choline-containing lipids, and molybdenum blue for phospholipids. Spray reagents were commercial formulations (Sigma, St. Louis, MO), or were prepared according to Skipsi and Barclay [25]. In some cases lipid extracts were subjected to mild alkali methanolysis prior to separation, to aid identifications [26]. Lipids labeled with ^{32}P were detected by autoradiography, and those labeled with ^{35}S or with ^{3}H were detected by fluorography; for the latter, the plates were sprayed with En^{3}Hance (DuPont/New England Nuclear) and exposure was at −70°C, using Kodak X-Omat X-ray emulsion.

The major glycolipid of *Nostoc* 584 heterocysts was resolved with the solvent system specified by Murry and Wolk (chloroform/methanol/acetic acid/water, 170:30:20:7.4, v/v) [27]. Extracts were obtained from cells grown in the presence and absence of combined nitrogen, and from the inner surface of N culture flasks as well as the cell-free culture supernatant [27]. The major glycolipid species was identified based upon its glycosylation, its non-saponifiable characteristic, and its R_f value relative to standards.

The incorporation of radioactivity detected through autoradiography, and the degree of charring or staining of components separated on plates, were assessed visually. Experiments were repeated in duplicate or triplicate and the chromatograms presented are representative of those obtained in repeat trials.

Storage lipid isolation

Samples of dry and of rehydrated cells were ground to a powder in liquid nitrogen and suspended in 50 mM Tris-HCl (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, 10 mM NaF. The slurry was passed twice through a chilled French Pressure Cell, at 110 MPa. The brie was centrifuged at 1000 × *g*, for 5 min, at 4°C, and the supernatant fraction was collected and adjusted to 0.5 M sucrose by addition of 2.5 M sucrose. This supernatant fraction was then centrifuged at 150000 × *g* for 60 min to float lipid droplets.

Electron microscopy

Cell material was fixed with glutaraldehyde, post-fixed with osmium tetroxide, and processed for electron microscopy, as described [10,11]. Fixation procedures using imidazole [28], *p*-phenylenediamine [29], or myrcene [30] were used to enhance the contrast of lipid inclusions.

Results

Total lipid and storage lipid

Desiccated and rehydrated cultures of *N. commune* contained nearly the same amounts of total lipid. We recovered 207 ± 63 μg ($n = 4$) of lipid per gram dry

weight from desiccated cells. After rehydration for 48 h, equivalent starting dry weights of cells yielded 230 ± 66 μg ($n = 4$). Efficiency of the lipid extraction method was evaluated by acid hydrolysis of insoluble residues recovered from 100 μg amounts of starting cell mass. Fatty acids in hydrolysates were below the limits of detection, suggesting that the extraction procedure removed most of the fatty acid-containing lipids present in the cells. Electron microscopic examination of *N. commune* cells fixed conventionally [10,11], or with lipid mordants, revealed no evidence for lipid droplets or lipid inclusion bodies within the desiccated or rehydrated cells (data not shown). Floating lipids were not recovered upon the ultracentrifugation of cell lysates; instead, lipids in the starting material were recovered quantitatively in pellets obtained by ultracentrifugation.

Neutral lipid synthesis

Upon separation on thin-layer plates, *N. commune* had a complex neutral lipid pattern. There was an accumulation of material migrating at or near the front of the TLC plates; these materials have the mobility characteristics of hydrocarbons and wax esters – no further characterization of these components was attempted (Fig. 1). Three yellow, and two green pigmented materials were resolved. One sterol, with mobility similar to that of cholesterol, was identified by virtue of a positive reaction with antimony trichloride and with Liebermann-Burchard reagent. By virtue of TLC mobility relative to standards, and modification upon mild alkali-catalyzed methanolysis, triacylglycerols (TG), 1,2- and 1,3-diacylglycerols (DG), and fatty acids were identified in lipid extracts (Fig. 1). A relatively large amount of a green pigment migrated with 1,2-diacylglycerols. Dehydrated, and rehydrating cells had identical neutral lipid patterns, and the relative amounts of neutral lipid constituents in desiccated and rehydrated cultures were judged to be very similar (data not shown).

In pulse-chase experiments with [^{3}H]glycerol, radiolabel was incorporated rapidly into constituents migrating with 1,2- and 1,3-DG, and with TG (Fig. 2a). All three of these glycerolipid constituents were labeled modestly at the end of the pulse, but were labeled heavily after 2 h chase. At 6 h chase, most label was gone from DG, but TG remained labeled through 24 h chase. After 72 h of the chase the signals detected for TG were significantly reduced. During long-term labeling with [^{3}H]glycerol, there was a progressive increase in the incorporation of isotope into DG and TG over 7 days in culture (Fig. 2b). Over the time-course of this experiment, there was a shift in mobility of TG from less polar to more polar species. These data suggest the possibility of a change in fatty acid composition of TG during the growth of *N. commune*. Over the time-course of this experiment, there was a shift in mobility of TG from less polar to more polar species. These data suggest the possibility of a change in fatty acid composition of TG during the growth of *N. commune*. Over the long-term labeling time-course, radioactivity appeared in one, then in two, constituents which migrated between DG and TG (Fig. 2b). Positions of these constituents corresponded with the two uppermost yellow pigments (Fig. 1). Mild alkali did not affect the migration of these pigments, suggesting that they did not contain ester-bonded fatty acids. Pérhaps label from glycerol is metabolized into a precursor which is then incorporated into these constituents.

Fig. 1. Thin-layer chromatographic separation of neutral lipids from rehydrated *N. commune*. The plate was developed in hexane/diethyl ether/acetic acid (45:5:1, v/v) and separated constituents were detected by charring with cupric acetate-phosphoric acid reagent. Arrowheads, in ascending order, indicate the origin, a green pigment, 1,2-diacylglycerols, 1,3-diacylglycerols, sterol, yellow pigment, unesterified fatty acids and triacylglycerols. Lipid constituents were identified through co-mobility with standard lipids, reaction with selective spray reagents, lability to alkali, and by labeling with lipid precursors. The mass of material migrating near and at the solvent front has mobility like hydrocarbons or wax esters; sterol esters apparently are absent since material near the solvent front did not yield a positive reaction with Liebermann-Burchard or antimony trichloride reagents. A second green pigment ran in the region of diacylglycerols, and a third yellow pigment migrated to a position just under the constituent identified as sterol.

Synthesis of polar lipids and heterocyst glycolipid

Four major and one minor constitituents identified as glycolipids by virtue of a positive reaction with orcinol, were observed upon separation of *N. commune* polar lipids (Fig. 3). Monogalactosyl- (MGDG) and digalactosylglycerols (DGDG) were identified based upon comigration with standards, and by alkali lability. Sulfoquinovosyl diacylglycerol (SQDG) was identified based on TLC mobility in comparison with other reports of identification of this lipid [31], by alkali lability, and by labeling with radioactive sulfur (discussed below). The other glycolipid was identified as the major heterocyst species of *N. commune* and *Nostoc* 584 on the basis of its R_f in the solvent system of Murry and Wolk [27], its non-saponifiable nature, and the lower amounts present in cells grown with combined nitrogen. In addition to this major alkali-stable glycolipid, a minor alkali-labile glycolipid was also present; the alkali-labile glycolipid was identified as phosphatidylinositol (PI) based on co-migration with authentic PI, and by virtue of a positive reaction for phosphorus. Polar lipid fractions contained also three additional phosphorus-containing lipids. By virtue of alkali lability and co-migration with the authentic standard, phosphatidylglycerol (PG) was identified as the major phospholipid. Relatively small amounts of phospholipids migrating with phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which gave positive reactions for choline and a free amino group, respectively, and which were alkali labile, also were present in extracts. One alkali-stable constituent which did not give a positive reaction with any selective spray reagent used was present in lipid extracts, and a minor constituent which gave a ninhydrin positive reaction, but was alkali-labile and which did not give a positive reaction with any other selective reagent used, was detected (Fig. 3). Separation of polar lipids in the methyl acetate-containing solvent system of Heape et al. [23] confirmed tentative identifications assigned to constituents given above (not shown). No major quantitative differences in the distribution of polar lipids between desiccated and rehydrated cells were apparent.

Upon short-term labeling with [^{3}H]glycerol, constituents migrating as PG and SQDG were labeled heavily within 5 min of incubation (Fig. 4a). PG remained labeled intensely throughout 24 h of incubation, but at later time-points (2, 6 and 24 h) a constituent migrating just under PG became labeled, yielding a doublet pattern for this constituent. Whether this was due to label incorporation into a different constituent, or to the appearance of a second, more polar pool of PG, remains to be determined. In contrast to PG, the relative intensity of labeling of SQDG diminished after 60 min of incubation. Radiolabel became detectable in constitutents corresponding to MGDG and DGDG only after 60 min; intensity of label in MGDG increased through the 24 h of incubation, but in DGDG it peaked at 2 h and was decreased progressively at 6 and 24 h. The alkali-stable heterocyst glycolipid (Fig. 3) was first detectably labeled at 24 h. Trace amounts of three other labeled constitiuents also be-

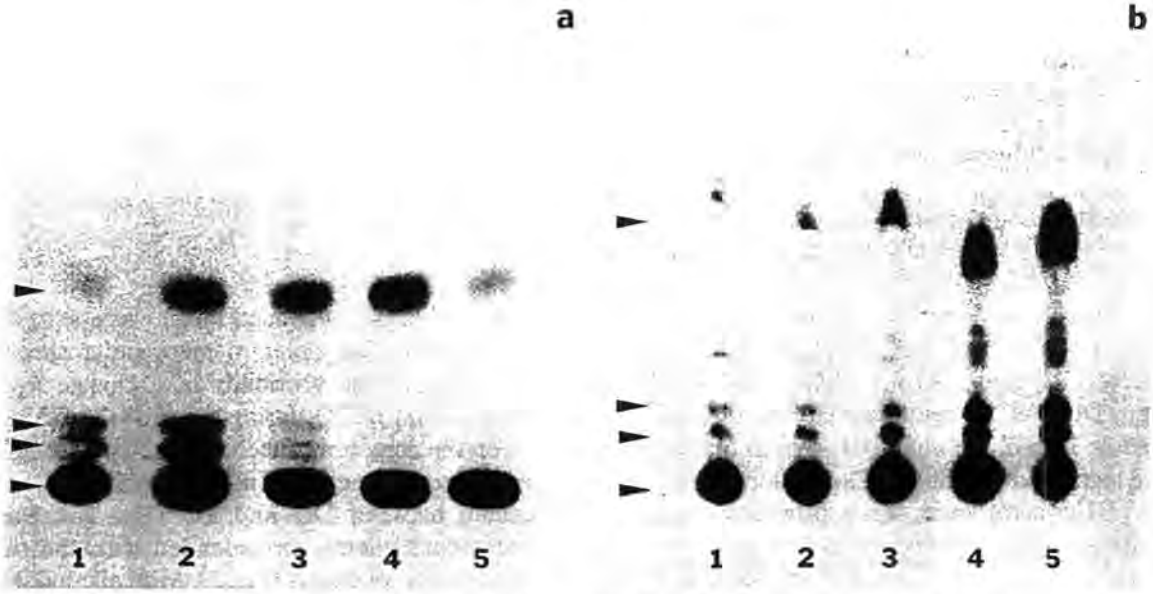

Fig. 2. Fluorograms of neutral lipids labeled by incorporation of [^{3}H]glycerol in pulse-chase (a) and long-term (b) incorporation experiments. Plates were developed as in Fig. 1. Arrowheads, in ascending order, indicate the origin and positions of 1,2-diacylglycerols, 1,3-diacylglycerols and triacylglycerols. In (a) lane 1 contains lipids extracted immediately after the pulse (no chase), 2 is 2 h chase, 3 is 6 h chase, 4 is 24 h chase, and 5 is 72 h chase. In (b) lane 1 contains lipids extracted just after rewetting, 2 is after 1 day, 3 is 2 days, 4 is 4 days, and 5 is 7 days incubation after rewetting.

came labeled, particularly at the 2 h time-point; two of these corresponded in mobility to PE and PC (Fig. 4a). In long-term [^{3}H]glycerol labeling, all major polar lipid constituents remained labeled heavily throughout the 7-day time-course (Fig. 4b), but there was diminution in labeling of constituents migrating near, and giving a doublet pattern to, MGDG, DGDG, PG and the alkali-stable glycolipid. With pulse-chase [^{3}H]glycerol labeling, SQDG and PG were labeled heavily after the pulse period and remained heavily labeled throughout the chase (Fig. 4c). Label first was detected in the alkali-stable glycolipid after 2 h chase, and this constituent remained labeled throughout the 72 h chase period. MGDG and DGDG were labeled after the pulse period, but relative intensity of labeling of these constituents increased during the first 2 h of chase.

Fig. 3. Thin-layer chromatographic separation of polar lipids from rewetted *N. commune*. The plate was developed in chloroform/methanol/water (35:11:1.5), and separated constituents were detected by charring with cupric acetate-phosphoric acid reagent. Arrowheads, in ascending order, denote the origin, phosphatidylcholine, sulfoquinovosyl diacylglycerol, phosphatidylglycerol, phosphatidylinositol, digalactosyl diacylglycerol, phosphatidylethanolamine, heterocyst glycolipid, an alkali-stable carbohydrate/phosphorus-negative lipid, and monogalactosyl diacylglycerol. Separated constituents were identified as in Fig. 1. Material at the solvent front is neutral lipids. The constituent just below phosphatidylcholine was ninhydrin-positive.

Sulfolipid synthesis

Radioactivity from [^{35}S]Na_2SO_4 was incorporated into polar material remaining at the origin of the TLC plates, and into three chromatographically distinct lipids (Fig. 5). After 5 min rehydration in the presence of isotope, material remaining at the origin, a constituent migrating with SQDG, and a constituent less polar than SQDG, were labeled (Fig. 5a and b). During the period between 5 and 30 min, label largely was absent from all constituents except those remaining at the origin. At 60 min, SQDG again was heavily labeled, as was a constituent with high mobility that was not labeled detectably at other time-points. SQDG remained heavily labeled between 60 and 24 h, but the label disappeared from the least polar sulfur-containing constituent. At 6 h, label was present in a constituent migrating between the least polar sulfolipid and SQDG. In a pulse-chase experiment, SQDG and the least polar sulfolipid were labeled at the end of the pulse, but at 2 h chase, radiosulfur in lipid extracts was detected by fluorography only in material remaining at the origin of the TLC plate (Fig. 5b). SQDG was the only constituent showing obvious labeling at 6, 24 and 72 h chase. These patterns were observed in repeat trials.

Phospholipid synthesis

The apparently equivalent labeling of PE and PI relative to PG noted in these experiments is not consistent with the small amounts of PE and PI relative to PG noted in Fig. 3. In addition, PE and PI were not detected after labeling with [^{3}H]glycerol (Fig. 4a). The identifications of PE and PI as given in Figs. 6a and b, therefore, while based upon co-migration with standards, must remain tentative at this time.

Upon short-term labeling with [^{32}P]orthophosphate (5 min), label was, primarily, in polar materials remaining at and streaking from the origin of TLC plates (Fig. 6a). At 10 min, some label was in PG, in PC, and in a constituent migrating between the origin and PC. At 30 and 60 min label had appeared in constituents corresponding to PE and PI, PG was labeled and a series of constituents between PG and the origin were labeled. At 2 and 6 h, label primarily was in PG, PI, PE, as well as material at the origin, and the intensity of labeling of PG was diminished relative to the 10 and 30 min time-points. At 24 h, nearly all label was in PE, PI and PG. From this experiment, it appeared that phospholipids more polar than PG were turned over relatively rapidly, that PE and PI were synthesized more slowly but were not turned over rapidly, and that PG may be synthesized cyclicly. Pulse-chase labeling with ^{32}P yielded results similar to those from short-term labeling (Fig. 6b), with label initially being most heavily incoporated into constituents more polar than PG, and later becoming concentrated in PG, PI and PE. By 72 h

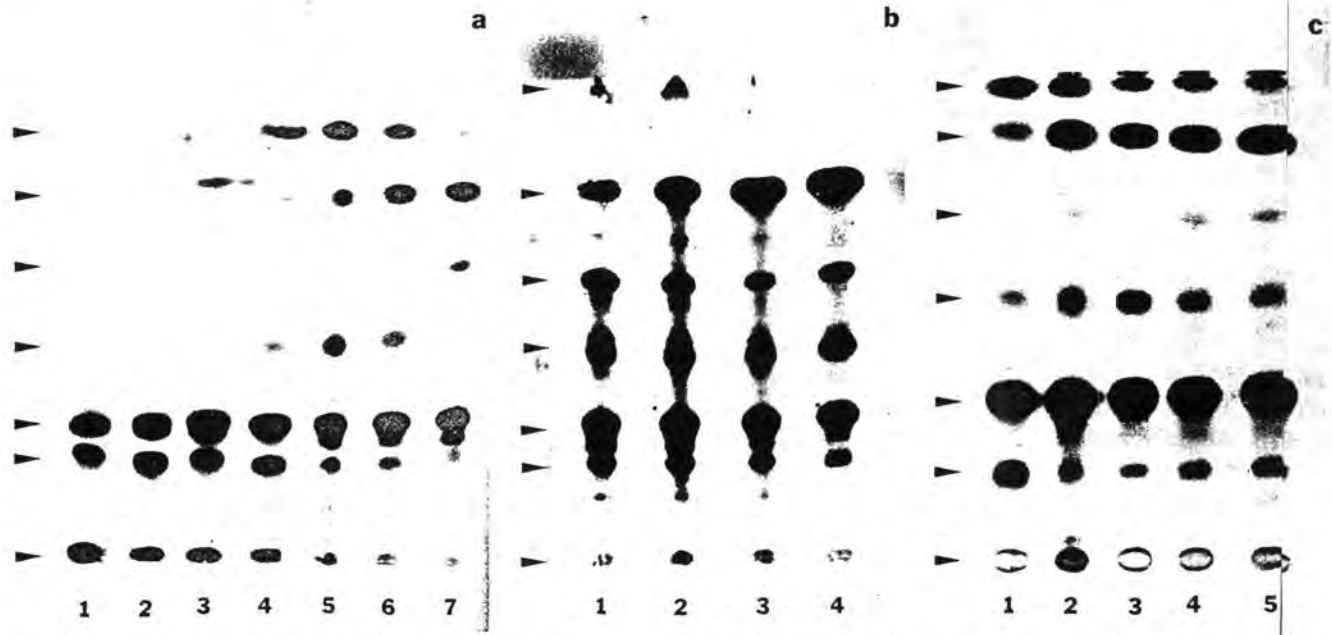

Fig. 4. Fluorograms of polar lipids labeled by incorporation of [^{3}H]glycerol in short-term (a), long-term (b), and pulse-chase (c) experiments. Plates were developed as in Fig. 3. Arrowheads, in ascending order, denote the origin, sulfoquinovosyl diacylglycerol, phosphatidylglycerol, digalactosyl diacylglycerol, heterocyst glycolipid, monogalactosyl diacylglycerol, and neutral lipids at the solvent front. In (a) lane 1 is 5 min, 2 is 10 min, 3 is 30 min, 4 is 1 h, 5 is 2 h, 6 is 5 h, and 7 is 24 h incubation after rewetting. In (b) lane 1 is 1 day, 2 is 2 days, 3 is 4 days, and 4 is 7 days incubation. In (c) lane 1 is just after the pulse, 2 is 2 h, 3 is 6 h, 4 is 24 h, and 5 is 72 h chase.

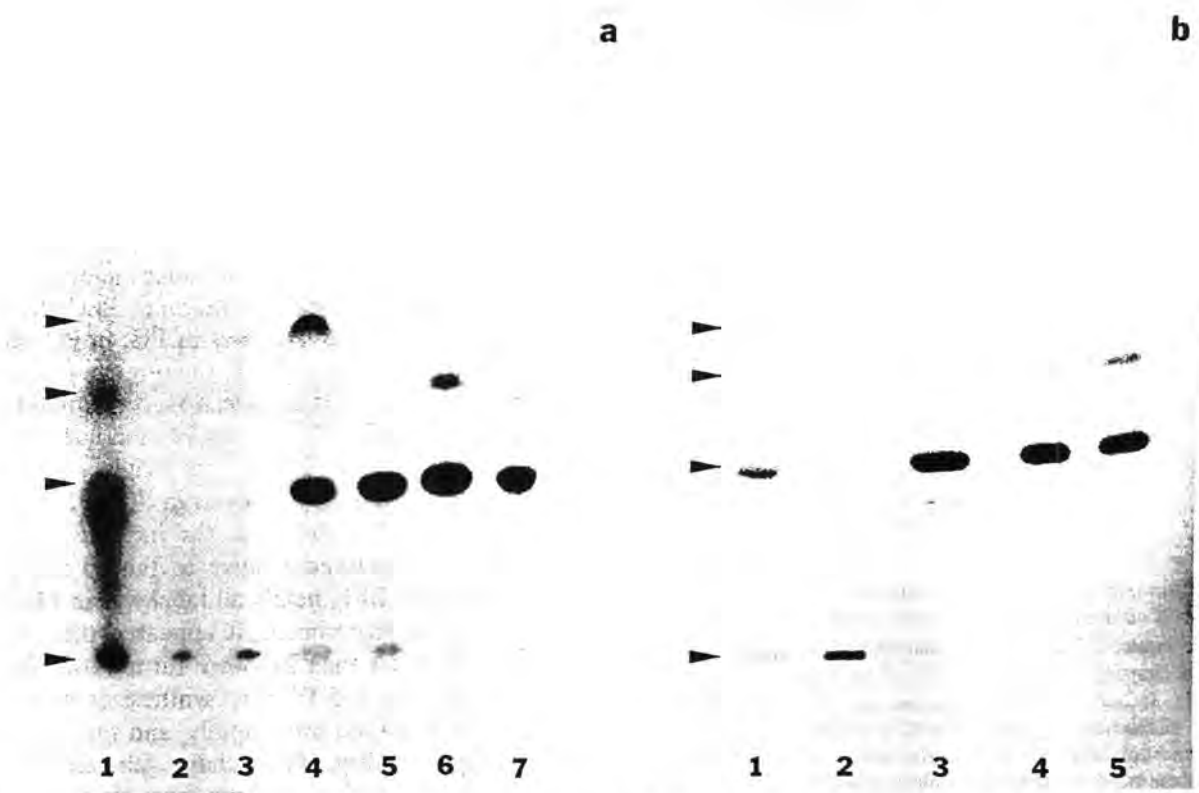

Fig. 5. Fluorograms of polar lipids labeled by incorporation of [^{35}S]sulfate in short-term (a), and pulse-chase (b) incubations. Plates were developed as in Fig. 3. Arrowheads, in ascending order denote the origin, sulfoquinovosyl diacylglycerol, and two unidentified sulfolipids. In (a) lane 1 is 5 min, 2 is 10 min, 3 is 30 min, 4 is 1 h, 5 is 2 h, 6 is 5 h, and 7 is 24 h incubation. In (b) lane 1 is immediately after the pulse, 2 is 2 h, 3 is 6 h, 4 is 24 h, and 5 is 72 h chase.

chase, label largely was gone from all constituents except PG.

Discussion

An advantage of the experimental approach used in this study is that a particular tracer enters desiccated cells simultaneously with water and, in principal, makes it possible to monitor very rapid (perhaps instantaneous) shifts in intracellular pools of macromolecules. The relative amount of mucilaginous sheath present in the samples is comparable to that present in field communities such that the speed of rehydration achieved in these experiments may be representative of that occurring in situ. However, the relative contribution of osmotic and matric stresses in the initial response of the cells to rewetting is difficult to assess. It should be noted that radioactive pool sizes were not measured here. As such it is not possible to state with certainty whether the radiolabeled precursor had equilibrated with all of a particular lipid. That is, the possibility of compartmentation must be considered with more than one pool of a particular lipid. As judged from short-term, long-term and pulse-chase labeling, with three different precursors, the synthesis and turnover of all major classes of complex lipids and their precursors underwent a rapid upshift upon the rewetting of desiccated cells. In previous studies it has been observed that *Nostoc commune* cells retain the structural integrity of their intra- and extracellular membrane systems during drying, prolonged desiccation, and after subsequent rehydration [10,11]. The dynamics of the biosynthesis and turnover of the major membrane lipids is, therefore, of interest. Furthermore, the metabolism, translocation and structure of phospholipids in *E. coli* is linked to protein secretion [32], a process which is not well understood in cyanobacteria. As a consequence of the two different pathways for phospholipid biosynthesis, the [^{32}P]phosphate incorporated in PE and PC derives from phosphoethanolamine and phosphocholine, respectively, while that in PG and PC is derived from phosphatidic acid (through *sn*-glycerol-3-phosphate; see Refs. 33 and 34). The heavy labeling of four major phospholipids within 30 min of rewetting of *Nostoc* cells suggests that both pathways are operable at this time. The radiolabeled pool of 1,2-diacyl-*sn*-glycerol rises very rapidly upon rewetting of cells, and is turned over within several hours, concomitant with the appearance of its products, TG and phosphoglycerides. This would be the expected result if phospholipid biosynthesis was fueled with rapidly-synthesized precursors. Of the other phospholipids detected in *N. commune*, PC has also been reported from the cytoplasmic membrane of *Synechocystis* sp. PCC 6714 [6]. PI, a disrupter of membrane stability is some eukaryotes, appears to be a minor

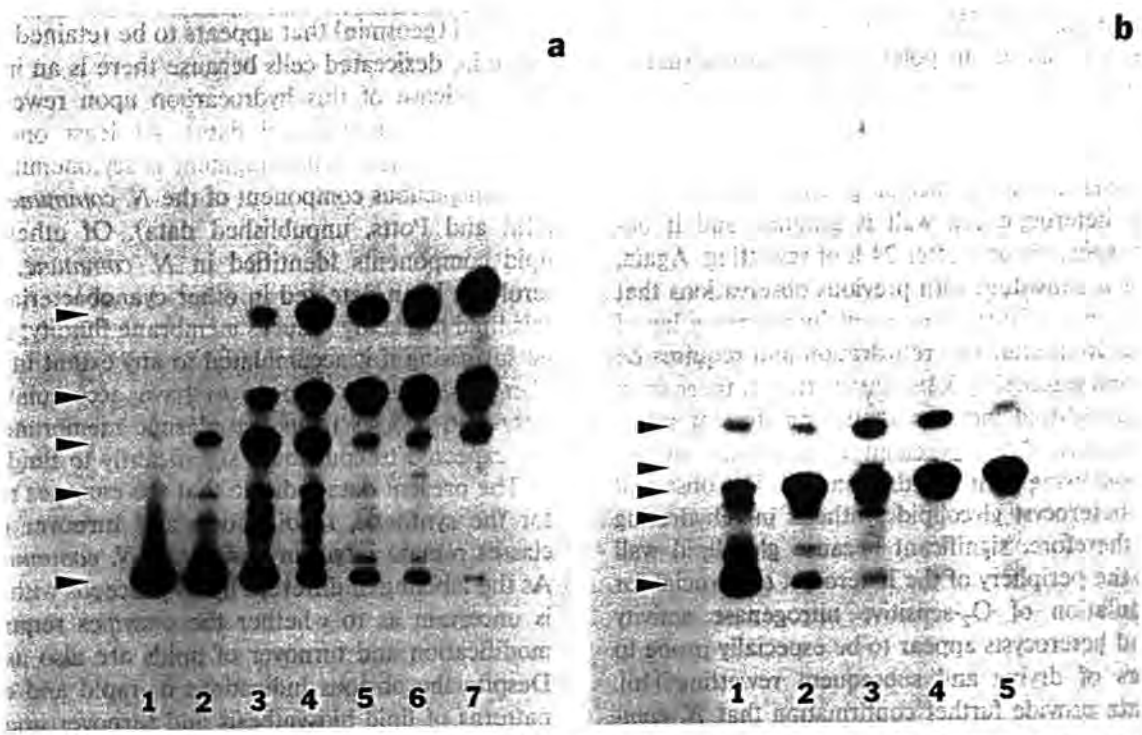

Fig. 6. Autoradiograms of polar lipids labeled by incorporation of [^{32}P]orthophosphate in desiccated colonies of *N. commune* through short-term (a) and pulse-chase (b) incubations. Plates were developed as in Fig. 3. Arrowheads, in ascending order, denote the origin, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, and phosphatidylethanolamine. In (a) lane 1 is 5 min, 2 is 10 min, 3 is 30 min, 4 is 1 h, 5 is 2 h, 6 is 5 h, and 7 is 24 h incubation. In (b) lane 1 is immediately after the pulse, 2 is 2 h, 3 is 6 h, 4 is 24 h, and 5 is 72 h chase. Note that the separations presented in Figs. 6a and b appear different becauces the total separation distances given by the solvent fronts are different.

component in *Nostoc*, while no evidence was obtained for the presence of cardiolipin, a derivative of PG and an essential phospholipid component of *E. coli* [35].

The synthesis of the principal thylakoid-membrane lipids SQDG, MGDG and DGDG, also commences rapidly upon rehydration. While all three are present in similar quantities under steady-state conditions, there is obvious specificity in their turnover during the initial stages of rehydration. The synthesis of SQDG is as rapid as that observed for PG, while there was a lag of several hours before the appearance of label in MGDG and DGDG. The subsequent accumulation of MGDG and DGDG, through an apparently low rate of turnover, is in contrast to the more rapid and almost complete turnover of label in SQDG within 5 h of rewetting of cells. There are few data about the synthesis of the sulfoquinovosyl moiety of SQDG. It is uncertain whether its biosynthesis is related to transfer of activated sulfur from 3′-phosphoadenosine 5′phosphosulfate, or oxidation from cysteine to cysteic acid and then deamination to 3-sulfolactate [36]. Results of both short-term and pulse-labeling experiments, suggest the presence of an activity which actively removes the $^{35}SO_3^{2-}$ from SQDG during the initial stages (30 min) of rewetting. That the activity is specific for the $^{35}SO_3^{2-}$group, and not the quinovose per se, is suggested by the observed accumulation of SQDG detected when labeling with [^{3}H]glycerol (see Fig. 4).

The major glycolipid of the cyanobacterial heterocyst cell wall, lipid III [37], is a non-saponifiable polar glucolipid with an R_f in polar solvent system that is intermediate between that of MGDG and DGDG [27]. In contrast to the rapid synthesis and turnover of membrane lipids upon rehydration, the accumulation of radiolabel in the principal glycolipid of the *N. commune* heterocyst cell wall is gradual, and it becomes conspicuous only after 24 h of rewetting. Again, this result is consistent with previous observations that the appearance of nitrogenase activity follows a lag of similar duration after cell rehydration and requires de novo protein synthesis [15,18]. Furthermore, there is an approximate 3-fold increase in the number of intercalary heterocysts of desiccated *N. commune* during the 48 h following their rehydration [19]. The observed onset of heterocyst glycolipid synthesis in rehydrating cells is, therefore, significant because glycolipid wall layers at the periphery of the heterocyst are crucial for the modulation of O_2-sensitive nitrogenase activity [3,27], and heterocysts appear to be especially prone to the rigors of drying and subsequent rewetting [16]. These data provide further confirmation that *N. commune* cells are unable to maintain the machinery for nitrogen fixation intact when subjected to long-term water stress.

Cells of *Nostoc* 584 maintain a steady-state intracellular ATP pool of approx. 200 pmol ATP μg chlorophyll a^{-1} (16 pmol ATP μg total protein^{-1}; see Refs. 17 and 18). Although nitrogenase activity is terminated within some 20 min of the immobilization and drying of cells, the ATP pool is maintained at control levels for a further 9 h under conditions of moderate water stress [17]. Under conditions of more extreme water stress ATP is depleted and subsequent rehydration of cells leads to an immediate rise in the ATP pool [19]. The rise is sensitive to CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) as well as sodium azide, dependent upon de novo protein synthesis, and the time taken to achieve steady-state levels corresponds to that required for the recovery of appreciable nitrogenase activity [1,16,17,19]. We questioned whether lipids could function as a storage energy source during the recovery of the ATP pool in rehydrating cells. While no indications were obtained here for storage lipid droplets, TG is a major component of the neutral lipid of *N. commune* and, as shown in labeling experiments, TG was shown to accumulate during long-term labeling, with turnover during 24 h, and with a probable change in fatty acid composition.

Cyanobacteria synthesize a range of simple and exotic hydrocarbons of undetermined function and origin [38]. The origin of the hydrocarbons and wax esters observed in this study have yet to be determined but it is possible that they may be associated with the extensive extracellular carbohydrate sheath. *Nostoc* 584 produces extensive amounts of *trans*-1, 10, dimethyl-*trans*-2-decalol (geosmin) that appears to be retained by, and stable in, desiccated cells because there is an instantaneous release of this hydrocarbon upon rewetting of cells (Potts, unpublished data). At least one major detergent-soluble yellow-pigment is scytonemin, which is a conspicuous component of the *N. commune* sheath (Hill and Potts, unpublished data). Of other minor lipid components identified in *N. commune*, cholesterol has been detected in other cyanobacteria [2]. As this lipid markedly reduces membrane fluidity, it would be surprising if it accumulated to any extent in cells of *Nostoc 584* which appear to have accumulations of fatty acid species in its cytoplasmic membrane which are expected to contribute significantly to fluidity [14].

The present data indicate that the enzymes required for the synthesis, modification and turnover of lipid classes remain intact in desiccated *N. commune* cells. As the labeling of different lipids proceeds with time, it is uncertain as to whether the enzymes required for modification and turnover of lipids are also as stable. Despite the obvious indications of rapid and complex patterns of lipid biosynthesis and turnover upon rehydration of *Nostoc*, the cells not only maintain their structural components (membranes) intact but they also maintain a total lipid pool which is quantitatively similar to that of desiccated cells. To permit such a restoration of steady-state lipid metabolism, to coordi-

nate membrane synthesis with processes such as ATP synthesis [1,17–19], protein synthesis [15], respiration [14], photosynthesis and nitrogen fixation [16,19] – all of which are recovered in an orderly, and reproducible, fashion-following rehydration – rehydrating cells must exercise very stringent controls over the stabilities and the activities of many different classes of proteins and enzymes. For example, desiccated cells clearly retain their capacities for the mobilization of sulfate (also see Ref. 15), orthophosphate, and glycerol and, upon rewetting they rapidly incorporate these substrates into lipids. Sulfate transport in *Synechococcus* sp. strain PCC 7942 requires a membrane-associated ATP-binding protein, a sulfate-binding protein, and two additional polypeptides whose synthesis is sulfur-regulated and which may be components of a cytoplasmic membrane channel [39]. Phosphate- and glycerol-uptake have not received such critical attention in cyanobacteria but they are likely to be equally complex processes. At least one major regulatory protein in cyanobacteria, ferredoxin NADP + oxidoreductase (FNR), may be acylated in *Synechococcus* sp. PCC 7002 [41].

We have yet to obtain any indication of extensive damage in *N. commune* cells after their exposure to acute water stress. The indications point to a highly-ordered system which may be dependent, in part, on specific mechanisms for protein targeting and recognition, and rigorous maintenance of membrane structure and function.

Acknowledgements

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Nostoc commune UTEX 584 Gene Expressing Indole Phosphate Hydrolase Activity in *Escherichia coli*

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A gene encoding an enzyme capable of hydrolyzing indole phosphate was isolated from a recombinant gene library of *Nostoc commune* UTEX 584 DNA in λgt10. The gene (designated *iph*) is located on a 2.9-kilobase *Eco*RI restriction fragment and is present in a single copy in the genome of *N. commune* UTEX 584. The *iph* gene was expressed when the purified 2.9-kilobase DNA fragment, free of any vector sequences, was added to a cell-free coupled transcription-translation system. A polypeptide with an M_r of 74,000 was synthesized when the *iph* gene or different *iph*-vector DNA templates were expressed in vitro. When carried by different multicopy plasmids and phagemids (pMP005, pBH6, pB8) the cyanobacterial *iph* gene conferred an Iph$^+$ phenotype upon various strains of *Escherichia coli*, including a *phoA* mutant. Hydrolysis of 5-bromo-4-chloro-3-indolyl phosphate was detected in recombinant *E. coli* strains grown in phosphate-rich medium, and the activity persisted in assay buffers that contained phosphate. In contrast, indole phosphate hydrolase activity only developed in cells of *N. commune* UTEX 584, when they were partially depleted of phosphorus, and the activity associated with these cells was suppressed partially by the addition of phosphate to assay buffers. Indole phosphate hydrolase activity was detected in periplasmic extracts from *E. coli* (Iph$^+$) transformants.

Our current understanding of the role of phosphorus in the control of cell function in microorganisms derives largely from studies with *Escherichia coli* (22). Four different phosphatases have been identified in the periplasm of *E. coli*, each showing hydrolytic activity with a range of substrates that do not penetrate the cytoplasmic membrane (4, 29). The major criteria used in the characterization of these four phosphatases are substrate specificity and pH optimum (4, 29). Considerable data have accumulated on the genes and enzymes involved in phosphate transport (23, 24, 26). As a consequence, current opinion is that the regulation of phosphate transport is complex.

Cyanobacteria warrant particular attention, because of the key role played by the availability and turnover of phosphorus in determining the development of water blooms or extent of economically important nitrogen-fixing communities such as those in rice fields. A range of cyanobacteria have been reported to show phosphatase activity (10), but little is known about the regulation of phosphate metabolism or the enzymes or genes involved. The availability of a recombinant gene libary of *Nostoc commune* UTEX 584 (W.-Q. Xie, K. Jäger, and M. Potts, submitted for publication) provided an opportunity to attempt the isolation of a cyanobacterial phosphatase gene for the further investigation of phosphate metabolism in this ecologically significant, nitrogen-fixing cyanobacterium. In the present study we report the isolation of a gene coding for an indole phosphate hydrolase (designated *iph*) from *N. commune* UTEX 584.

MATERIALS AND METHODS

Microorganisms and growth conditions. *N. commune* UTEX 584 was grown as described previously (16) in liquid BG 11_0 medium (18). *Anabaena variabilis* PCC 7118 was grown in BG 11 medium under the same conditions of growth. The strains of *E. coli* used in this study are listed in Table 1. All strains were grown in LB liquid medium (12) at 37°C, with or without the addition of ampicillin (200 μg ml^{-1}, final concentration). In certain experiments a minimal medium (8) was used; this was supplemented with different concentrations of KH_2PO_4. Where necessary, liquid media were solidified by the addition of 1.2% (wt/vol) agar.

Recombinant DNA analyses. Unless stated otherwise, routine methods were used for the manipulation of DNA (9, 12). Restriction endonucleases were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and were used according to the specifications of the manufacturer. The plasmid pGEM-4 and bacteriophage λgt10 were obtained from Promega Biotec (Madison, Wis.). The phagemid pBluescript M13+ (Stratagene, La Jolla, Calif.) was a gift from T. Larson.

Construction of recombinant DNA library. During the isolation of *rpo* genes from *N. commune* UTEX 584 (Xie et al., submitted), a recombinant library of *N. commune* UTEX 584 genomic DNA was constructed in the phage vector λgt10 (*imm*^{434} *b527*) and propagated in *E. coli* C600 (*hfl*) by standard methods (9). The library was constructed with *N. commune* UTEX 584 genomic DNA-*Eco*RI restriction fragments (size range, 3 to 7 kilobases [kb]). The genomic DNA was prepared as follows. A culture was grown to a density of approximately 20 g (wet weight) of cells per liter, the cells were harvested by centrifugation, and the pellet was washed once (through suspension of the cells) in 50 mM Tris hydrochloride buffer (pH 8.0). The cells (40 g of wet weight) were frozen under liquid nitrogen, ground to a powder, and suspended in 40 ml of lysis buffer (15% [wt/vol] sucrose, 10 mM EDTA, 25 mM Tris hydrochloride [pH 8.0]). This suspension was frozen (under liquid nitrogen) and thawed a total of five times. Solid lysozyme (10 mg ml^{-1}, final concentration) was added to the suspension, which was then incubated at 37°C with gentle agitation for 4.5 h. The solution

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TABLE 1. Bacterial strains, plasmids, and bacteriophages

Strain, plasmid, or phage	Relevant characteristics	Source or reference
E. coli		
HB101	F⁻ *hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galk2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44* (λ^-)	15
LE392	F⁻ *hsdR514* (r_K^- m_K^+) *supE44 supF58 lacY1* or Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55* (λ^-)	15
ECL8	HfrC *phoA8 glpD3 glpR2 relA1 spoT1 fhuA22 ompF627 fadL701 pit-10* (λ)	T. Larson
C600Hfl	*hflA150* [Chr::Tn*10*]	Promega Biotec
DH5-α	F' *endA1* (r_K^- m_K^+) *hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* ϕ80d*lacZ*Δ*M15* (λ^-)	Bethesda Research Laboratories
ATCC 23601	Derivative of *E. coli* B	American Type Culture Collection
Plasmids		
pGEM-4	2.87 kb, Ap^r	Promega Biotec
pMP004	5.78 kb, Ap^r, *iph* (subclone of a 2.9-kb *Nostoc* genomic DNA *Eco*RI insert fragment [from λgt10P1] in pGEM-4)	This study
pMP005	5.78 kb as pMP004, *iph* insert in reverse orientation (Fig. 1).	This study
pGAL85	7 kb, Ap^r, *lacZ*	DuPont
pB8	5.9 kb, Ap^r, *iph* (subclone of a 2.9-kb *Nostoc* genomic DNA *Eco*RI insert fragment [from λgt10P1] in pBluescript M13+: orientated with unique *Ava*I site proximal to *lacZ* promoter [Fig. 1])	This study
pBH6	5.9 kb, Ap^r, *iph* (same as pB8, insert in reverse orientation)	This study
Phagemid pBluescript M13+	2.96 kb, Ap^r, *lacZ*	Stratagene Inc.
Bacteriophages		
λgt10	*srl* 1° *b527 srl* 3° *imm*^{434} (*srl*434$^-$) *srl* 4° *srl* 5°	Promega Biotec
λgt10P1	Recombinant of λgt10 and a 2.9-kb *Eco*RI fragment of *Nostoc commune* UTEX 584 genomic DNA (*iph*)	This study

became brown and viscous within 2 h after the addition of the lysozyme. The solution was then stored at 4°C overnight; then 0.4 g of *N*-lauroylsarcosine and 0.8 mg of proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.; 20 μg ml^{-1}, final concentration) were added to the solution, and incubation was continued at 50°C for 4 h with gentle agitation. Approximately 1 ml of 1 M Tris hydrochloride (pH 9.0) was added to the solution to achieve a pH of 7.0 (to compensate for the drop in pH due to addition of *N*-lauroylsarcosine). The lysate was diluted with 30 ml of buffer (50 mM EDTA, 50 mM Tris hydrochloride [pH 7.5]); then 80 ml of preequilibrated phenol (12) was added to the mixture, which was then shaken gently overnight at room temperature. The aqueous phase was recovered after centrifugation of the solution and was extracted further with equal volumes of phenol, phenol-chloroform (1:1), and then chloroform (two extractions at room temperature). The phenol phases were extracted with distilled water (30 ml), and all the aqueous phases were pooled before mixing with an equal volume of isopropanol (−20°C) in the presence of 0.3 M sodium acetate. The DNA was collected by spooling, and the pellet was washed first in 70% (vol/vol) ethanol and then in 90% (vol/vol) ethanol (−70°C). The pellet was dissolved in 1 mM EDTA, 10 mM Tris hydrochloride (pH 7.0) and purified further by cesium chloride density gradient ultracentrifugation (12).

Purification of plasmid and phage DNAs. Plasmid DNAs were purified from 1-ml liquid cultures (grown overnight) by using an alkaline hydrolysis technique (GemSeq K/RT Technical Manual; Promega Biotec). The preparation of high-titer liquid lysates and the purification of phage DNA were achieved as described by Silhavy et al. (21).

Southern analysis. A biotinylated RNA probe was synthesized by using the 2.9-kb *N. commune* UTEX 584 DNA fragment as a template (in pB8; Table 1). The synthesis was performed with T7 RNA polymerase (Promega Biotec), and precautions were observed during all manipulations of RNA. General procedures for Southern transfer were as described previously (5). Hybridization was performed under stringent conditions: the hybridization buffer contained 45% (wt/vol) deionized formamide, 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate), 0.5% (wt/vol) sodium dodecyl sulfate, 2 mM disodium EDTA, 10 mM Tris hydrochloride (pH 7.5), 2× Denhardt solution, and 5% (wt/vol) polyethylene glycol (type 8000). Hybridization was performed at 50°C for 16 h. After hybridization the filter was washed first in 1× SSC–0.1% (wt/vol) sodium dodecyl sulfate and then in 0.2× SSC–0.1% (wt/vol) sodium dodecyl sulfate for 40 min at 50°C. Finally, the filter was washed in 1× SSC–0.1% (wt/vol) sodium dodecyl sulfate. Blocking of the filter with bovine serum albumin was performed at 60°C for 20 min in the presence of vanadyl-ribonucleoside complex. Biotinylated RNA-DNA hybrids were visualized with a colorimetric assay (Bethesda Research Laboratories).

Construction of deletion clones. Plasmids carrying deletions of the 2.9-kb *Eco*RI-*Eco*RI fragment were constructed through processive deletion of pB8 and pBH6 (Table 1) with exonuclease III, exonuclease VII, and the Klenow fragment as described previously (W.-Q. Xie and M. Potts, Gene Anal. Tech., in press).

Coupled in vitro transcription-translation assay. A cell-free system for coupled transcription-translation of DNA was obtained from DuPont NEN Research Products. The system was supplemented with carrier-free L-[^{35}S]methionine (1,134 Ci mmol^{-1}; DuPont). Conditions for the measurement of ^{35}S incorporation in translation products, gel electrophoresis, and autoradiography were as described previously (11). Plasmid and phage DNAs to be used for the transcription-

translation assays were purified through high-performance liquid chromatography. Chromatography was performed with a Gen-Pak FAX column, a DuPont Instruments series 8000 Gradient controller, and Spectro series 8000 high-performance liquid chromatography pump/detector system. A dual buffer system of 50 mM Tris hydrochloride (pH 8.1) and 1 M LiCl (in 50 mM Tris hydrochloride) was used with a gradient from 40 to 80% (vol/vol). Fractions with peaks at A_{260} were collected and mixed with 2 volumes of 95% (vol/vol) ethanol and then stored at −20°C. After centrifugation the pellets of DNA were washed in 70% (vol/vol) ethanol, dissolved in water, and stored at 4°C until needed.

Transformation. Competent cells of the various strains of *E. coli* were transformed with plasmid DNA in the presence of calcium chloride, rubidium chloride, and 3-[*N*-morpholino]propanesulfonic acid (12).

Detection of enzyme activities. To detect the presence of enzyme activities in plaques and bacterial colonies, 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma Chemical Co.) was dissolved in dimethylformamide (50 mg ml^{-1}), and 20 μl of the solution was spread over the surface of an agar plate (25). The plate was allowed to dry and then streaked with cells. The other substrates used to detect enzyme activities were 4-*p*-nitrophenyl phosphate (PNPP) (28), bis-PNPP, and 5-bromo-4-chloro-3-indolyl acetate (Sigma). Unless stated otherwise the detection of enzyme activities in whole cells and cell fractions, spectrophotometric assays, and the use of the different substrates followed general procedures (1, 2, 4, 29).

RESULTS

Isolation of a gene showing BCIP hydrolase activity. The use of BCIP permitted the detection of a single blue plaque after screening ~8,000 plaques in the recombinant library of *N. commune* UTEX 584 genomic DNA in phage λgt10. The single positive plaque was subjected to three rounds of plaque purification. DNA from the recombinant phage (λgt10P1) was purified from a 1-liter lysate of *E. coli* C600Hfl (Table 1); after digestion with *Eco*RI, the DNA insert (2.9 kb) was purified through electroelution and subcloned through ligation in *Eco*RI-digested pGEM-4 and pBluescript M13+ (Table 1). Several strains of *E. coli* were transformed with recombinant plasmids that carried the 2.9-kb fragment in different orientations (Table 1; Fig. 1). All transformants generated bright blue colonies when they were plated on LB agar plates in the presence of BCIP. Nontransformed cells plated under identical conditions gave rise to white colonies. The gene of *N. commune* UTEX 584 encoding the indole phosphate hydrolase (phosphatase) activity has been termed *iph* until such time that a definitive assignment (e.g., *pho*) can be given. Indole phosphate hydrolase activity in *E. coli*(pMP005) was found associated with whole cells, periplasmic extracts, intact spheroplasts (after osmotic schock treatment), and sonicated spheroplasts (data not shown). Activity was not detectable in culture supernatants or in the cytoplasmic membrane-cell debris fraction, which was obtained after high-speed centrifugation of the sonicated spheroplasts.

Southern analysis. Since endogenous phosphatases are synthesized by the strains of *E. coli* used in this study, it was essential to confirm that the 2.9-kb fragment originated from *N. commune* UTEX 584 genomic DNA. A biotinylated RNA probe synthesized with pB8 DNA (Table 1) was used to screen *Eco*RI digests of genomic DNA from *N. commune* UTEX 584, *A. variabilis* PCC 7118, and *E. coli* C600Hfl and ATCC 23601 (Fig. 2a). A single band, corresponding to an *Eco*RI-*Eco*RI *N. commune* UTEX 584 DNA fragment of 2.9 kb, was detected (Fig. 2b). The digests of genomic DNA from *A. variabilis* PCC 7118 and the two strains of *E. coli* gave no hybridization signals with the RNA probe.

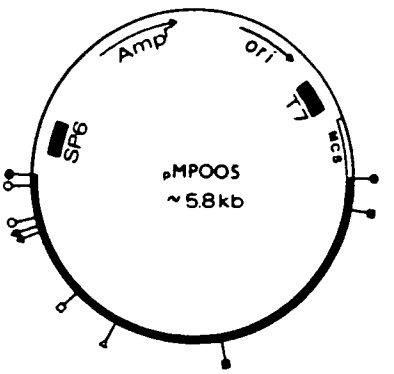

FIG. 1. Restriction map of the 2.9-kb DNA fragment from *N. commune* UTEX 584 carrying *iph* in pMP005 (Table 1). The positions of the phage-specific promoters SP6 and T7 are indicated. Not shown are multiple sites of *Hin*cII cleavage; an additional site for *Pst*I is located between the two *Pst*I sites that are indicated. Sites for the following endonucleases are absent in the 2.9-kb fragment: *Bam*HI, *Bgl*II, *Hin*dIII, *Sac*I, *Sma*I, and *Xba*I. Symbols: ○, *Acc*I; ▲, *Ava*I; ●, *Eco*RI; ▽, *Kpn*I; ■, *Pst*I; ◆, *Sal*I; □, *Sph*I.

In vitro transcription-translation. Different phage and phagemid preparations (Table 1) were used in coupled transcription-translation reactions to obtain information on the number and size of the gene products encoded by the 2.9-kb fragment. In parallel experiments the DNA was digested with *Eco*RI before the transcription-translation reaction to excise the 2.9-kb fragment and to uncouple any *iph* transcription from the potential control of vector-specific promoters. At the completion of the transcription-translation reactions, BCIP was added to the solutions (0.5 mM, final concentration). Indole phosphate hydrolase activity was detected in every transcription-translation reaction which contained *iph* DNA, including the reaction that contained

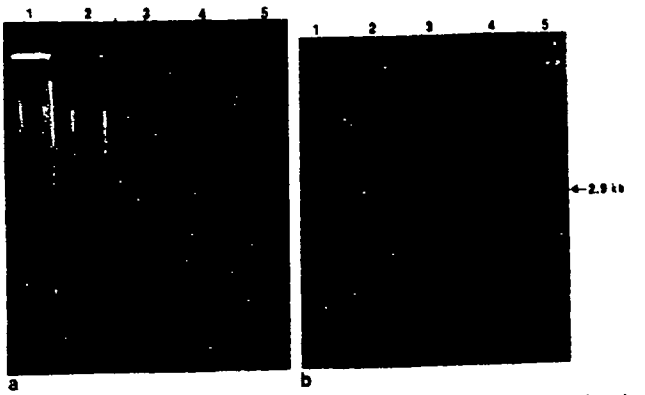

FIG. 2. Southern hybridization analysis with biotinylated *iph* riboprobe. (a) Agarose (0.8% [wt/vol]) gel electrophoresis of different DNA preparations each digested with *Eco*RI for 2 h at 37°C. Lanes: 1, genomic DNA of *E. coli* ATCC 23601 (10 μg); 2, genomic DNA of *A. variabilis* PCC 7118 (6 μg); 3, genomic DNA of *N. commune* UTEX 584 (10 μg); 4, λgt10P1 DNA (80 ng; *Eco*RI fragments of 32.7, 10.6, and 2.9 kb (*iph*); 5, λ *Hin*dIII size markers (0.1 μg). (b) Southern transfer of gel in Fig. 2a.

TABLE 2. Expression of *iph* in a cell-free coupled transcription-translation system

DNA template	Vector	*Eco*RI digest	Lane in Fig. 3	Iph activity
None (control)		−	1	−
pGAL85 (control)	pBR322	−	2	−
λgt10P1	λgt10	−	3	−
λgt10P1	λgt10	+	4	−
2.9-kb *iph* fragment		−	5	+
pB8	pBluescript M13+	−	6	+
pB8	pBluescript M13+	−	7	+
pB8	pBluescript M13−	−	8	+
pB8	pBluescript M13+	+	9	−
pBH6	pBluescript M13+	−	10	+
pBH6	pBluescript M13+	+	11	−

the 2.9-kb fragment free of any vector DNA (Table 2). Activity persisted when λgt10P1, pB8, or pBH6 DNA was digested with *Eco*RI before use. A single polypeptide with an M_r of approximately 74,000, not present in control assays, was the most obvious reaction product common to the different transcription-translation assays (Fig. 3). The intensity of the band at M_r 74,000 was equivalent for most of the assays, with the exception of the weaker signals for λgt10P1 DNA, where the molar concentration of *iph* DNA was reduced relative to the input DNA (Fig. 3, lanes 3 and 4). An additional polypeptide with an M_r of approximately 38,000 was synthesized only in the reaction supplemented with pBH6 DNA but not when the pBH6 DNA was predigested with *Eco*RI (Fig. 3, lanes 10 and 11).

Deletion analysis. Derivatives of pB8, with deletions of approximately 1.2 kb or less at the end of the 2.9-kb insert distal to the *lac* promoter, conferred an Iph$^-$ phenotype upon transformants of *E. coli* DH5-α. pB8 derivatives with larger deletions in this region of the insert or with small deletions at the end of the insert proximal to the *lac* promoter failed to confer an Iph$^-$ phenotype in transformants (data not shown).

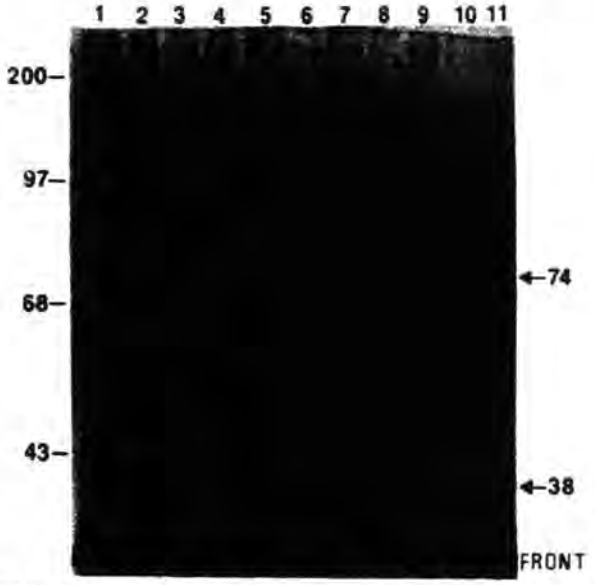

FIG. 3. Autoradiogram of reaction products from cell-free coupled transcription-translation reactions with different *iph* DNA templates (Table 2). Identical quantities of radioactivity (10^6 cpm) were loaded for each of the different samples. Numbers refer to sizes of polypeptides (in kilodaltons). The major band of reference in lane 2 (control) represents LacZ (M_r, 115,000).

Transformation of *E. coli* ECL8 (*phoA*). Plasmid DNA isolated and purified from *E. coli* LE392 (r_K^- m_K^+)(pMP005) was used to transform *E. coli* ECL8 (*phoA*), a strain that lacks alkaline phosphatase. *E. coli* strains LE392(pMP005) and ECL8(pMP005) both hydrolyzed BCIP when plated on LB agar plates, whereas *E. coli* strains LE392 and ECL8 gave rise to white colonies. Hydrolysis of BCIP by strains of *E. coli* transformed with pMP005 persisted when growth media were supplemented with 10 to 100 mM NaH_2PO_4-Na_2HPO_4.

Substrate specificity. Hydrolysis of BCIP by *E. coli* HB101(pMP005) was unaffected when indole was included in assay buffers at concentrations up to 10 times the BCIP substrate concentration (40 to 200 μM, final concentration of indole). No color reaction was detected when 5-bromo-4-chloro-3-indolyl acetate was used in place of BCIP in standard assays with whole cells or cell extracts of *E. coli* HB101(pMP005).

DISCUSSION

We have isolated a gene (*iph*) that encodes an indole phosphate hydrolase from *N. commune* UTEX 584 as an early step in a long-term study of phosphatase activities of cyanobacteria. Southern analysis indicated that a single copy of *iph* is present within the genome of *N. commune* UTEX 584. In addition to the *iph* coding sequence, the 2.9-kb *Eco*RI fragment of *N. commune* UTEX 584 DNA carries regulatory sequences that permit the expression of the *iph* gene both in *E. coli* and in a cell-free coupled transcription-translation system derived from *E. coli*. Concerning the latter, the ability to synthesize active enzyme from only the purified 2.9-kb fragment suggests the presence of both an *iph* promoter sequence in the DNA and a ribosome-binding site on the *iph* transcript. However, the possibility that a fortuitous *E. coli*-like promoter was used cannot be discounted at this time.

In vitro transcription-translation analyses demonstrated that two polypeptides were synthesized from the 2.9-kb fragment when it was incorporated in different DNA templates. The larger of the two polypeptides (M_r, 74,000) was synthesized with each of the different DNA templates, including those predigested with *Eco*RI. Synthesis of the smaller polypeptide (M_r, 38,000) occurred only in reactions with pBH6 DNA, but synthesis was prevented when pBH6 DNA was digested with *Eco*RI before the transcription-translation reaction. Synthesis of the smaller polypeptide was not observed when pB8 DNA (2.9-kb fragment in reverse orientation with respect to *lac* promoter) was used under identical conditions. Furthermore, deletions of up to 1 kb at the end of the insert distal to the *lac* promoter (in pB8) did not prevent synthesis of indole phosphate hydrolase. In these respects, indole phosphate hydrolase activity cannot be attributed to the smaller polypeptide, which is most likely a LacZ fusion product. Synthesis of a fusion protein of this size would be expected if, as indicated from deletion analyses, *iph* regulatory sequences (and the 5′ end of the coding sequence) are located approximately 1 kb downstream of the *lac* promoter (in pBH6). Autoradiographic analysis showed the smaller (fusion) protein to have a stronger signal than the larger polypeptide (Fig. 3, lane 10). It is uncertain whether this represents increased synthesis of the smaller protein or its greater enrichment in [^{35}S]methionine residues. Increased synthesis of the smaller protein would result from a more

efficient use of the ribosome-binding site for *lacZ*, as opposed to that for *iph*, in the *E. coli*-derived transcription-translation system. Although the present data suggest that the protein with an approximate M_r of 74,000 is the gene product of *iph*, final confirmation must await DNA sequence analysis and purification of Iph.

Indole phosphate hydrolase activity in whole cells of *N. commune* UTEX 584 was regulated by the availability of phosphate. However, P_i was unable by itself to repress the indole phosphate hydrolase activity either in whole cells or in cell extracts of *E. coli* (Iph^+). This was not unexpected, since phosphatases are known to be subject to complex regulation. The alkaline phosphatase activity of the cyanobacterium *Anabaena cylindrica* was increased sevenfold after growth of the cells in phosphate-free medium, whereas *Coccochloris peniocytis* appeared to be constitutive for this activity as no induction was observed upon starving the cells for phosphate (6). Regulation of alkaline phophatase in *E. coli*, for which there is the best understanding (24–27), is specifically induced by phosphate starvation, as are other alkaline phosphatases (30), but there is evidence for multiple positive regulators (28), and the scope of negative control by *phoR* is not resolved completely (25, 27). The expression of *E. coli* periplasmic acid phosphatase (pH optimum of 2.5) is also subject to complex regulation (2, 4). The activity of this enzyme is influenced by phase of growth, presence or absence of oxygen, the concentration of P_i in the medium, and the level in the cells of cyclic AMP as well as its receptor protein (2). Evidence for multiple regulatory elements for the acid phosphatase was suggested after observing that in standard assays with PNPP, P_i was not inhibitory (K_i, 13 mM) (4).

Hydrolysis of PNPP by the indole phosphate hydrolase in the *E. coli* transformants was obscured by a much higher background level of PNPP hydrolysis due to host periplasmic phosphatases (29). The high PNPP background hydrolysis was observed for all the strains tested, the apparent pH optima were between 5.0 to 6.0, and the activity persisted in the presence of phosphate. It appears that these host acid phosphatases have less affinity for BCIP than does the cyanobacterial indole phosphate hydrolase (or the host alkaline phosphatase). This explains how it was possible to detect the single blue positive plaque of λgt10P1 on the lawn of *E. coli* C600 (*hfl*).

The indole phosphate hydrolase does not cleave 5-bromo-4-chloro-3-indolyl acetate, suggesting that activity is not due to an esterase, and the hydrolysis of BCIP was unaffected by excess indole. The latter result confirms that BCIP hydrolysis is not an artifact of some recognition of the cloned gene product for the indole ring system of BCIP.

The cellular localization of phosphatases, even those with rather similar properties, varies with the organism. For the prokaryotes *E. coli* and *Micrococcus sodonensis* the alkaline phosphatases are located in the periplasm and extracellularly, respectively, whereas for the yeast *Neurospora crassa* the enzyme is intracellular (30). The phytoflagellate *Ochromonas danica* secretes an acid phosphatase that shows properties different from those of the intracellular enzyme (15). Different intra- and extracellular acid phosphatases have also been described for fungi (19). Phosphatase activity in *A. cylindrica* was found to be associated with the cell wall (6), in contrast to *C. peniocytis*, where phosphatase was secreted. Indole phosphate hydrolase activity in *E. coli* transformants was found exclusively external to the cytoplasmic membrane and was associated predominantly with the periplasm. This suggests that the *iph* gene product may carry sequences that permit the translocation of the protein across the cytoplasmic membrane of *E. coli* (7, 14, 17).

In summary, we have cloned a nitrogen-fixing cyanobacterium *N. commune* UTEX 584 phosphatase gene which can be expressed, possibly from its own promoter, in *E. coli*. The gene product is found predominantly in the periplasm, where it is active, and the expression of *iph* both in whole cells of *E. coli* and in cell-free extracts can be visualized readily by hydrolysis of BCIP.

The *iph* gene is useful for the study of phosphatase regulation in cyanobacteria and also may prove useful for the study of cyanobacterial promoter function and the processing of cyanobacterial membrane proteins, areas of study for which the data are limited (3, 13, 20).

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A Protein-tyrosine/serine Phosphatase Encoded by the Genome of the Cyanobacterium *Nostoc commune* UTEX 584*

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Protein-tyrosine phosphorylation has long been regarded as an exclusively eukaryotic phenomenon. Although some non-eukaryotes, mainly viruses, possess genes encoding protein-tyrosine kinases or protein-tyrosine phosphatases, these were probably appropriated from the eukaryotic hosts that constitute the sites of action of these enzymes. Herein we identify a gene, *iphP*, from the chromosome of the cyanobacterium *Nostoc commune* UTEX 584 that contains the His-Cys-Xaa-Ala-Gly-Xaa-Xaa-Arg sequence characteristic of known protein-tyrosine phosphatases. The expressed gene product, IphP, displayed protein-tyrosine phosphatase activity toward phosphotyrosine residues on reduced, carboxyamidomethylated, and maleylated lysozyme with optimum activity at pH 5.0. In addition, IphP dephosphorylated the phosphoseryl groups on casein that had been phosphorylated by the cAMP-dependent protein kinase. Cell lysates of *N. commune* probed with antibodies to phosphotyrosine indicated the presence of a tyrosine-phosphorylated protein of M_r ≈85 kDa. This tyrosine-phosphorylated protein was detected in cells grown in the presence of combined nitrogen but not in nitrogen-deficient media that induces the formation of differentiated N_2-fixing cells (heterocysts). Together, these data suggest a role for protein-tyrosine phosphorylation in regulating cellular functions in this cyanobacterium. IphP is the first protein-tyrosine phosphatase to be discovered that is encoded by the chromosomal DNA of any prokaryote. Given the free-living nature of *N. commune* and the phylogenetic antiquity of the cyanobacteria, these findings suggest for the first time the existence of a protein-tyrosine phosphatase of genuine, unambiguous prokaryotic ancestry, thus raising fundamental questions as to the origin and role of tyrosine phosphorylation.

Many key events during the growth and function of eukaryotic cells are controlled and coordinated through the reversible phosphorylation of proteins on tyrosine (1). An important component of protein-tyrosine phosphorylation networks is the growing family of receptor-like and cytosolic protein-tyrosine phosphatases (PTPases),[1] which have been implicated as important modulators of a number of fundamental cellular processes (2). Dysfunctions in this network of tyrosine phosphorylation can have deleterious effects on the cell. Aberrant tyrosine phosphorylation induced by oncogenic protein-tyrosine kinases can lead to transformation, while an increasing number of pathogens, such as vaccinia virus, have been found to carry PTPases (3, 4). Despite reports suggesting the existence of phosphotyrosine in prokaryotes (Ref. 5 and references therein), the phosphorylation of proteins on tyrosine and the possession of protein-tyrosine kinases and PTPases is still generally regarded as exclusive to eukaryotic cells. Although certain viral and pathogenic bacteria possess genes for protein-tyrosine kinases or PTPases, it is highly probable that these genes were originally acquired from eukaryotic host organisms and subsequently adapted for the pathogens' use. In this regard it is noteworthy that the targets of these enzymes are proteins in the infected eukaryotic host and not proteins endogenous to the pathogen itself. Thus, these examples fall under the eukaryotic umbrella by virtue of their genetic ancestry and sites of action. In this paper we describe the cloning, expression, and initial characterization of IphP, the first chromosomally encoded PTPase from a prokaryotic organism, the cyanobacterium *Nostoc commune* strain UTEX 584. To our knowledge, this represents the first identification of a PTPase of genuine, unambiguous prokaryotic ancestry.

EXPERIMENTAL PROCEDURES

Materials—Purchased materials included: restriction endonucleases, T7 and SP6 RNA polymerases, RQ1 RNase-free DNase, RNase (Promega Biotec, Madison, WI); Klenow fragment (Life Technologies, Inc., Gaithersburg, MD); and L-[^{35}S]methionine (Du Pont-New England Nuclear). Cultures of a clonal axenic culture of *N. commune* strain UTEX 584 were grown as previously described (6). All other reagents and materials were from previously listed sources (6–9).

Sequence Analysis of iphP—Plasmid pMP005, containing a 3415-base pair fragment of genomic *N. commune* DNA encoding IphP phosphomonoesterase activity, was constructed, selected, and isolated as described earlier (6). DNA sequencing was performed using deletion clones (8) and the dideoxy chain termination method (10). Homology searches were performed using the GenBank data base. Sequence alignments were optimized using the FASTA program (11).

Expression and Partial Purification of IphP—Escherichia coli strain BL21 DE3 (12) was transformed with plasmid pMP005 and grown in M9 medium, then induced for 3 h by the addition of IPTG (0.5 mM final concentration). Cell-free media were obtained by centrifugation, supplemented with 10 mM EDTA, 2 μg/ml leupeptin, 1 mM benzamidine, and 1 mM dithiothreitol, then concentrated by ultrafiltration using an Amicon concentrator (12-kDa membrane). Concentrated media were passed through a Mono Q HR5/5 column equilibrated in 50 mM Tris·HCl, pH 7.0. The Mono Q flow-through was concentrated

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™ Data Bank with accession number L11392.

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[1] The abbreviations used are: PTPase, protein-tyrosine phosphatase; IPTG, isopropylthiogalactopyranoside; ORF, open reading frame; RCM-lysozyme, reduced, carboxyamidomethylated, and maleylated lysozyme; PAGE, polyacrylamide gel electrophoresis.

using a Centricon 10 centrifugal concentrator, then applied to a Superose 12 HR10/30 column equilibrated in 50 mM Tris·HCl, pH 7.0, containing 50 mM KCl. The column was then eluted with the same buffer, and 1-ml fractions were collected and assayed for *p*-nitrophenylphosphatase or 5-bromo-4-chloro-3-indolyl phosphatase activity. Active fractions were pooled and stored at 4 °C as IphP activity was found to be sensitive to freeze-thawing.

Assay of Protein Phosphatase Activity—[^{32}P]Phosphotyrosyl RCM-lysozyme and [^{32}P]phosphoseryl casein were prepared as described (7, 13). Protein phosphatase assays were carried out in 50 mM acetate buffer, pH 5, supplemented with 1 mg/ml bovine serum albumin, and 2 mM dithiothreitol. The identity and concentration of phosphoprotein substrates used in particular experiments are indicated in the individual figure legends. Reactions were terminated by the addition of 4% (w/v) Norit A charcoal suspended in a solution containing 0.9 M HCl, 90 mM $Na_4P_2O_7$, and 2 mM NaH_2PO_4, followed by vigorous mixing. The charcoal was sedimented by centrifugation and a portion of the supernatant liquid counted for released [^{32}P]phosphate.

Amino Acid Sequence Analyses—Following SDS-PAGE, proteins were transferred to an Immobilon P membrane as described (14), and sections of the membrane containing individual proteins were subjected to Edman degradation using an Applied Biosystems model 477A gas phase sequenator.

In Vitro Translation and Transcription-Translation—Linear DNA templates were generated from plasmid pMP005 by cleavage with *Aat*II or *Mlu*I restriction endonucleases. The linearized plasmid was deproteinized by phenol/chloroform extraction and recovered by ethanol precipitation. A portion, 5 μg, of each linearized DNA was used to prepare an RNA template for *in vitro* translation (15). Coupled transcription-translation was performed using an S30 system using supercoiled pMP005 as template.

RESULTS

Previous studies revealed that cells of *N. commune* strain UTEX 584 secreted a phosphomonoesterase activity, designated IphP, that hydrolyzed *p*-nitrophenyl phosphate or 5-bromo-4-chloro-3-indolyl phosphate (16). The gene for this enzyme resided on a 3415-base pair *Eco*RI fragment of genomic *N. commune* DNA and was identified by screening a λgt 10 library in *E. coli* for expressed 5-bromo-4-chloro-3-indolyl phosphatase activity (6).

Sequencing of the DNA fragment encoding IphP phosphomonoesterase activity revealed the presence of three open reading frames, ORFs 1, 2, and 3, potentially encoding polypeptide products of 294, 110, and 159 amino acids, respectively (Fig. 1*A*). Plasmids in which either ORF 2 or ORF 3 had been deleted still conferred 5-bromo-4-chloro-3-indolyl phosphatase activity upon *E. coli* transformants, while those from which all or part of ORF 1 had been deleted did not. To confirm that ORF 1 did indeed code for IphP, the DNA fragment containing ORF 1 was transcribed from either direction under the direction of unique promoters. When these mRNAs were translated *in vitro* and subsequently analyzed by SDS-PAGE, only a single polypeptide was detected whose apparent molecular mass, ≈30 kDa, corresponded to the expected size of the ORF 1 gene product (Fig. 1*B*, *lanes 1* and *2*). Appearance of this 30-kDa polypeptide was accompanied by a marked increase in the *p*-nitrophenylphosphatase and 5-bromo-4-chloro-3-indolyl phosphatase activity of the *in vitro* translation mixture. These data identified ORF1 as the gene for IphP.

The DNA-derived amino acid sequence of IphP (Fig. 2) showed no obvious homologies with alkaline, acid, or serine/threonine-specific protein phosphatases of prokaryotic origin. However, a striking correspondence was noted between the amino acids surrounding Cys^{182} in IphP and the conserved His-Cys-Xaa-Ala-Gly-Xaa-Xaa-Arg sequence present in the active site of eukaryotic PTPases (2) such as the human placental PTPase 1B (17) or the VH1 gene product of vaccinia virus that has both protein-tyrosine and protein-serine phosphatase activity (3) (Fig. 3). We therefore investigated whether IphP displayed protein-phosphatase activity.

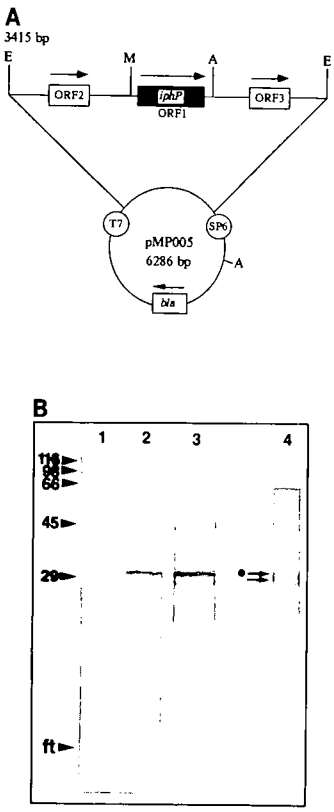

FIG. 1. **The *iphP* gene cluster of *N. commune* UTEX 584 and expression of the *iphP* gene product.** *A*, schematic diagram of plasmid pMP005. Shown are the positions of ORFs 1, 2, and 3; restriction sites for *Eco*RI (*E*), *Mlu*I (*M*), and *Aat*II (*A*); and the promoter sites for RNA polymerases T7 (*T7*) and SP6 (*SP6*). The direction of transcription of ORFs 1–3 and the β-lactamase gene (*bla*) of the pGEM-4 vector are indicated by *arrows*. *bp*, base pairs. *B*, SDS-PAGE of *iphP* gene product. Shown are an autoradiogram of an SDS-polyacrylamide gel of [^{35}S]methionine-labeled proteins (*lanes 1–3*) and a second, Coomassie-stained gel that was run under identical conditions (*lane 4*). The positions of molecular weight standards are indicated at the *far left*. *Lane 1* shows the radiolabeled products of *in vitro* translation of the mRNA obtained when plasmid pMP005 was linearized with restriction endonuclease *Mlu*I, transcribed using SP6 RNA polymerase, and translated using an *E. coli* S30 system (Promega Biotec). *Lane 2* shows an *in vitro* translation performed under identical conditions using the mRNA obtained when plasmid pMP005 was digested with restriction endonuclease *Aat*II and then transcribed using RNA polymerase T7. *Lane 3* shows the radiolabeled products obtained when supercoiled plasmid pMP005 was subjected to *in vitro* coupled transcription/translation with an *E. coli* S30 system. *Lane 4* shows the Coomassie Blue-stained gel of the partially purified IphP phosphatase.

Transformation of *E. coli* strain BL21 DE3 with a plasmid carrying the *iphP* gene, pMP005 (Fig. 1*A*), led to the appearance of high levels of *p*-nitrophenylphosphatase activity (40 nmol of *p*-nitrophenyl phosphate hydrolyzed ml^{-1} min^{-1}) in the extracellular medium when expression of plasmid-encoded genes was induced using IPTG. The cell-free media were then tested for PTPase activity using [^{32}P]phosphotyrosyl RCM-lysozyme as substrate. Dephosphorylation of RCM-lysozyme was observed with a pH optimum of 5. Little or no PTPase activity was found in media from cells transformed with vector alone under similar conditions of IPTG induction, indicating that the activity was encoded by DNA specific to plasmid pMP005. Elevated PTPase and *p*-nitrophenylphosphatase activities were accompanied by the appearance of two polypeptides of 29 and 30 kDa, respectively, as determined by SDS-PAGE, which were of similar mobility to the polypeptides produced when plasmid pMP005 was used as template for coupled *in vitro* transcription-translation reactions (Fig. 1*B*, *lane 3*). Only the 29-kDa polypeptide could be detected in the

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1143 GGCCGTCAACTTCTGATTCGACTGGAGAACCCCATGAAGACCCACCACGCCAACCTTGCC
   1                                 MetLysThrHisHisAlaAsnLeuAla

     CTGGCCCTGATGCTGGGCCTGTCGTCGTCCGCAACCGCGGTTGCCGCCGACGCACCGCAG
  10 LeuAlaLeuMetLeuGlyLeuSerSerSerAlaThrAlaValAlaAlaAspAlaProGln
                                                  ▲
     GCCGTTGCCACCAAGGCGGCCGCGCCGAATGTGAAGCCCGTCGCTGCCGATGCGCACGGG
  30 AlaValAlaThrLysAlaAlaAlaProAsnValLysProValAlaAlaAspAlaHisGly

     GTGATCCCCGATGGGGCGCCTGGAATGTGCGCACGTTCGCCGGCCTGCAGGGCCACGGCG
  50 ValIleProAspGlyAlaProGlyMetCysAlaArgSerProAlaCysArgAlaThrAla

     ATTCCGGCCGATGCGTTCGTACGTACCGCCGACCTCGGCCGCCTGACCGATGCCGACCGT
  70 IleProAlaAspAlaPheValArgThrAlaAspLeuGlyArgLeuThrAspAlaAspArg

     GATGCCCTGGCCGCGTTGGGCGTGAAGCTGGACATCGACCTGCGCACCGCCGATGAGGAA
  90 AspAlaLeuAlaAlaLeuGlyValLysLeuAspIleAspLeuArgThrAlaAspGluGlu

     GCGCAGTCGCCGGACCTGCTGGCCAGGGACGACCGCTTCGACTACCAGCGCATTTCGCTG
 110 AlaGlnSerProAspLeuLeuAlaArgAspAspArgPheAspTyrGlnArgIleSerLeu

     ATGGGCACCGAGAAGATGGACCTGCAGAAGATGATGACCAGCTTCCCGGATTCGCTGGGC
 130 MetGlyThrGluLysMetAspLeuGlnLysMetMetThrSerPheProAspSerLeuGly

     GAAGCCTATGTGCAGTGGTTGGGCCACAGCCAGCCGCAGTTCAAGCAGGTGTTCCAGCGC
 150 GluAlaTyrValGlnTrpLeuGlyHisSerGlnProGlnPheLysGlnValPheGlnArg

     ATTGCCGCGCAGCAGGACGGTGCGGTGCTGTTCCATTGCACGGCCGGCAAGGACCGCACC
 170 IleAlaAlaGlnGlnAspGlyAlaValLeuPheHisCysThrAlaGlyLysAspArgThr
                                             *
     GGCATCATCGCCGGCCTGCTGCTGGACCTGGCCGGCGTGCCGAAGGCCGAGATCGTGCAC
 190 GlyIleIleAlaGlyLeuLeuLeuAspLeuAlaGlyValProLysAlaGluIleValHis

     AACTACGCGATCTCCGCGCACTACCTGGAAGGCCAGCCGAAGGACAGCGATGAACGCGCA
 210 AsnTyrAlaIleSerAlaHisTyrLeuGluGlyGlnProLysAspSerAspGluArgAla

     GATCATGGCGCTGGTCAGGCAGAACCCGGAGATCGGCCGCAGGATGGCGGGCATGGCCGG
 230 AspHisGlyAlaGlyGlnAlaGluProGlyAspArgProGlnAspGlyGlyHisGlyArg

     TACCGCGCCGGACAACATGGAGCAGTTCTGGCGGCCTTGCACAGCCAGTACGGTGGCGCG
 250 TyrArgAlaGlyGlnHisGlyAlaValLeuAlaAlaLeuHisSerGlnTyrGlyGlyAla

     GAGGGGTACCTGAAGTCGATCGGGGTCAGCGAACAGGAGATCCAGCAGTTGAAGGTGCGG
 270 GluGlyTyrLeuLysSerIleGlyValSerGluGlnGluIleGlnGlnLeuLysValArg

     CTGGGGCAGGCCGGCTGA
 290 LeuGlyGlnAlaGly -
```

FIG. 2. **Nucleotide and predicted amino acid sequence of IphP.** Shown is the DNA sequence of ORF 1 with the predicted amino acid sequence of the IphP protein, 294 amino acids in length, immediately below it. A potential ribosome-binding sequence preceding the first ATG is both *over-* and *underlined*. The first 24 amino acids comprise a potential N-terminal signal sequence (score 12.5), as identified using the PSIGNAL program of the program PCGENE (Intelligenetics). The predicted site of proteolytic cleavage of the putative signal sequence, as determined by the −3, −1 rule of Von Heinje (19), is indicated by an *arrow*. *Boldface underlining* indicates the segment corresponding to the conserved PTPase active site sequence. Cys^{182} is marked by an *asterisk*.

IphP	Q	D	G	A	V	L	F	H	C	T	A	G	K	D	R	T	G	I	I
VH1	N	E	·	P	V	L	V	H	C	A	A	G	V	M	R	S	G	A	M
1B	E	H	G	P	V	V	V	H	C	S	A	G	I	G	R	S	G	T	F
LAR	Q	D	G	P	M	V	V	H	C	S	A	G	V	G	R	S	G	V	F
CD45	F	S	G	P	I	V	V	H	C	S	A	G	V	G	R	T	G	T	Y
YopH	K	L	R	P	-	V	I	H	C	R	A	G	V	G	R	T	A	Q	·

FIG. 3. **Comparison of the amino acid sequence surrounding Cys^{182} of IphP with sequences surrounding the active site cysteines of known PTPases.** Shown is the amino acid sequence surrounding Cys^{182} in IphP aligned with those around the active site cysteines of the H1 gene product of vaccinia virus (*VH1*, Cys^{110} (3)), human placental PTPase 1B (*1B*, Cys^{215} (17)), cytoplasmic domain 1 of *Drosophila melanogaster* LAR (*LAR* Cys^{1861} (25)), cytoplasmic domain 1 of leukocyte common antigen (*CD45*, Cys^{817} (26)), and *Y. pseudotuberculosis* YopH (*YopH*, Cys^{403} (4)). Identities between IphP and these other PTPases are indicated by *boxes*.

extracellular media of cultures tranformed with vector alone. When the source of the phosphatase activity was partially purified by ion-exchange and gel filtration chromatography using *p*-nitrophenylphosphatase or 5-bromo-4-chloro-3-indolyl phosphatase activity as a marker, the active fractions were found to be enriched both for these polypeptides (Fig. 1*B*, *lane 4*) and for PTPase activity. N-terminal amino acid sequence analysis revealed that the smaller polypeptide was β-lactamase (18), a product of the vector, pGEM-4. The N-terminal sequence of the 30-kDa polypeptide was identical to residues 25–37 of the predicted amino acid sequence of the *iphP* gene product. As the sequence of the first 24 amino acids of IphP (Fig. 2) constitutes a recognizable prokaryotic signal sequence as defined by the criteria of Von Heinje (19), these data suggest that IphP was proteolytically processed during its secretion from transformed *E. coli.*

The partially purified IphP protein readily dephosphorylated RCM-lysozyme (Fig. 4). While it exhibited no detectable phosphatase activity toward phosphoseryl phosphorylase *a*, it dephosphorylated casein that had been phosphorylated on seryl residues by cAMP-dependent protein kinase (Fig. 4*A*). The activity of IphP toward both RCM-lysozyme and casein exhibited a pH optimum of 5 and was inhibited by sodium orthovanadate or the sulfhydryl-modifying reagent *N*-ethylmaleimide, both of which are known to inhibit PTPases of eukaryotic origin. In this regard, IphP most closely resembles another dual specificity protein phosphatase, VH1 (3), since both of these enzymes utilize a similar mechanism involving an active site cysteine (20) to dephosphorylate both phosphotyrosyl and phosphoseryl substrates. Okadaic acid or the cyanobacterial toxin microcystin-LR, potent inhibitors of protein-serine/threonine phosphatases 1 and 2A, did not inhibit IphP (Fig. 4*B*). IphP was also insensitive to tetramisole, an inhibitor of alkaline phosphatases, or tartrate, an acid phosphatase inhibitor (Fig. 4*B*), further indicating that the enzyme was encoded by *iphP* and was not due to a contaminating *E. coli* alkaline or acid phosphatase activity. Active IphP eluted from a Superose 12 gel filtration column with an apparent molecular mass of ≈30 kDa, as compared with protein standards, indicating that the protein existed as a monomer.

We next examined whether *N. commune* strain UTEX 584 contained endogenous tyrosine-phosphorylated proteins that might be the targets for the protein-tyrosine phosphatase activity of IphP. As shown in Fig. 5, Western blots of total cellular proteins from *N. commune* probed with antibodies to phosphotyrosine revealed the presence of a prominent band

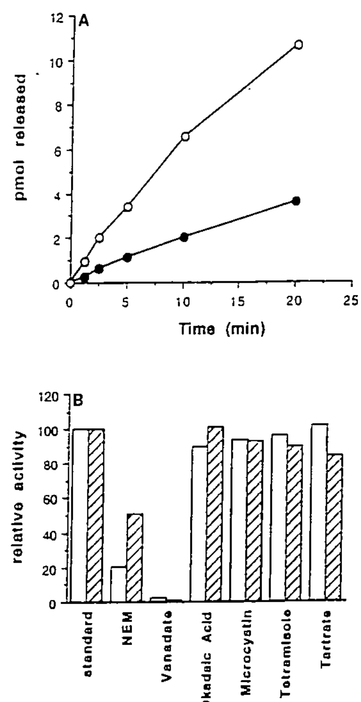

FIG. 4. **Characterization of IphP PTPase activity.** *A*, time course. Partially purified IphP was assayed for protein-phosphatase activity using phosphotyrosyl RCM-lysozyme (○) or phosphoseryl casein (●) (1 μM phosphoprotein each) as substrate. Shown is the release of $[^{32}P]$phosphate as a function of time. *B*, effect of various agents on IphP PTPase activity. Protein-phosphatase activity was measured with phosphotyrosyl RCM-lysozyme (*open bars*) or phosphoseryl casein (*hatched bars*) in the presence of 10 mM *N*-ethylmaleimide (*NEM*), 1 mM sodium orthovanadate, 1 μM okadaic acid, 0.1 μM microcystin-LR, 1 mM tetramisole, or 5 mM sodium tartrate. Activity is reported as the percentage of that observed in the absence of added agents (*standard*).

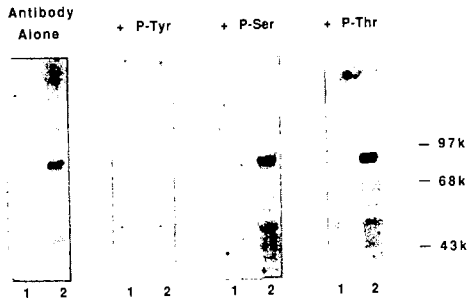

FIG. 5. **Western blot analysis with antiphosphotyrosine antibodies.** Total cell lysate from *N. commune* UTEX 584 cells grown in $BG11_0$ medium (*lane 1*) or in BG11 medium (*lane 2*) was analyzed by SDS-PAGE and transferred to nitrocellulose filters as previously described (9). Anti-phosphotyrosine monoclonal antibody 4G10 (UBI, Lake Placid, NY) was then applied to the filters either directly (*Antibody Alone*) or following pretreatment with and subsequent incubation in the presence of 1 mM phosphotyrosine (+ *P-Tyr*), phosphoserine (+ *P-Ser*), or phosphothreonine (+ *P-Thr*). The positions of molecular weight standards are shown at *far right*.

with an apparent molecular mass of ≈85 kDa. The immune reaction producing this signal could only be depleted by competition with exogenous phosphotyrosine (Fig. 5, + *P-Tyr*). Phosphoserine or phosphothreonine were completely ineffective, indicating that the 85-kDa protein did indeed contain phosphotyrosine. The appearance of this band was observed to be developmentally regulated. The band was very prominent in cells grown in media enriched for combined nitrogen, *i.e.* $NaNO_3$ (Fig. 5, *Antibody Alone, lane 2*). However in nitrogen-deficient media, under which conditions the cyanobacterium is induced to differentiate nitrogen-fixing heterocysts, no trace of a corresponding immunoreactive band could be detected (Fig. 5, *Antibody Alone, lane 1*). At this juncture it is impossible to determine whether this reflects the complete absence of the protein itself or a dramatic decrease in its phosphotyrosine content.

DISCUSSION

We have identified a chromosomally encoded gene, *iphP*, from the cyanobacterium *N. commune* that encodes an enzyme with protein-tyrosine/serine phosphatase activity. This protein phosphatase, IphP, represents the first example of a PTPase of genuine, unambiguous prokaryotic origin. Although the YopH protein of the pathogenic bacterium *Yersenia pseudotuberculosis* is also a PTPase (4), it appears doubtful that its ultimate ancestor was prokaryotic in nature. Unlike IphP, which is chromosomally encoded, YopH is encoded by an extrachromosomal element, a megaplasmid (4). Moreover, the YopH PTPase targets tyrosine-phosphorylated proteins in the cells of the infected eukaryotic host (21). In fact, a mutation abolishing the protein phosphatase activity of YopH had no discernible effect on the ability of *Y. pseudotuberculosis* to grow in culture (21). Taken together, these findings strongly suggest that this virulence determinant arose through lateral gene transfer from a eukaryotic host in a manner analogous to that which gave rise to the retroviral protein-tyrosine kinases, as was first suggested upon its discovery (4). By contrast, IphP is encoded by genomic DNA. Furthermore, *N. commune* is a free-living, obligate photoautotroph. It does not exist in association with eukaryotic organisms in nature, as is the case with *Y. pseudotuberculosis*. Moreover, *N. commune* contains at least one endogenous tyrosine-phosphorylated protein, suggesting that protein-tyrosine phosphorylation, and thus, potentially, the PTPase activity of IphP, does play a role within the confines of the cyanobacterial cell. Thus, the weight of evidence strongly indicates that the ultimate origins of IphP were prokaryotic in nature.

The discovery of the IphP PTPase in this cyanobacterium raises fundamental questions as to the origins and functions of tyrosine phosphorylation in both prokaryotes and eukaryotes. *N. commune* is a representative of an ancient assemblage of prokaryotes whose fossil record spans some 3.5 billion years (22). This obligate photoautotroph represents an ubiquitous, and often conspicuous, component of terrestrial N_2-fixing microbial communities. Although it is possible that the emergence of tyrosine phosphorylation in this organism was a late evolutionary event, it seems at least equally likely that tyrosine phosphorylation may have arisen at a much earlier point in evolutionary time than previously suspected, *i.e.* before the emergence of the eukaryotes as a distinct group. As to the function tyrosine phosphorylation may serve in this organism, the observed dependence of the appearance of the 85-kDa tyrosine-phosphorylated protein on immunoblots upon the source of nitrogen in the media, although as yet unexplained, is striking. In addition, computer analysis has revealed that the sequences of the open reading frames flanking the *iphP* gene in *N. commune*, ORF 2 and ORF 3, possess significant homology to portions of the FhuA and FhuB proteins of *E. coli* (23, 24). FhuA and FhuB comprise the outer membrane receptor and inner (cytoplasmic) membrane transporter, respectively, for ferrichrome-mediated high affinity iron transport in *E. coli*. Since prokaryotes often group genes of related function in spatially contiguous operons, it is tempting to speculate that the IphP PTPase may be involved in some aspect of sequestration and transport of iron by *N. commune* UTEX 584.

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PROTEIN PHOSPHATASES IN PROKARYOTES: REFLECTIONS OF THE PAST. WINDOWS TO THE FUTURE?

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INTRODUCTION

This series now spans eight full volumes, yet this chapter represents one of less than a handful devoted to the protein phosphatases of prokaryotic organisms, i.e. to the *Bacteria* and *Archaea*. The question that naturally springs to mind upon confronting such a statement is: Why talk about them now?" The answer is that in recent years we have witnessed several unexpected developments in this area. These observations challenge long-standing perceptions concerning the origins and role of protein phosphorylation-dephosphorylation, and protein phosphatases, not only in prokaryotic organisms, but in their eukaryotic brethren as well. Thus, the leitmotif of this essay will be one of evolution, both the Darwinian process that has molded the protein phosphorylation networks themselves and the intellectual one that has shaped our understanding of their form and function.

IN THE BEGINNING THERE WERE MAMMALS

In the beginning there were mammals. Our first glimpses of the molecular labyrinths of protein kinases, protein phosphatases, and phosphoproteins that comprise the protein phosphorylation networks of living cells were the offspring of efforts to dissect the molecular mechanisms by which neural and hormonal inputs impinging upon the exterior of cells influenced the rate and direction of the metabolic processes going on within them (Reviewed in 1). The experimental systems of choice for these seminal explorations were the livers, muscles, etc., of rats, rabbits, and other mammals.

The initial context in which protein phosphorylation events were viewed

has had a profound and lasting impact. The first protein phosphorylation-dephosphorylation cascades appeared in the guise of intracellular extensions of the neuroendocrine system. Each hormone or nerve impulse impacting the exterior surface of a target cell triggered a series of molecular events in the interior, a signal transduction cascade, in which protein kinases and protein phosphatases were prominently featured. The activation of these cascades ultimately resulted in the alteration of the phosphorylation state and, as a consequence, functional properties of intracellular enzymes catalyzing key, rate-limiting steps in metabolic and other processes. This elegant functional partnership, along with the perceived parallels between the anatomy of the neuroendocrine system and the molecular architecture of these signal transduction cascades (for example, the manner in which second messengers such as cAMP and Ca^{2+} functioned as cytoplasmic hormone surrogates), suggested that the two systems were inextricably linked in function and ancestry. Mutual complimentarity provided a compelling argument that the modification of protein function by phosphorylation-dephosphorylation had evolved in response to the special needs of organisms comprised of multiple, differentiated cells.

If protein phosphorylation networks were the progeny of the neuroendocrine system that provided their raison d'etre, it naturally followed that protein phosphorylation networks did not exist in prokaryotes. This did not seem particularly surprising. Simple, unicellular organisms had no apparent need for such a complex and subtle regulatory mechanism. Perhaps a few bacterial proteins contained covalently-bound phosphate for structural purposes or as a form of nutrient storage. However, it was considered extremely unlikely that phosphorylation events of any regulatory consequence took place in these organisms.

The first observations concerning protein phosphorylation in prokaryotes seemed to confirm the common sense view. A few scattered reports appeared during the late 1960's and early 1970's concerning the possible existence of protein kinase activity in bacteria, but their impact quickly dissipated from a lack of either detailed follow-up studies or confirmatory evidence from other laboratories (Reviewed in 2). Not until the waning years of the 1970's would convincing evidence for the covalent modification of prokaryotic proteins by phosphorylation appear (3-5). Yet, as the first details of the protein phosphorylation networks of prokaryotic organisms came to light, their appearance was distinctly alien. The protein phosphorylation events carried on by the sugar phosphotransferase (Reviewed in 6) and two-component regulatory systems (Reviewed in 7) in *Bacteria* involved phosphohistidine and phosphoapartate, rather than the

familiar phosphoserine, phosphothreonine, and phosphotyrosine encountered in the eukaryotic world. In *Salmonella typhimurium*, isocitrate dehydrogenase was phosphorylated by a protein kinase whose polypeptide chain was contiguous with that of the protein phosphatase responsible for the phosphoprotein's subsequent dephosphorylation (8), a hermaphrodite without precedent among the *Eucarya*. Ironically, the first evidence indicating that prokaryotes and eukaryotes shared this elegant mechanism for regulating protein function only served to widen the perceived gulf between them, since their protein phosphorylation networks appeared to be mutually exclusive entities born at different times and in different places.

THE PROKARYOTIC WORLD. A BRIEF OVERVIEW OF PHYLOGENY

The definition of the term prokaryote is essentially negative in nature. Quite literally, a prokaryote is any cellular organism that is not a eukaryote. The distinguishing feature by which the eukaryotes were originally defined was the possession of an internal, nuclear membrane that segregated their genomic material away from the bulk of the cytoplasm (9). Those organisms lacking a compartmented nucleus, the leftovers so to speak, were grouped together as the prokaryotes (9). For many years it was generally accepted that this morphologically-based taxonomy reflected genetic ancestry, that the living world was bipartite in nature, consisting of eukaryotic and prokaryotic phylogenetic domains that had diverged from one another eons ago. Today, thanks to the ability to isolate and sequence genes at a prodigious rate, phylogeny can be analyzed from a truly genetic perspective (10). Somewhat surprisingly, these examinations have revealed that the living world is tripartite in nature (11). The eukaryotes first identified on the basis of their cellular morphology have proved to comprise a single coherent phylogenetic domain — the *Eucarya*. However, the same cannot be said of the prokaryotes. Rather, the organisms grouped under the prokaryote umbrella are members of two different, and quite distinct, phylogenetic domains — the *Bacteria* and the *Archaea*, sometimes referred to as the *Eubacteria* and *Archaebacteria*, respectively (Reviewed in 10, 12).

The *Bacteria* include those types of the organisms traditionally encountered in a typical survey course in microbiology: *Escherichia coli*, *Pseudomonas aeruginosa*, *Paracoccus denitrificans*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Haemophilus influenzae*, etc. At first glance, the *Archaea* appeared to be specialized *Bacteria* adapted to life in

extreme environments, such as those characterized by high temperatures, extreme salinity, zero oxygen tension, acidic pH, or some combination thereof. However, such superficial comparisons have proved quite deceptive. *A priori*, one might predict that, since the *Bacteria* and *Archaea* share many common morphological features, they would reside on one "branch" of a rooted phylogenetic tree, i.e. a tree that assumes the existence of a single common ancestor, with the *Eucarya* alone on the other. In fact, the opposite now appears to be the case (10, 12). The *Archaea* and *Eucarya* sprout from the same branch of the tree while the *Bacteria* stand alone. The vast majority of known archaeal genes and gene products resemble their eukaryotic counterparts much more closely than their bacterial ones. These include proteins as diverse as 3-hydroxy-3-methylglutaryl-coenzyme A reductase (13), DNA-dependent RNA polymerase (14), initiation factor 5A (15), elongation factor 2 (16, 17), F_0F_1ATPase (18), and ribosomal proteins L10, L12, and L39/46 (19, 20). In addition, the *Archaea* use methionyl tRNA, rather than N-formyl-methionyl tRNA, to initiate translation (21); their promoters contain TATA boxes (22-24); and they possess introns within their genome (25, 26) — all "eucaryal" features. The frequency with which such similarities have been encountered has triggered speculation that the *Archaea* number among their ancestors the proto-eukaryote that gave rise to the nuclear/cytoplasmic portions of present day *Eucarya* (27).

PARALLELS AND INTERSECTIONS

Recently, several gaps have opened in the previously impenetrable wall separating the protein phosphorylation networks of eukaryotic and prokaryotic organisms — unanticipated functional parallels and structural intersections between their protein phosphatases and protein kinases. For example, bacteriophage lambda encodes a protein-serine/threonine phosphatase that is homologous to large portions of the catalytic regions of the protein-serine/threonine phosphatases of the type 1/2A/2B superfamily in eukaryotes (28, 29). Another bacteriophage, phi 80, contains an open reading frame potentially encoding a similar enzyme (28). Bis(5`-nucleotidyl)-tetraphosphatase from *Escherichia coli* also contains sequences homologous with both these bacteriophage genes and, to a lesser extent, their eukaryotic counterparts (30). In our own laboratory, we have observed the presence of a protein-serine/threonine phosphatase activity in the methanogenic archaeon *Methanosarcina thermophila* strain TM-1 the activity of which was sensitive to a set of compounds — okadaic acid,

calyculin A, and microcystin-LR — thought to be specific inhibitors of the protein phosphatase 1/2A/2B superfamily in eukaryotes (31).

These intersections are not confined to the protein-serine/threonine phosphatases. The pathogenic bacterium *Yersinia pseudotuberculosis* harbors a protein-tyrosine phosphatase, YopH, that possessed the same sequence of active site amino acids as do the protein-tyrosine and dual-specificity (i.e. tyrosine and serine/threonine) protein phosphatases of eukaryotic organisms (32). In our own laboratory, a dual-specificity protein phosphatase containing this same conserved active site signature was discovered in the cyanobacterium *Nostoc commune* strain UTEX 584 (33). The expression or activity of IphP may be tied to the availability, or lack thereof, of fixed nitrogen in the microbe's environment (33). Intriguingly, this has been observed to be the case for expression of a gene encoding a protein-tyrosine phosphatase in yeast (34).

Genes containing sequences homologous to eukaryotic protein-serine/threonine kinases have also been found in prokaryotes. *Myxococcus xanthus* (35) and *Anabaena* sp. strain PCC 7120 (36) both contain genes whose predicted products are homologous to eukaryotic protein-serine/threonine kinases. The sequence of an open reading frame in the *hisA* region of the genome of the archaeon *Methanococcus vannielii* predicts a protein product with a derived amino acid sequence that was homologous to subdomains VI and VII of eukaryotic protein kinases (37). In our laboratory Southern blots of *M. thermophila* strain TM-1 have revealed the presence of several sequences that hybridize with probes to the conserved subdomain IX sequence from eukaryotic protein kinases. The converse has also proved to be true. Recently, branched-chain alpha-ketoacid dehydrogenase kinase (38) and pyruvate dehydrogenase kinase (39) have been cloned from rat heart. While their predicted sequences possessed little homology with previously-described eukaryotic protein kinases, they bore significant resemblance to the so-called histidine kinases of bacteria. Similarly, genes encoding potential prokaryote-like histidine kinases have also been found in yeast (40) and plants (41).

REFLECTIONS AND INTERCONNECTIONS?

This sudden and unexpected burst of molecular and functional parallels raises some provocative questions. Do these represent random intersections, isolated events that are the products of convergent evolution and/or a few chance exchanges of genetic material? Are they evidence for ancestrally-based interconnections between the protein phosphorylation

networks of the entire phylogenetic spectrum of cellular organisms? In other words, are phosphorylation-based signal transduction cascades a late evolutionary development, as we have often consciously or unconsciously assumed, or do their origins reach back to the epoch of the "universal ancestor", an ancestor whose primitive protein phosphorylation network may have provided a common foundation for those found in present day Eucarya, Bacteria, and Archaea? To what extent do their phosphorylation networks represent distant mirrors of one another, and how can these mirrors be used to bring the form and function of each into sharper focus? Can the "alien" phosphorylation networks of simple prokaryotic organisms be used as tools for uncovering some of the fundamental principles by which their more complex eukaryotic counterparts operate? Can they serve as guides to as yet undiscovered signal transduction networks in higher animals, and *vice-versa*?

Definitive answers to these questions have yet to emerge, but it is tempting to try and glean hints of what the future will reveal. Certainly, the sheer number of encounters with phylogenetically foreign protein kinases and protein phosphatases reported of late indicates that many more cases will be uncovered in future. It seems significant that these exchanges, or invasions, are bidirectional in nature. Presumptive prokaryote-like genes or gene products have been found in eukaryotes, and vice-versa. It is also noteworthy that this phenomenon appears to be quite general, involving prokaryotes and eukaryotes of all morphologies, physiologies, and habitats.

It is clear that the presence of certain phylogenetically foreign protein kinases and protein phosphatases can be readily accounted for by the direct transfer of DNA between members of different phylogenetic domains at some relatively recent period in evolutionary time. The YopH protein-tyrosine phosphatase from *Yersinia pseudotuberculosis*, for example, is encoded by a megaplasmid, not the bacteria's genome (32). Thus, it seems likely that YopH was acquired by *Y. pseudotuberculosis* as a consequence of its close association with eukaryotic organisms (32). This supposition is supported by the observation that the enzyme is not known to act within the bacterium itself. Deletion of YopH does not noticeably affect the viability or growth of *Y. pseudotuberculosis* in culture (42). Rather, YopH is secreted into and targets phosphotyrosyl proteins endogenous to the infected host (42). The existence of virally-encoded protein phosphatases and protein kinases also provides strong circumstantial evidence for the transfer of protein phosphatase genes within, and perhaps even between, phylogenetic kingdoms.

The exchange of DNA between phylogenetically distinct, but intimately associated organisms would appear to account for only a fraction of the

phylogenetically foreign protein kinases and protein phosphatases encountered thus far. The dual-specificity protein phosphatase of the cyanobacterium *Nostoc commune* strain UTEX 584, IphP, possesses the same active site consensus sequence as YopH (33). However, the former appears to be of genuine, unambiguous prokaryotic ancestry. IphP is encoded by the cyanobacterium's genome (33), not a mobile and malleable extrachromosomal genetic element like a plasmid. Also, *Nostoc commune* a free-living organism that does not associate with or depend upon eukaryotes during any portion of its life cycle (Although it should be noted that other strains of *Nostoc* and *Anaboena* enter into a range of associations with higher and lower plants.) (43). In addition, at least one tyrosine phosphorylated protein is present in this cyanobacterium, providing a potential site of action for IphP endogenous to the cyanobacterium itself (33).

Since the homology between IphP and similar phosphatases from eukaryotic organisms was not comprehensive in nature, but rather was confined to the region around the presumed active site cysteine, the possibility that this reflects evolutionary convergence cannot be ruled out. However, this is not the case for those genes homologous to the so-called protein-histidine kinases of the bacterial two component regulatory system that have been cloned from several eukaryotes. Their chromosomal location and widespread distribution among organisms as diverse as yeast (40), plants (41), and mammals (38,39) indicates that these genes were acquired either before the eukaryotes emerged as a distinct group, or shortly thereafter. The latter could have taken place when proto-eukaryotes enveloped the bacterial endsymbionts followed by incorporation into the "hosts" genome. While this model readily accounts for prokaryote to eukaryote transmission, "retrograde" events cannot be accounted for in this manner.

A multiplicity of mechanisms could, and in all likelihood do, account for the presence of eukaryote-specific and prokaryote-specific protein kinases and protein phosphatases in prokaryotes and eukaryotes, respectively. However, it seems clear that the (great)^{nth} grandparents of present day protein kinases and protein phosphatases were proteins of extreme antiquity. At least some of their number were in existence, and perhaps even functioning as protein modifiers/biological regulators, prior to the emergence of the three phylogenetic kingdoms extant today, i.e. during the epoch of the so-called universal ancestor. In retrospect this is not surprising, since the biopolymers found in the first living organisms relied on phosphoester, phosphoramide, and phosphoanhydride bonds for their basic structure, not peptide bonds (44). Given that these proteins emerged

in an environment dominated by phosphate-based polynucleotides and polypeptide-polynucleotide hybrids, the making and breaking of phosphoester and phosphoramide bonds represent natural functions for early polypeptide catalysts.

Regardless of whether these molecular parallels reflect divergence from a common ancestor or subsequent genetic exchanges, their existence suggests that the terms prokaryote or eukaryote-specific, as applied to protein kinases and protein phosphatases, will in many instances prove to be misnomers. Although distinguishable, prokaryotic and eukaryotic protein phosphorylation networks may not be distinct. Rather, all organisms may utilize — in whole or in part — variations upon a set of common molecular themes, the protein phosphorylation networks of the Bacteria, Archaea, and Eucarya representing distant mirrors of one another. To the extent that this is true, each represents a potential tool for understanding fundamental principles operative in the others, each with its own distinct advantages and shortcomings. The "unique" signalling systems discovered in one phylogenetic domain may represent the harbinger of things to come in the others.

ARCHAEAL PROTEIN-SERINE / THREONINE PHOSPHATASES

The suggestion that the *Archaea* and *Eucarya* may represent variations on what began as a common evolutionary theme provided the impetus for our current studies of archaeal protein-serine/threonine phosphatases. The work of Spudich and coworkers (45,46) and that of Skorko (47,48) established that the halophilic archaeon *Halobacterium halobium* and the acidothermophilic archaeon *Sulfolobus acidocaldarius*, respectively, contained phosphorylated proteins and endogenous protein kinase activity. More recently, we have made similar observations with regards to the acidothermophilic archaeon *Sulfolobus solfataricus*. In addition, we observed that soluble extracts from this archaeon contained significant levels of protein phosphatase activity toward phosphoseryl casein that had been prepared using the cAMP-dependent protein kinase (49). This activity proved to be derived from a single, divalent metal ion-stimulated protein phosphatase with a molecular weight of approximately 30 kDa. Analysis of the purified protein by SDS-polyacrylamide gel electrophoresis revealed a subunit molecular weight of approximately 31 kDa, demonstrating that the phosphatase was a monomer.

Challenges with a spectrum of potential substrates indicated that the *S. solfataricus* protein phosphatase is phosphoserine-phosphothreonine

specific, lacking appreciable activity toward phosphotyrosine-containing proteins or peptides. Although it dephosphorylated a wide range of protein and peptide substrates, the archaeal protein phosphatase would dephosphorylate neither histone H1 nor phosphoseryl-containing reduced, carboxyamidomethylated and maleylated lysozyme. Studies with synthetic peptides suggest that the presence of basic amino acids near, more specifically N-terminal to, the targeted phosphoserine or phosphothreonine was a positive determinant for substrate recognition, while the presence of nearby acidic residues had negative effects (Donella, A., Leng, J., Kennelly, P. J., and Pinna, L. A., manuscript in preparation). Mn^{2+}, Ni^{2+}, and Co^{2+} all activated the enzyme with K_{act} values of a few tenths millimolar. Mg^{2+}, by contrast, is only a weak activator at best, and then only at millimolar concentrations. It is unknown at present whether these divalent metal ions act as stimulators of catalytic activity or as indispensable participants in the catalytic process itself. Circumstantial evidence suggests the former, since significant basal activity (generally 2-7% of metal-ion stimulated levels) can be detected in some enzyme preparations. Moreover, the level of basal activity present in a particular preparation appeared to correlate inversely with the care taken to inhibit proteolytic activity during isolation. Protein phosphatase activity was sensitive to diethylpyrocarbonate, a known modifier of histidine (and tyrosine) residues, while a spectrum of sulfhydryl-modifying reagents proved innocuous.

Surveys of cell extracts from *S. solfataricus* with a number of other phosphorylated proteins including mixed histones, phosphorylase a, and phosphorylase kinase consistently revealed the presence of this single, divalent metal ion-stimulated protein phosphatase and no other. No tyrosine phosphatase activity was apparent using poly (glu_4:tyr) as a probe, nor were proteins reactive with antiphosphotyrosine antibodies apparent on Western blots. Incubation of cell extracts with [^{32}P]ATP resulted in the radiolabelling of approximately a dozen proteins. Incubation of renatured SDS-polyacrylamide gels with [^{32}P]ATP also revealed the presence of four self-phosphorylating polypeptides, i.e. potential autophosphorylated protein kinases, all of which contained phosphothreonine and only one of which contained phosphoserine as well. It is tempting to speculate that the observed preponderance of phosphothreonine over phosphoserine, which has also been observed in the major phosphoproteins in *S. acidocaldarius* (47,48), may in some way be related to the extremely high temperature, 65 - 85°C, at which these organisms grow.

The *S. solfataricus* protein phosphatase appears to be one of member of a family of divalent metal ion-stimulated archaeal protein-serine/threonine

phosphatases. Extracts from both a methanogenic archaeon, *Methanosarcina thermophila* strain TM-1 (31), and a halophilic archaeon, *Haloferax volcanii* (50), possessed protein-serine/threonine phosphatase activities that closely resembled that from *S. solfataricus* in their gross functional properties. These included activation by Mn^{2+}, their chromatographic behavior, their apparent substrate preferences, and their sensitivity to the histidyl-modifying reagent diethylpyrocarbonate, but not sulfhydryl-modifying reagents. Since the divergence of the *Crenarchaeotes*, which include *S. solfataricus*, from the *Euryarchaeotes*, which include both *M.thermophila* strain TM-1 and *H. volcanii*, represents the earliest discernable event in the evolution of the *Archaea* (10,12), this suggests that the forbear of these phosphatases was extant prior to this event, in the most ancient of the *Archaea*.

Thus far, the archaeal phosphatases have resisted facile classification under the now-standard system developed by Cohen (51) for the identification and classification of the eukaryotic protein-serine/threonine phosphatases. Their dependence upon divalent metal ions for expression of significant catalytic activity is reminiscent of the type-2C protein-serine/threonine phosphatases. However, their size, 30 kDa, is enticingly close to that of the structurally homologous catalytic subunits of protein phosphatases 1 and 2A, and much lower than that of protein phosphatase 2C, which belongs to a separate genetic superfamily distinct from that of protein phosphatases 1, 2A, and 2B. Moreover, the bacteriophage lambda protein phosphatase, another member of the protein phosphatase 1/2A/2B superfamily, is quite similar to the archaeal enzyme in size, is also activated by Mn^{2+}, and also displays high catalytic activity toward casein (29).

Most eukaryotic protein-serine/threonine phosphatases, including the members of the 1/2A/2B superfamily, and protein-tyrosine phosphatases are quite sensitive to sulfhydryl modifying reagents. However, each of the archaeal enzymes surveyed in our laboratory was unaffected by treatment with a variety of such reagents, although a sulfhydryl reagent sensitive, Mn^{2+}-stimulated alkaline phosphatase with activity toward casein has been reported in *Halobacterum halobium* (52). This suggests some fundamental divergence in structure and perhaps even catalytic mechanism. Although the identity and functional significance of the chemically-sensitive sulfhydryl group(s) of the protein-serine/threonine phosphatases remains cryptic, the chemically sensitive sulfhydryl group in the protein-tyrosine phosphatases is known to act as a nucleophile during catalysis, forming a phosphocysteinyl intermediate (53, 54).

A very surprising and provocative observation made during the course

of these studies was that the protein-serine/threonine phosphatase activity of *M.thermophila* strain TM-1 was inhibited, at least in partially purified form, by okadaic acid, microcystin-LR, and calyculin A (31). Sensitivity to these compounds has been regarded as a functional hallmark of the members of the major family of eukaryotic protein-serine/threonine phosphatases, the protein phosphatase 1/2A/2B superfamily (51). The archaeal protein phosphatase was inhibited by micromolar concentrations of these compounds, which generally places it on the low end of the sensitivity scale. For example, protein phosphatase 2A is generally inhibited by sub-nanomolar concentrations of okadaic acid, and protein phosphatase 1 by concentrations in the mid-nanomolar range. Their homolog protein phosphatase 2B, however, has an IC_{50} for okadaic acid of 5 μM — somewhat above that estimated for the protein phosphatase from *M. thermophila* strain TM-1. Moreover, the structural diversity of these compounds — okadaic acid is a polyether fatty acid (55), microcystin-LR is a cyclic heptapeptide (56), and calyculin A is a polyhydroxylated fatty acid containing a phosphomonoester and two gamma-amino acids (57) — argues that the inhibition observed was specific in nature. On the other hand, neither of the other archaeal phosphatases tested, those from *S. solfataricus* and *H. volcanii*, proved sensitive to any of these compounds. However, examples of okadaic acid-insensitive protein phosphatases from the type 1/2A/2B superfamily do exist, including the phage lambda enzyme (29), so such behavior is not without precedent. The ultimate resolution of this issue must await the determination of the amino acid sequence of an archaeal protein phosphatase, a process that is currently underway.

IphP, THE CYANOBACTERIAL DUAL-SPECIFICITY PROTEIN PHOSPHATASE

Even after it had become apparent that prokaryotes were the sites of protein phosphorylation-dephosphorylation, it was widely believed that the molecular building blocks of these phosphorylation networks were eukaryote- and prokaryote-specific in nature. One element of this dogma held that phosphotyrosine was the exclusive province of eukaryotic organisms (58). Thus, it was came as a great surprise when the DNA-derived amino acid sequence of the protein responsible for a p-nitrophenyl phosphatase activity in the cyanobacterium *Nostoc commune* strain UTEX 584 (59, 60), IphP, was found to contain the His-Cys-Xaa-Ala-Gly-Xaa-Xaa-Arg sequence that encompasses the active site cysteinyl residues of eukaryotic protein-tyrosine phosphatases (33).

Isolation and characterization of IphP revealed that it functions as both a protein-serine/threonine and protein-tyrosine phosphatase, placing it in the newly emerging class of dual-specificity protein phosphatases. These enzymes share the same active site sequence as the protein-tyrosine phosphatases and include viral representatives from vaccinia (61) and baculovirus (62). IphP was sensitive to the classic tyrosine phosphatase inhibitor vanadate, could be inactivated by treatment with the sulfhydryl modifying reagent N-ethylmaleimide, but was insensitive to the protein-serine/threonine phosphatase inhibitors okadaic acid and microcystin-LR, the last-named of which is a natural product of other forms of cyanobacteria (56).

IphP dephosphorylated a wide range of protein and synthetic peptide substrates containing phosphotyrosine, phosphoserine, or both phosphotyrosine and phosphothreonine. The first-named included reduced and carboxyamidomethylated lysozyme, myelin basic protein, poly (glu_4:tyr), (val_5)angiotensin I, and ENDY(P)INASL peptide. The second named also included reduced and carboxyamidomethylated lysozyme, as well as casein. The last named was MAP kinase. However, IphP was not completely promiscuous. Neither glycogen phosphorylase a nor histones H2a or H2b were dephosphorylated at an appreciable rate by IphP. Kemptide proved to be a poor substrate as well. Recently, we have observed that the inclusion of polyanionic macromolecules such as heparin or poly-aspartic acid either alone or in combination with bovine serum albumin (BSA) can enhance dephosphorylation of some substrates by IphP by 2-8 fold. For example, dephosphorylation of casein, myelin basic protein, and MAP kinase were dramatically stimulated by heparin. Heparin actually inhibited dephosphorylation of ENDY(P)INASL and both phosphotyrosyl and phosphoseryl RCM-lysozyme. However when added along with BSA a dramatic and synergistic stimulation of this dephosphorylation of RCM-lysozyne or poly (glu_4:tyr) took place. Dephosphorylation of (val_5)angiotensin-II or p-nitrophenyl phosphate, by contrast, exhibited no significant stimulation with any of these added factors. Given the substrate-specific nature of this behavior, it would appear that these escorting" macromolecules acted, in whole or in part, through complexation with substrate macromolecules. This suggests that IphP recognizes portions of its protein substrates distant from the phosphoamino acid itself, and that these regions are charged in nature, or that enzyme and substrate encounter one another through the mediation of some ionic matrix.

Although IphP represents the first genuine prokaryotic protein phosphatase with activity against protein-bound phosphotyrosine *in vitro*, it

is not yet known whether this particular aspect of it catalytic abilities has physiological importance. The enzyme also was active toward phosphoseryl and phosphothreonyl proteins and, in common with other enzymes with protein-tyrosine phosphatase activity, small molecular weight substrates such as p-nitrophenyl phosphate and 5-bromo-4-chloro-3-indolyl phosphate as well. In this regards it is highly significant, however, that *N. commune* strain UTEX 584 contains at least one protein that contains within it phosphotyrosine (33). This protein has a molecular weight of approximately 85 kDa on SDS-polyacrylamide gels and can be detected with antiphosphotyrosine antibodies in cells of *N. commune* strain UTEX 584 grown in the presence of combined nitrogen (sodium nitrate). Phosphotyrosine-containing proteins of similar molecular weight have also been detected in other *Bacteria*, including *Acinetobacter calcoaceticus* (63) and *Pseudomonas solanacearum* (64). When *N. commune* strain UTEX 584 was grown in nitrogen deficient media, however, under which conditions it fixes dinitrogen from the atmosphere in specialized, differentiated cells called heterocysts, the immunoreactive signal disappears. It is not known at present whether this is the consequence of the protein's degradation, dephosphorylation, or some combination of the two.

SUMMARY

Our knowledge of the identities and role of the phosphoproteins, protein kinases, and protein phosphatases in prokaryotic organisms lags substantially behind that with regards to eukaryotic organisms. However, one thing has become apparent in recent years — the wall that has divided the protein phosphorylation networks of prokaryotes from the eukaryotes appears to be on the verge of tumbling down. The first casualty of this scientific Jericho is the meaning and validity of the concept of prokaryote- and eukaryote-specific protein kinases and protein phosphatases. Another likely casualty is the concept that protein phosphorylation represents a relatively recent addition to the cell's arsenal of regulatory mechanisms, one junior to allosterism and gene regulation. The widespread nature of the emerging parallels between prokaryotic and eukaryotic organisms suggest that the building blocks of present day protein phosphorylation networks are in fact quite ancient in nature, and may have been operative as regulators in the primitive cells extant before the divergence of our present phylogenetic domains. If this is true, even to a limited extent, then the alien" protein phosphorylation networks of prokaryotic organisms may

serve as windows into as yet undiscovered portions of the signaling networks of their eukaryotic brethren. Time will tell.

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Cloning of *nifHD* from *Nostoc commune* UTEX 584 and of a Flanking Region Homologous to Part of the *Azotobacter vinelandii nifU* Gene

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The heterocystous cyanobacterium *Nostoc commune* UTEX 584 contains two *nifH*-like sequences (*nifH1* and *nifH2*) in addition to *nifHD*. A region of DNA 1 kilobase upstream from the 5′ end of *nifH* showed considerable sequence similarity to part of the published *nifU* sequences of *Azotobacter vinelandii* and *Klebsiella pneumoniae*.

Two distinct arrangements of the *nifHDK* genes have been described in a number of taxonomically diverse nitrogen-fixing cyanobacteria (2, 3, 7, 12, 13, 28). During differentiation of heterocysts of *Anabaena* sp. strain PCC 7120, at least two rearrangements of DNA occur within the genome (8, 9, 15; M. E. Mulligan and R. Haselkorn, Abstr. Symp. Mol. Biol. Photosynthetic Procaryotes, p. 35, 1987). One of these rearrangements involves excision of approximately 55 kilobases (kb) near the *nifS* gene, resulting in the joining of *nifB* and a gene for a ferredoxin to *nifS*, which is then expressed (8, 9; Mulligan and Haselkorn, Abstr. Symp. Mol. Biol. Photosynthetic Procaryotes). The functional significance of the rearrangement near *nifS* is unknown, and its occurrence in other strains of cyanobacteria has not been determined. The second rearrangement results from excision of an 11-kb element (excison) which in vegetative cells separates *nifK* from *nifHD* and splits *nifD* 43 codons from its 3′ end (9). The physiological significance of the latter rearrangement is unclear, since an excisonlike element was not detected in DNA from vegetative cells of the heterocystous form *Fischerella* sp. (28). In addition to these different arrangements and rearrangements of *nif* genes, multiple copies of *nifH*-like sequences have been described for several cyanobacteria (12, 13) and analyzed more particularly in *Anabaena* sp. strain PCC 7120 (23). We report that the heterocystous cyanobacterium *Nostoc commune* UTEX 584 has a *nifU*-like sequence located approximately 1 kb upstream from *nifH* and that this strain contains two *nifH*-like sequences in addition to *nifHD*.

Cyanobacteria were grown in BG-11 medium (containing sodium nitrate; 25) as described previously (22). Cells of *N. commune* are resistant to methods for gentle lysis (1). To purify DNA, the cells were ground to a powder under liquid nitrogen, suspended in lysis buffer (15% [wt/vol] sucrose, 25 mM Tris hydrochloride [pH 8.0], 10 mM disodium EDTA) and subjected to five freeze-thaw cycles. Solid lysozyme (Sigma Chemical Co., St. Louis, Mo.) was added to the solution (final concentration, 15 mg ml^{-1}), and incubation was continued first at 37°C for 5 h with shaking and then at 4°C overnight. Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and *N*-laurylsarcosine (Sigma) were added (final concentrations, 10 μg ml^{-1} and 5 mg ml^{-1}, respectively), and the suspension was incubated at 50°C for 4 h. The suspension was diluted and deproteinized (17), and the DNA was recovered by precipitation in the presence of isopropanol (−20°C) and further purified by cesium chloride density gradient ultracentrifugation (17). Nick translation (24) was used to label *nif*-specific DNA probes with biotin-11-dUTP (Table 1). The biotin-11-dUTP, DNA polymerase I, DNase I, and restriction endonucleases were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Southern analyses were performed by the procedure described by Mason and Williams (18). Baked nitrocellulose filters were incubated for 2 h at 42°C in prehybridization solution (30% [wt/vol] deionized formamide, 5× SSC [1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate], 0.1% [wt/vol] sodium dodecyl sulfate, 1 mM disodium EDTA, 10 mM Tris hydrochloride [pH 7.5], 1× Denhardt solution, 0.5 mg of salmon sperm DNA ml^{-1}; 17). Filters were then incubated in hybridization solution (same as prehybridization solution but with the addition of 5% [wt/vol] polyethylene glycol [type 8000] and a concentration of 0.2 mg of salmon sperm DNA ml^{-1}) after addition of the heat-denatured biotinylated DNA probe (100 ng ml^{-1}). Hybridization was performed at 42°C for 18 h. After hybridization, the filters were washed first in 2× SSC–0.1% (wt/vol) sodium dodecyl sulfate, then in 1× SSC–0.1% (wt/vol) sodium dodecyl sulfate, and then in 0.5× SSC–0.1% (wt/vol) sodium dodecyl sulfate for 90 min at 50°C. Finally, the filter was rinsed briefly in a solution of 2× SSC. Biotinylated DNA-DNA hybrids were visualized by use of a colorimetric assay (Bethesda Research Laboratories).

Two distinct bands, corresponding to *Eco*RI fragments of 18 and 11 kb, were detected after Southern analysis of *Anabaena variabilis* PCC 7118 DNA with the *nifH*-specific probe (Fig. 1a, lane 1). The 11-kb fragment gave a stronger hybridization signal. These results are in agreement with those reported for vegetative cells of *Anabaena* sp. strain PCC 7120 (9). *Anabaena variabilis* PCC 7118 is considered to be a nomen species of the much better characterized cyanobacterium *Anabaena* sp. strain PCC 7120 (14). Three distinct bands of different sizes were detected when *N. commune* UTEX 584 DNA was digested with *Eco*RI, *Hin*dIII, or *Eco*RI and *Hin*dIII (double digest) and hybridized with the *nifH*-specific probe (Fig. 1a, lanes 2 and 3, and b, lanes 1 and 2). In these analyses, the largest DNA fragment consistently gave the weakest signal of the three

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TABLE 1. Strains and plasmids

Strain or plasmid	Description or genotype	Reference or source
Strains		
Nostoc commune UTEX 584	Het^+	22
Anabaena variabilis PCC 7118	Het^-	25
Escherichia coli HB101	F^- *hsd20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44* (λ^-)	17
Plasmids		
pMJH5	606-base-pair *Bgl*II-*Eco*RI fragment from within *nifH* of *Azotobacter vinelandii* (nucleotide positions 208 to 814)	5
pDB6	827-base-pair *Kpn*I-*Kpn*I fragment from within *nifD* of *Azotobacter vinelandii* (nucleotide positions 304 to 1131)	5
pBR322	Ap^r Tc^r 4.36 kb	Promega Biotec
pGEM-4	Ap^r 2.87 kb	Promega Biotec
pGEM-blue	Ap^r *lacZ* 2.75 kb	Promega Biotec
pND001	3.5-kb *Hin*dIII-*Hin*dIII fragment in *Hin*dIII site of pBR322 (Fig. 3)	This study
pNDD1	1.1-kb *Eco*RI-*Eco*RI fragment from pND001 (including an adjacent 31 base pairs of pBR322) in pGEM-blue (Fig. 3)	This study
pNDH1	2.4-kb *Eco*RI-*Hin*dIII fragment from pND001 in pGEM-4 (Fig. 3)	This study

when hybridized with the *nifH* probe. Strong hybridization signals were noted in Southern analyses of *N. commune* UTEX 584 DNA with the *nifD*-specific probe (Fig. 2, lanes 1 to 3). In these hybridization analyses, a strong signal corresponding to a band at 1.1 kb was the only signal detected after hybridization of the genomic DNA (digested with *Hin*dIII and *Eco*RI) with the *nifD* probe.

A library of *N. commune* UTEX 584 DNA was constructed in pBR322 (11, 17) by using genomic *Hin*dIII DNA fragments of 2 to 6 kb to permit isolation of the single 3.5-kb *Hin*dIII fragment which hybridized to both *nif* probes. The recombinant plasmids were used to transform *Escherichia coli* HB101 (Table 1), and the recombinant library was stored as suggested by Mason and Williams (18). Filters supporting bacterial colonies were processed for screening with biotinylated probes (M. J. Haas and D. W. Fleming, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, I-155, p. 198). The recombinant plasmid from one transformant contained a DNA insert of 3.5 kb which hybridized strongly to both of the *Azotobacter vinelandii nif* probes. The 3.5-kb *Hin*dIII fragment contained a single site for *Eco*RI, with no internal sites for *Hin*dIII (Fig. 3). These results, together with those obtained in Southern analyses, suggest that *N. commune* UTEX 584, like other diazotrophs (6, 21, 26), contains multiple (two) *nifH*-like sequences in addition to *nifH*. Digestion of the 3.5-kb *Hin*dIII fragment with *Eco*RI generated two fragments of 1.1 and 2.4 kb. The *nifH*-specific probe hybridized only with the 2.4-kb fragment, while the *nifD*-specific probe hybridized only with the 1.1-kb fragment. These data suggested that the *nifD* sequence was

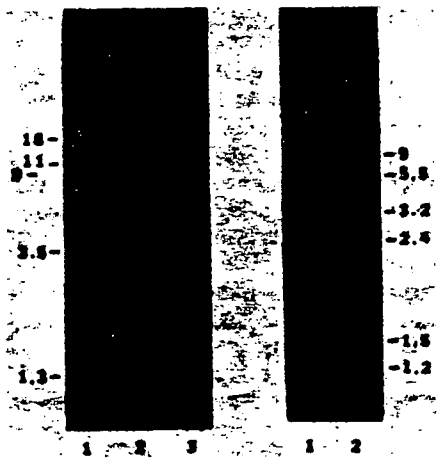

FIG. 1. Southern hybridization analyses with a biotinylated *nifH*-specific probe from *A. vinelandii* (Table 1). The sizes indicated are in kilobases. The color reaction with streptavidin-alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indolylphosphate (Bethesda Research Laboratories) was allowed to proceed for 10 min. (a) Lanes: 1, *Anabaena variabilis* PCC 7118 DNA digested with *Eco*RI; 2, *N. commune* UTEX 584 DNA digested with *Hin*dIII for 60 min at 37°C; 3, as lane 2 but for 15 h at 37°C. (b) Lanes: 1, *N. commune* UTEX 584 DNA digested with *Eco*RI and *Hin*dIII; 2, *N. commune* UTEX 584 DNA digested with *Eco*RI.

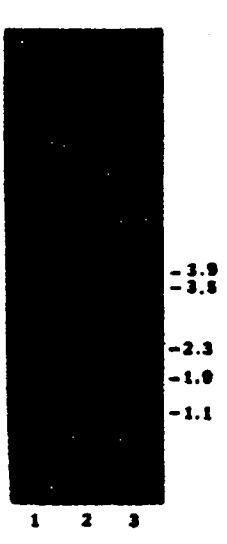

FIG. 2. Southern hybridization analyses of *N. commune* UTEX 584 genomic DNA with a biotinylated *nifD*-specific probe from *A. vinelandii* (Table 1). Restriction enzyme digestions were performed at 37°C for 60 min. For detection of biotinylated DNA-DNA hybrids (Fig. 1a and b), the color reaction was allowed to proceed for 10 min. The weaker signals observed in lanes 1 and 2 were noted consistently in repeat hybridization experiments. Lanes: 1, genomic DNA digested with *Hin*dIII; 2, genomic DNA digested with *Eco*RI; 3, genomic DNA digested with *Hin*dIII and *Eco*RI. The numbers on the right indicate molecular sizes in kilobases.

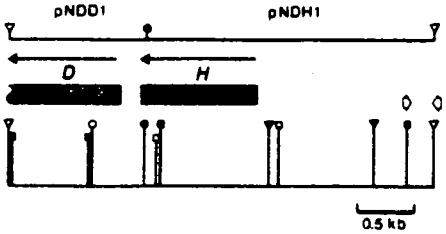

FIG. 3. Restriction map of the 3.5-kb *Hin*dIII fragment isolated from the *N. commune* UTEX 584 library and subcloned in pBR322 (pND001; Table 1). The location of the *nifHD* cluster was determined from partial DNA sequencing and Southern analyses of restriction enzyme digests with *nif*-specific probes. Not shown are an additional *Pvu*I site between the 5′ end of *nifH* and the *nifU*-like sequence and frequent *Hpa*II sites within *nifH*. Arrows indicate the direction of transcription of *nifHD*. The direction of transcription of the *nifU*-like sequence is the same as that of *nifHD*. The location of the *nifU*-like sequence is indicated by opposing open arrows. Symbols: ▼, *Hin*dIII; ▼, *Pvu*I; ▼, *Eco*RI; , *Acc*I; ▼, *Hpa*II; ▼, *Hin*cII. The *nifD* (D; partial) and *nifH* (H) sequences were subcloned separately (Table 1).

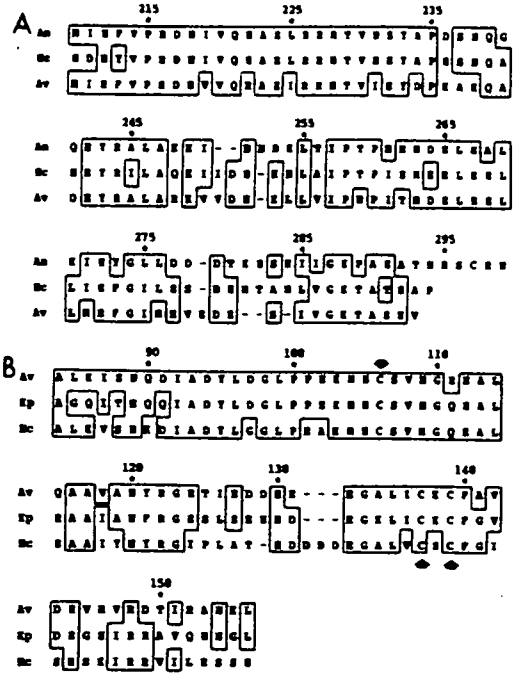

FIG. 4. (A) Comparison of partial derived amino acid sequences from the carboxy-terminal region of NifH from *Anabaena* sp. strain PCC 7120 (An; 20), *A. vinelandii* (Av; 5), and *N. commune* UTEX 584 (Nc; this study). Numbers refer to the amino acid residues in the NifH sequence of *Anabaena* sp. strain PCC 7120. Regions of sequence similarity are boxed. (B) Alignment of a region of the amino acid sequences of NifU from *A. vinelandii* (Av) and *K. pneumoniae* (Kp; 4) with the derived amino acid sequence from the *N. commune* UTEX 584 *nifU*-like DNA sequence (Nc). Conserved cysteinyl residues are indicated by arrows, and numbers refer to residues in the *A. vinelandii* derived amino acid sequence (4).

adjacent to *nifH* in vegetative cells of *N. commune* UTEX 584. This result is consistent with the findings for other *Nostoc* strains (12). The fragments were subcloned separately in pGEM-4 (Table 1; Fig. 3), and the 5′ and 3′ regions of each insert were sequenced with the Klenow fragment of DNA polymerase I and the dideoxy-chain termination method of Sanger et al. (27). The partial sequence analysis confirmed the identities of the *N. commune* UTEX 584 *nifHD* sequences and confirmed that the two genes were adjacent (Fig. 4A). The single *Eco*RI site in the 3.5-kb *Hin*dIII fragment occurred 64 base pairs from the 3′ end of the *N. commune* UTEX 584 *nifH* sequence. This *Eco*RI site is absent in the *Anabaena* sp. strain PCC 7120 *nifH* sequence as a consequence of a single-base change at position 818 (20).

In an attempt to localize additional *nif* genes, especially *nifS*, which occurs approximately 1 kb upstream from *nifH* in *Anabaena* sp. strain PCC 7120 DNA (8, 9; Mulligan and Haselkorn, Abstr. Symp. Mol. Biol. Photosynthetic Procaryotes), we compared our sequence data with the published sequences for *nifS*, *nifV*, and *nifU* from *A. vinelandii* and *Klebsiella pneumoniae* (4). A high degree of DNA and derived amino acid sequence similarity was found between a region of the 3.5-kb fragment distal to and upstream from *nifH* (Fig. 3) and the published *nifU* sequences. The derived amino acid sequence of 72 residues from *N. commune* UTEX 584 contained three conserved cysteinyl residues which, in the *A. vinelandii nifU* sequence, are located between residues 106 and 139 (Fig. 4B; 4).

The most detailed studies of *nif* gene organization and expression have been performed with *Anabaena* species (10, 15, 16, 19, 20, 23). Our sequence data, although only partial, suggest that *N. commune* has a *nifU*-like sequence upstream from *nifH* in the position where *nifS* is located in *Anabaena* sp. strain PCC 7120 (8, 9; Mulligan and Haselkorn, Abstr. Symp. Mol. Biol. Photosynthetic Procaryotes). *nifU* has not been isolated from a cyanobacterium, and the functions of this gene, as well as those of *nifS*, have not been fully resolved (4). It has been suggested that *nifU* is a metalloprotein in view of the pattern of conservation of cysteinyl residues (4), while recent work has shown that *nifU* expression is required for maturation of both the Fe protein and MoFe protein of nitrogenase (D. Dean, personal communication). For the purposes of this study, cells of *N. commune* UTEX 584 were grown on a source of combined nitrogen, and the cultures contained no heterocysts. The positions of the *nifU*-like sequence, adjacent sequences, and multiple *nifH*-like sequences in differentiated cells of *N. commune* remain to be determined.

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Analysis of the sequences within and flanking the cyanoglobin-encoding gene, *glbN*, of the cyanobacterium *Nostoc commune* UTEX 584*

(Recombinant DNA, *nif* genes; repetitive sequences; *nifH* promoter; BifA)

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SUMMARY

A 3.5-kb segment of DNA containing *nifU glbN nifH nifD* was cloned from a gene library of *Nostoc* 584 and sequenced. The *nifU-glbN* intergenic region contains short tandemly repeated repetitive sequences (5′-AATTACG). A sequence corresponding to a NifA-like upstream activator sequence (with the consensus recognition sequence for BifA in *Anabaena* 7120), elements of a *nifH* promoter and a sequence that may function as a transcription terminator, were identified downstream from *glbN*. GlbN, unique to certain *Nostoc* spp., is more homologous to protozoan myoglobins than to any other prokaryotic, vertebrate or plant globins.

The *glbN* gene encoding cyanoglobin, the only known prokaryotic myoglobin (Potts et al., 1992), is positioned immediately downstream from *nifU* and immediately upstream from *nifH* in the genome of *Nostoc* 584 (Defrancesco and Potts, 1988). The *nifUHD* cluster has been characterized in other prokaryotes but in no case has an ORF, *glbN*, or a *glbN*-related sequence been identified. Here we discuss features of the regions flanking *glbN*.

The 268-bp *nifU-glbN* intergenic region contains five direct repeats of 5′-AATTACG with four of these in tandem (Fig. 1). The four repeats are flanked by two 11-bp inverted repeats which have the potential to form part of the stem of a stable secondary RNA structure ($\Delta H = -22.4$ kcal/mol). Identical short tandem repeats (*STRR*) have been identified in the genomes of other cyanobacteria (Mazel et al., 1990; Ewart et al., 1990; U. Monnerjahn and H. Böhme, unpublished data). Repetitive sequences are ubiquitous in the genomes of prokaryotes and simple eukaryotes. Although the precise function of these sequences has not been determined, the consensus opinion is that they play an important role in the structure and evolution of genomes (Lupski and Weinstock, 1992).

The 268-bp *nifU-glbN* intergenic region showed no obvious homologies or structural similarities with the 348-bp *glbN-nifH* intergenic region (Fig. 1). The latter contains the *STRR* 5′-GACAAA within a region with the potential to form a stable secondary RNA structure ($\Delta H = -15.4$ kcal/mol, 17-bp stem) that is flanked by two different palindromic sequences. The stem-loop appears to function as a transcription terminator (data not shown). Alignments of the *glbN-nifH* intergenic region of *Nostoc* 584 with the *nifH* promoter region of *Anabaena*

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*On request, the authors will supply detailed experimental evidence for the conclusions reached in this Brief Note.

Abbreviations: aa, amino acid(s); *Anabaena* 7120, cyanobacterium *Anabaena* sp. strain PCC 7120; bp, base pair(s); BifA, DNA-binding factor in *Anabaena* 7120; *glbN*, gene (DNA) encoding GlbN (cyanoglobin); *HIP1*, highly iterated palindromic repeat (5′-GCGATCGC); kb, kilobase(s) or 1000 bp; *nifD*, gene (DNA) encoding NifD (α-subunit of dinitrogenase); *nifH*, gene (DNA) encoding dinitrogenase reductase; *Nostoc* 584, cyanobacterium *Nostoc commune* strain UTEX 584; nt, nucleotide(s); ORF, open reading frame; STRR, short tandem repeat(s); *UAS*, upstream activator sequence(s).

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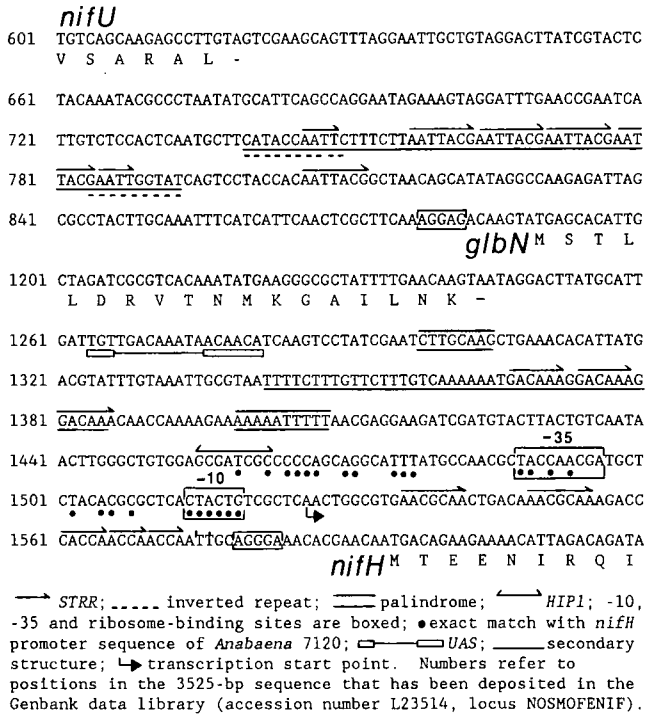

Fig. 1. *nifU-glbN* and *glbN-nifH* intergenic regions.

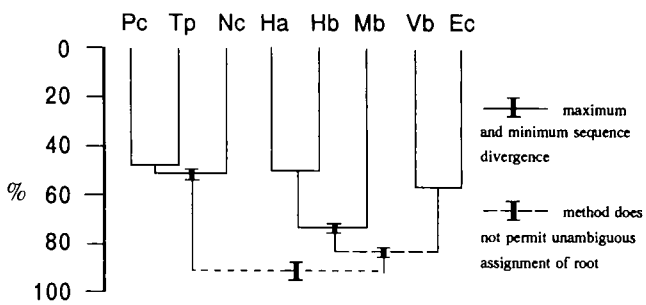

Fig. 2. Percentage difference (cladogram) between aligned globin protein sequences. Alignments were refined using the ALIGN and CLUSTAL programs of Intelligenetics (Mountain View, CA, USA). Sequences compared and accession Nos. in the Swiss-Prot data library were: Pc, *Paramecium caudatum* myoglobin, P15160; Tp, *Tetrahymena pyriformis* myoglobin, P17724; Nc, cyanoglobin of *Nostoc* UTEX 584, Q00812; Ha, a chain of human hemoglobin, P01922; Hb, β chain of human hemoglobin, P02023; Mb, sperm-whale myoglobin, P02185; Vb, *Vitreoscilla* sp. hemoglobin, P04252; Ec, *Escherichia coli* hemoprotein (Hmp), P24232.

7120 (Mulligan and Haselkorn, 1989) identified the conserved −10 region (CTACTG), a less conserved −35 region (TANCNA or ACNAAC) and a conserved C-rich upstream region. The latter, in *Nostoc* 584, includes one *HIP1* sequence (Gupta et al., 1993). A *HIP1* element is also present within the coding region of *glbN* (data not shown). A sequence very similar to the consensus NifA *UAS* (Lee et al., 1993) was identified 18 bp downstream from the stop codon of *glbN* (5′-TGTN$_9$ACA and 5′-TGTN$_{12}$ACA; Fig. 1). The *Nostoc UAS* show more extensive homology to a NifA *UAS* from *Rhodobacter capsulatus* (Willison et al., 1993) with the consensus 5′-TTGTTN$_8$AACAA. The 5′-TGT(N$_{9\ or\ 10}$)ACA sequence is also the consensus recognition sequence for BifA, a transcriptional regulator in *Anabaena* 7120, but it is absent in the *nifH* promoter (Ramasubramanian et al., 1994). The *glbN* promoter was not identified but transcription may terminate after *glbN* and is uncoupled from *nifH* transcription.

Cyanoglobin shows minimal sequence correspondence with plant leghemoglobins (Arredondo-Peter and Escamilla, 1991), the cytochromes, or the other known 'prokaryotic' globins (Wakabayashi et al., 1986; Vasudevan et al., 1991), with clear dichotomy emphasizing the potentially unique ascent of cyanoglobin (Fig. 2).

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SCIENCE

Myoglobin in a Cyanobacterium

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Myoglobin in a Cyanobacterium

Malcolm Potts,* Stephen V. Angeloni, Richard E. Ebel, Deeni Bassam

Myoglobin was found in the nitrogen-fixing cyanobacterium *Nostoc commune*. This cyanobacterial myoglobin, referred to as cyanoglobin, was shown to be a soluble hemoprotein of 12.5 kilodaltons with an amino acid sequence that is related to that of myoglobins from two lower eukaryotes, the ciliated protozoa *Paramecium caudatum* and *Tetrahymena pyriformis*. Cyanoglobin is encoded by the *glbN* gene, which is positioned between *nifU* and *nifH*—two genes essential for nitrogen fixation—in the genome of *Nostoc*. Cyanoglobin was detected in *Nostoc* cells only when they were starved for nitrogen and incubated microaerobically.

Cyanobacteria may have figured in the evolution of Earth's oxygenic atmosphere (*1*). Many cyanobacteria have nitrogenase, an enzyme that catalyzes the reduction (fixation) of dinitrogen; as a group, these photosynthetic microorganisms are responsible for much biological dinitrogen fixation (*2*). Nitrogenase is sensitive to gaseous oxygen, and cyanobacteria use various mechanisms to achieve aerobic nitrogen fixation, including attenuation of the oxygen tension in the immediate vicinity of nitrogenase. For example, some cyanobacteria can effect either a spatial or temporal separation, or both, of their oxygen-evolving and nitrogen-fixing activities (*3, 4*), whereas others restrict their nitrogenase activity to a specialized differentiated cell, the heterocyst. During the course of its differentiation, the heterocyst is modified structurally and biochemically in order to provide a reducing environment conducive to nitrogen fixation (*5*). Marked changes in gene expression accompany heterocyst differentiation and include the induction of the *nif* operons (*6*).

Of the heterocystous cyanobacteria, species of *Nostoc* are especially prevalent in terrestial environments from the tropics to the polar regions, where they often enter into associations with a variety of higher and lower plants (*7*). One of these species, *Nostoc commune*, forms visually conspicuous colonies in the shallow natural depressions of limestone rock in karst areas. We isolated the *nifUHD* gene cluster from the strain *Nostoc commune* UTEX 584 (*8*). DNA sequence analysis revealed an open reading frame (ORF) of 118 codons, potentially encoding a 12.5-kD protein, between *nifU* and *nifH* that showed no obvious similarity in either DNA or amino acid sequence with any known *nif* genes or Nif proteins, respectively (Fig. 1A). However, marked similarity was apparent, both in size and in amino acid sequence, to the myoglobins of the ciliated protozoa *Paramecium caudatum* and *Tetrahymena pyriformis*, which contained 116 and 121 amino acids, respectively (*9, 10*). We used the polymerase chain reaction (PCR) (*11*) to amplify *glbN*, subcloned *glbN* into an expression vector (pT7-7), and overexpressed the recombinant protein in *Escherichia coli* (Fig. 1A) (*12*). A 12-kD protein was revealed by electrophoresis of whole-cell lysates from IPTG (isopropyl-β-D-thiogalactopyranoside)-induced (Fig. 1B, lanes C and D) cells carrying the cloned *Nostoc* gene (*13*); the protein was not revealed in cells uninduced by IPTG (Fig. 1B). The cell pellets from GlbN$^+$ transformants were bright red in color. Examination of induced cells with light microscopy did not reveal inclusion bodies.

The recombinant protein partitioned to the soluble fraction after disruption of GlbN-expressing *E. coli* in a French pressure cell. During the course of the purification, the color of the protein preparation changed from bright red to orange-brown, probably the result of auto-oxidation of the protein to the ferric (met)-form. This is a characteristic of myoglobins and hemoglobins isolated from other sources. The orange-brown fraction, obtained after ion-exchange chromatography, ultrafiltration, and gel-exclusion chromatography, was judged to be homogenous when examined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (Fig. 1B). The molecular sizes of GlbN estimated from gel filtration and SDS-PAGE were 15 kD and 12 kD, respectively. These data indicate that recombinant GlbN is a monomer.

The ultraviolet (UV)-visible spectrum of the recombinant GlbN was consistent with that of a high-spin ferric hemoprotein, with a maximum absorbance in the Soret region at 412 nm (Fig. 2). Reduction with sodium dithionite shifted the Soret peak to 422 nm, whereas addition of carbon monoxide to the reduced protein caused a shift in the Soret peak to 419 nm. These data demonstrate that the reduced protein has an open ligand position that can bind carbon monoxide and probably molecular oxygen. As no cysteine residues are present in

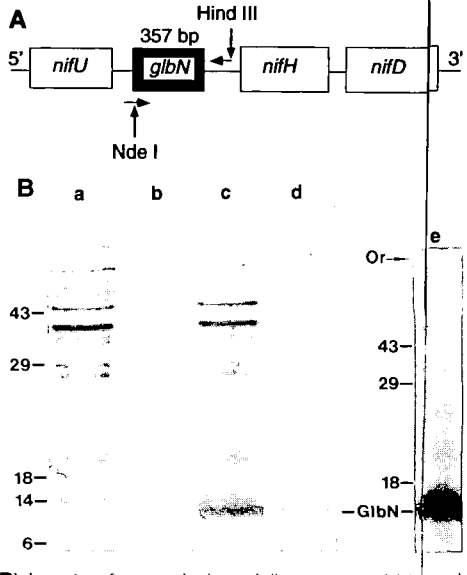

Fig. 1. Chromosomal location of *glbN* and purification of recombinant GlbN protein. (**A**) The position of *glbN* in the *nifUHD* cluster of *Nostoc commune* (*8*) and the region amplified by PCR are represented schematically. Note that the *nifU-nifH* intergenic region of the related cyanobacterium *Anabaena sp.* strain PCC7120 is devoid of any ORF (*22*). The two oligonucleotides used were 5′-CTGGCATATGAGCACATTGTACG-3′ and 5′-GCGAAGCTTCGATAGGACTTGAT-3′. These are complementary to the 5′ end of *glbN* and to a region 50 bp downstream of its 3′ terminus, respectively. Recognition sites for Nde I and Hind III were introduced into the oligonucleotides to permit subsequent subcloning of the PCR product in the desired orientation in pT7-7. The sequence of *glbN* is 357 bp; the engineered PCR product was 422 bp. The first five amino acid residues of GlbN—represented by bases 8 to 22 in the first oligonucleotide—are Met, Ser, Thr, Leu, and Tyr. The sequence of *glbN* has been submitted to GenBank (accession number M92437). (**B**) Lysates from uninduced (lanes a and b) and induced cells (lanes c and d); purified GlbN prepared by liquid chromatography and examined by SDS-PAGE (lane e). Lanes b and d were loaded with one-tenth the amount of total protein present in lanes a and c. Molecular sizes in kilodaltons are shown at the left. Proteins were detected with Coomassie blue stain (lanes a to d) or silver stain (lane e). GlbN, recombinant cyanoglobin; Or, origin.

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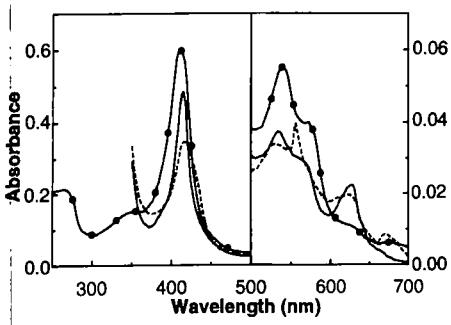

Fig. 2. Soret and visible spectra of recombinant GlbN. Spectra of purified native recombinant GlbN were obtained from a solution in 50 mM Hepes (pH 7.5) (solid curve with symbols); from the identical solution after the addition of crystalline sodium dithionite (dashed curve); and after saturation of the solution with carbon monoxide (solid line only). Spectra were obtained with a Cary 219 spectrophotometer (Varian, Palo Alto, California) and 1-cm pathlength quartz cuvettes.

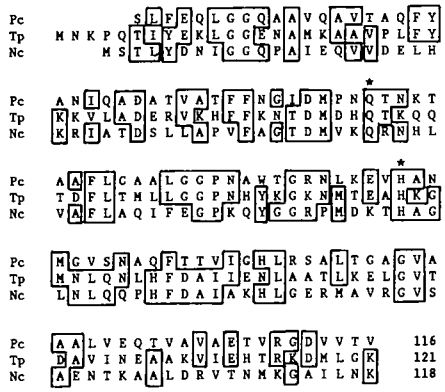

Fig. 4. Deduced amino acid sequence (*23*) of cyanoglobin and comparison with other globins. Alignment of GlbN with the sequences of the monomeric hemoglobins of *Paramecium* (Pc) and *Tetrahymena* (Tp). Nc, *Nostoc* cyanoglobin. For presentation of the comparison of Nc, Pc, and Tp, only exact correspondences are boxed. Numbers = the total residues in each protein. Residues in Pc and Tp thought to function in heme-binding (*9, 10*) are indicated with an asterisk.

the amino acid sequence of GlbN, the heme group is probably noncovalently attached to the monomeric apoprotein—a characteristic of myoglobins and hemoglobins (*14*). The UV spectrum of the ferric form of the protein and the spectra in the Soret region of the ferrous and carbon monoxy forms of the protein are typical of myoglobins and hemoglobins isolated from other sources, including those from the two protozoans. However, the spectral characteristics of the ferrous and carbon monoxy forms in the visible region are atypical. To reflect the globin-like properties of GlbN, we refer to this cyanobacterial hemoprotein as cyanoglobin.

Cyanoglobin, with a molecular size identical to that of the recombinant protein (12 kD), was detected in cells of *Nostoc commune* UTEX 584 only when they were grown in the absence of combined nitrogen (when they had differentiated heterocysts) and then only after a 24- to 56-hour period of incubation under microaerobic conditions (Fig. 3) (*15*). Under these conditions, induction of cyanoglobin synthesis was demonstrated with cultures in the early and late logarithmic phase of growth (Fig. 3).

Nostoc cyanoglobin is smaller than other known myoglobins but appears to be related to the monomeric protozoan globins for which, at present, no functions have been assigned. The calculated isoelectric points for the *Nostoc* and *Tetrahymena* proteins are virtually identical (9.7 and 9.5, respectively), and all three proteins show very similar hydropathic profiles. These three monomeric globins constitute a distinct homogenous group when their sequences are aligned (*16*) and compared with the mammalian globins as well as with the dimeric hemoglobin reported from a species of the bacterium *Vitreoscilla* (*17*) (Fig. 4). Cyanoglobin shares only limited sequence similarity with the mammalian and *Vitreoscilla* proteins and no obvious similarity with other hemoproteins including the leghemoglobins and cytochromes.

The marked conservation between cyanoglobin and related protozoan myoglobins, in contrast to the limited similarity between cyanoglobin and the eubacterial *Vitreoscilla* hemoglobin, raises questions of whether these different monomeric hemoproteins have a common origin and whether they can provide clues to early globin evolution. The observation that the synthesis of cyanoglobin is induced by nitrogen starvation, and the positioning of *glbN* between *nifU* and *nifH*, may suggest that GlbN is involved in some aspect of nitrogen fixation. That aspect may involve a scavenging of oxygen because the induction of cyanoglobin synthesis required incubation of *Nostoc* cells under low oxygen tension.

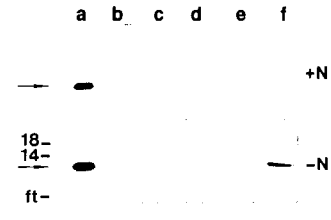

Fig. 3. Cyanoglobin in *Nostoc*. Immunoblotting was used to detect cyanoglobin in cells at different developmental stages grown in the presence (+N) or absence (−N) of combined nitrogen. Lane A, 20 ng of recombinant cyanoglobin; lane B, hormogonia (24 hours of aerobic growth); lane C, 48 hours of aerobic growth (heterocysts differentiated in −N culture); lane D, 48 hours of aerobic growth and 24 hours of anaerobic induction; lane E, 9 days of aerobic growth; and lane F, 9 days of aerobic growth and 56 hours of anaerobic induction. Equivalent amounts of *Nostoc* proteins (approximately 5 μg) were loaded in each lane of two gels, which were processed under identical conditions. Molecular size standards in kilodaltons are at the left; ft, unresolved protein front. Arrows, 12-kD cyanoglobin.

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12. The PCR reactions were performed in a Coy Tempcycler model 50. Before its expression in *E. coli*, the nucleotide sequence of the PCR product was determined and found to be identical with the genomic sequence of *glbN*. Ligation of the Nde I–Hind III–digested PCR product to Nde I–Hind III–digested pT7-7 created the recombinant plasmid pGlbN (2895 bp), in which the translational initiation codon of GlbN was positioned 7 bp downstream from the ribosome-binding site, an orientation that favored efficient translation. The pGlbN was used to transform *E. coli* BL21DE3 (F⁻, *ompT*), a strain that contains a chromosomal copy of the gene for T7 DNA–dependent RNA polymerase, with expression of that gene under the control of the *lac* repressor. IPTG was used to induce transcription from the T7 promoter upstream of pGlbN, with concomitant overproduction of the GlbN protein.
13. The *glbN* transformants were grown in Luria-Bertani liquid medium in the presence of ampicillin (100 μg/ml) at 37°C to the logarithmic phase of growth monitored at an absorbance of 550 nm. When the absorbance at 550 nm reached 0.4, IPTG was added to a final concentration of 0.4 mM, and cells were collected after 2 hours at 37°C. For analytical purposes, cells from induced, and uninduced cultures were lysed in Laemmli buffer (*18*) and boiled for 5 min, and the clarified protein extracts were examined directly by SDS-PAGE (12% w/v polyacrylamide). Protein concentrations were determined with a protein determination kit (Pierce) with bovine serum albumin as the standard. For preparative purposes, the colored cell pellet harvested from induced cultures was washed in 50 mM Hepes (pH 7.5), resuspended in the same buffer (containing 1 mM phenylmethylsulfonyl fluoride), and then passed through a chilled French pressure cell at 100 MPa. The suspension was clarified by centrifugation at 30,000*g* (SS-34 rotor; Sorvall, Wilmington, DE) for 30 min. The cleared lysate was applied directly to a Mono S HR 5/5 FPLC (fast protein liquid chromatography) column (Pharmacia LKB) equilibrated with 50 mM Hepes (pH 7.5) with a flow rate of 1 ml/min. Absorbance was monitored simultaneously at 280 and 418 nm. Under these conditions, GlbN appeared in the flow-through fraction. This orange-colored fraction was concentrated with Centriprep 10 cartridges (Amicon, Danvers, MA)

and applied to a Superose 12 HR10/30 column equilibrated with the same buffer. The fractions that showed maximum absorbance at 418 nm were collected and concentrated again by ultrafiltration. Aliquots were denatured in Laemmli buffer (*18*) and applied to 12% w/v polyacrylamide mini-gels (Hoefer) to assess their purity. The fractions were judged to be >95% homogenous when examined by SDS-PAGE and silver staining. To determine the native molecular size of the recombinant protein, we equilibrated the Superose 12 column with 50 mM tris-HCl (pH 8.0) and 100 mM NaCl and calibrated it after individual resolution of the following standards: carbonic anhydrase (29 kD), equine-heart myoglobin (18.8 kD), cytochrome c (12.4 kD), and aprotinin (6.5 kD) (Sigma). Purified recombinant GlbN (Fig. 1B) was used to generate polyclonal antibodies in rabbit.

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15. Cells of *Nostoc commune* UTEX 584 in stationary phase were subcultured and grown in the presence or absence of combined nitrogen, in BG-11 or BG-11_0 liquid media, respectively (*19*). Incubation was at 32°C, with a photon flux density at the surface of the culture vessels of 60 μmol photons m^{-2} s^{-1} and with continuous and vigorous sparging with sterile air. Under these conditions, induction of hormogonia (motile filaments that lack heterocysts) occurred within 22 hours. After 48 hours, those hormogonia that were induced in BG-11_0 cultures had completed heterocyst differentiation. Aliquots of the cultures were harvested, resuspended in fresh BG-11 or BG-11_0, and transferred to Erlenmeyer flasks provided with gas-tight Suba seals (Fisher, Pittsburgh, PA). To achieve microaerobic conditions, we flushed the gas phase once with argon (100 v/v) and continued incubation for 24 hours under the same conditions of light and temperature. Cultures that had grown to a higher cell density (over 9 days) were also subjected to microaerobic conditions. In this case, the gas phase was flushed intermittently (for 5 min at approximately 12-hour intervals) during a 56-hour period of incubation. The developmental growth stage of cultures was monitored by light microscopy, and proteins were extracted when necessary by grinding of cells in liquid nitrogen and then in Laemmli buffer (*18*). Protein extracts were processed for SDS-PAGE and immunoanalysis as described (*20*). To reduce nonspecific cross-reactions in immunoblotting analysis, we diluted the antiserum 1:10 in tris-buffered saline buffer and incubated it overnight with nitrocellulose filters that had been saturated with protein extracts from *E. coli* BL21DE3 (pT7-7).
16. Using a published alignment of sperm whale myoglobin (SMb), the alpha (Ha) and beta (Hb) polypeptides of human hemoglobin, and *Vitreoscilla* dimeric hemoglobin (Vb) (*17*), we compared protein sequences with the alignment of the monomeric hemoglobins of *Paramecium* (Pc) (*9*) and *Tetrahymena* (Tp) (*10*). The derived sequence of the *Nostoc* cyanoglobin (Nc) was added to the alignment after sequence comparison of Nc with Pc and Tp with the use of the FASTA program (*21*), available through GenBank.
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23. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
24. Supported by the National Science Foundation (grants DCB 8803068 and DCB 9103232 to M.P.). We thank T. Larson for the synthesis of oligonucleotides and for critically reading the manuscript, D. Dean for pT7-7, and D. Hill for photography.

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Original Papers

Chroococcus S24 and *Chroococcus* N41 (cyanobacteria): morphological, biochemical and genetic characterization and effects of water stress on ultrastructure*

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Abstract. Two strains of desiccation-tolerant coccoid cyanobacteria, *Chroococcus* S24, a marine form, and *Chroococcus* N41, a cryptoendolith isolated from a hot-desert rock, have been characterized.

The mol % DNA base compositions of the strains are 47.1 and 48.9 % respectively. Plasmid DNA was not detected in either strain. The pigment contents and nutritional characteristics of the strains are identical. Both lack phycoerythrinoid pigments and, in culture, behave as slow-growing halotolerant marine forms with elevated requirements for Na^+, Cl^-, Mg^{2+} and Ca^{2+}. Sucrose was the only carbon source of those tested that supported photoheterotrophic growth. Each strain synthesizes nitrogenase under anaerobic conditions but not in air. Morphologically the two strains are indistinguishable. They are considered to be independent isolates of the same cyanobacterial species.

Chroococcus N41 was studied in detail with the electron microscope. When brought to equilibrium at matric water potentials of −168 MPa and lower (to −673 MPa = $c0.12\,a_w$) the protoplast shrinks, but the cells maintain the same size and diameter as those at −2,156 kPa (MN medium; control); the sheath expands and remains attached to the cell wall outer membrane by fibrils. The cell wall, cell membrane, thylakoid membranes, cyanophycin granules and carboxysomes appeared intact in desiccated cells.

Key words: Cyanobacteria – Ultrastructure – Nitrogen fixation – Water stress – Taxonomy – DNA – Plasmids

Many cyanobacteria possess the ability to withstand extended periods of desiccation and have the capacity to revive from the air-dry state (Bewley 1979; Brock 1975; Whitton et al. 1979). Information on those structural and/or biochemical mechanisms that may be involved is, however, scant. In an earlier study, we demonstrated that two morphologically-identical strains of cyanobacteria, *Chroococcus* N41, a cryptoendolith isolated from a hot-desert rock, and *Chroococcus* S24, a marine form, while particularly tolerant of desiccation, fixed $^{14}CO_2$ within only a very narrow range of matric water potentials (Potts and Friedmann 1981). The present account provides data on the morphological, biochemical and genetic characterization of these two strains and the effects of matric water stress on the ultrastructure of *Chroococcus* N41.

Abbreviations: DCMU, 3-3,4-dichlorophenyl 1,1-dimethylurea; SCE, 18 % w/v sorbitol, 0.1 M sodium citrate, 10 mM EDTA; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 15 mM sodium citrate, PH 7.0; STET, 8 % w/v sucrose, 5 % w/v Triton X-100, 50 mM EDTA, 50 mM TRIS, pH 8.0; TSED, 25 % w/v sucrose, 1 mM EDTA, 50 mM TRIS, pH 8.0

* Certain of these data were reported to the XIII International Congress of Microbiology, Boston, 1982

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Material and methods

Geographical location of sample sites

Chroococcus N41 was isolated by R.O.F. from a sandstone rock (no. N41) collected by Y. Lipkin in 1964 from an area near Timna, in the Negev Desert, Israel. The cryptoendolithic cyanobacteria growing within rock sample N41 have been described in detail by Friedmann (1971) and Friedmann et al. (1967); in these publications they were assigned to the genus *Gloeocapsa*. *Chroococcus* S24 was isolated initially by I. Baldinger from a limestone rock collected from an intertidal area of the Gulf of Elat, Israel.

Cultures of both strains were obtained from the Culture Collection of Microorganisms from Extreme Environments maintained in the laboratory of E. I. Friedmann at Florida State University.

Chroococcus turgidus

Chroococcus turgidus UTEX 123 was obtained from Dr. R. Starr, the University of Texas Culture Collection of Algae.

Enrichment techniques and isolation

Rock samples with visually obvious growth were fractured under sterile conditions to yield fragments of 1 – 8 cm^3. The fragments were transferred to 7-cm diameter crystallizing dishes (fitted with glass covers) and sterile distilled water was added until the fragments were three quarters submerged. Samples were incubated at 25° C, without agitation, and illumination was provided by cool-white fluorescent lights operated on a cycle of 16 h light:8 h dark. The photon flux density at the surface of the culture vessels was *c* 40 μmol photons $m^{-2}\ s^{-1}$. Incubation was continued for up to 4 months until growth became visually conspicuous. The water level during incubation was kept more or less constant by periodic additions of sterile distilled water. Clonal axenic isolates were obtained from single colonies, after repeated cycles of transfer between solid and liquid media and "streaking" on agar plates (Allen 1968). Currently both strains are maintained on MN medium (Rippka et al. 1979).

Light microscopy

Samples of the microbial community were obtained from rock fragments using mounted needles. The samples were suspended in distilled water and photographed using a Zeiss Standard microscope with Neofluar objectives. For photography, a Leitz Orthoplan microscope (Leitz ×100 plan apochromatic objective n.a. 1.32; 5 lens polarizing condenser with immersion n.a. 1.4) equipped with an Orthomat 35-mm camera was used. Cell wall birefringence was studied and photographed with a Leitz polarizing microscope. Cells from axenic cultures were viewed through a green filter with phase contrast illumination using a Leitz ×40 n.a. 0.65 phaco 2 objective and were photographed with a Nikon FX-35A camera and ancillary Nikon microflex UFX control unit.

Scanning electron microscopy

Rock fragments approximately 1 cm^2 in area, were removed from the original samples for observation using scanning electron microscopy. The unfixed, dry fragments were coated with a gold/palladium film and were studied using a Cambridge Instruments MK IIA scanning electron microscope.

Equilibration of cells at different matric water potentials

Cells in the late logarithmic phase of growth were harvested by filtration through 0.45 µm millipore filters. Filters were then transferred to glass desiccators, each containing a saturated salt solution of a particular water potential: $CaCl_2 \cdot 6H_2O$ ($\Psi_m = -168$ MPa), CH_3COOK ($\Psi_m = -206$ MPa), $LiCl \cdot H_2O$ ($\Psi_m = -673$ MPa). The desiccators were stored at 25° C for 14 days, in subdued light, to allow the filters to reach equilibrium (see Potts and Friedmann 1981).

Transmission electron microscopy

Cell material was prepared for study with transmitted electron microscopy using a modification of the method outlined by Rippka et al. (1974). Some culture material was processed immediately after filtration without storage at a given water potential. This material is referred to elsewhere as the control ($\Psi_m = -2.165$ kPa = water potential of MN medium at 25° C). Cells were pre-fixed at room temperature with 0.5% w/v glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 30 min. The glutaraldehyde concentration was then increased to 3.5% w/v for a further $3\frac{1}{2}$ h at room temperature, after which time the cells were rinsed six times (15 min for each rinse) in 0.1 M sodium cacodylate buffer. Post-fixation was carried out with 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 min at room temperature. The cells were washed five times in buffer and once in distilled water (all operations at 4° C), then dehydrated through an ethanol series. Ethanol was exchanged with acetone and the samples were infiltrated with Spurr's low viscosity embedding resin. After staining in 2% uranyl acetate and Reynold's lead citrate, thin sections were examined in a Phillips model EM20 electron microscope.

Photoheterotrophy

The ability of the strains to grow under photoheterotrophic conditions was assessed using the technique of Rippka (1972). Media consisted of MN liquid (containing 10^{-5} M DCMU) supplemented with one of ten different carbon sources (acetate, glycerol, ribose, arabinose, glucose, galactose, fructose, maltose, sucrose, lactose) at a concentration of either 0.5% w/v or 0.1% w/v. Illumination was provided by incandescent lights with a photon flux density of *c* 40 µmol photons m^{-2} s^{-1} at the surface of each vessel.

Vitamin B_{12} requirement

Aliquots of a concentrated solution of vitamin B_{12} [filtered previously through a 0.45 µm filter (Millipore)] were added to liquid media to give a final concentration of 10 µg l^{-1} B_{12}.

NaCl requirement

Cells were grown in MN supplemented with either 0.2, 0.4, 0.6, 0.8, 1.0 or 2.0 molal sodium chloride. Growth was measured turbidimetrically (Stacey et al. 1977) at 627 nm (Frischknecht and Schneider 1979). Over the range of growth the A_{627} was found to be a reliable indication of cell concentration. Cells were added also to BG11 containing 0.7 molal sodium chloride – the water potential of this osmoticum ($\Psi_o = -3{,}407$ kPa) corresponds to the water potential that was found to support maximum $^{14}CO_2$ uptake by these strains under matric conditions ($\Psi_m = -3{,}407$ kPa; Potts and Friedmann 1981).

Acetylene reduction assay technique

The ability of strains to synthesize nitrogenase under anaerobic conditions was tested using a modification of a technique developed by Rippka and Waterbury (1977). Reduction of acetylene under photoheterotrophic anaerobic conditions was tested for using the same method, except that the 24–36 h preincubation with 5% CO_2/air was omitted and sucrose (0.5% w/v) was added to the cell suspension before anaerobic induction. Aerobic nitrogenase activity was studied using the method of Potts et al. (1978).

Antibiotic sensitivity

The sensitivity of the strains to a range of different antimicrobial agents was tested in two ways. Four milliliters of MN medium containing the appropriate concentration of the agent to be tested were inoculated with 1 ml of a stock culture in exponential growth. Growth was estimated visually during 2 weeks incubation. A modification of the Kirby-Bauer test that has proved successful with other strains of cyanobacteria was used to study the sensitivity of cells growing on solid media. Approximately 50 ml of a stock culture were harvested by centrifugation and the pellet was resuspended in 25 ml molten 0.9% agar w/v in MN. The cell suspension was poured to form a 2-mm deep layer over 1.5% w/v hard agar in a 15-cm diameter petri dish. Antimicrobial agents, absorbed on paper discs (Sensi-Disc; Beckton, Dickinson and Co., USA) were applied to the surface of the agar and incubation was continued for up to 3 weeks.

Sensitivity to lyticase (β-1,3 glucanase/alkaline protease)

The presence of polysaccharides containing β-1,3 glycosidic linkages has been demonstrated in the sheath envelopes of

some cyanobacteria (Cardemil and Wolk 1979), suggesting that lyticase (a mixture of β-1,3 glucanase and an alkaline protease – isolated from *Oerskovia xanthineolytica*; Scott and Schekman 1980) may be of use in weakening and/or removing sheath material prior to standard lytic procedures for obtaining plasmid DNA (see below). Lyticase was obtained from Bethesda Research Laboratories (Rockville, MD, USA) and was used according to the manufacturer's recommendations, but with the following modifications: incubation was continued overnight at 30° C and duplicate assays were performed in SCE buffer at pH 5.8 and pH 8.75. After washing in 1 M sorbitol, the cells were resuspended in TSED buffer and placed on ice 25 min before plasmid analysis (see below).

Pigment analysis

Cells of a 50-ml culture in the late logarithmic phase of growth were disrupted at 110 MPa in a French Pressure Cell. The crude lysate was cleared by centrifugation at 27,000 × *g* for 20 min at 4° C, and the supernatant was then centrifuged at 110,000 × *g* for 60 min at 4° C. The absorbance of the pale blue supernatant was scanned between 380 and 720 nm using a Beckmann Acta III UV-visible spectrophotometer.

DNA extraction

A method was developed that relies upon lysis at 65° C – otherwise the extraction procedure is similar to those of Marmur (1961) and Stam and Venema (1977).

Plasmid analysis

A number of different methods were used to analyze strains for the presence of cccDNA. These included a rapid boiling method (Holmes and Quigley 1981), alkaline extraction procedures (Birnboim and Doly 1979; Crosa and Falkow 1981) and methods based on incubation with lysozyme and subsequent treatment with either ionic or non-ionic detergents (see Crosa and Falkow 1981). For certain procedures the cells were washed four times in 0.5 M NaCl and shaken in 0.5 M sucrose for 30 min, at room temperature, prior to lysozyme treatment. This treatment has been shown to be effective in removing the outer membrane of many marine bacteria (Laddaga and Macleod 1982).

Determination of mol% CG composition

Routinely 0.9 ml of a DNA solution was adjusted to × 1 SSC and then mixed with an equal volume of isopropanol in an Eppendorf microfuge tube and left at −20° C for 1 h. After centrifugation for 10 min at 12,000 × *g*, the DNA pellet was hydrolyzed at 100° C for 60 min using 10 µl 70% w/v $HClO_4$. The solution was adjusted to pH 6.6 using KOH and centrifuged at 12,000 × *g* for 10 min. Aliquots of the supernatant were analyzed using reverse-phase HPLC (Waters system). The column packing was µ Bondapak C_{18}. A mobile phase of 4% acetonitrile: 96% 10 mM KH_2PO_4 buffer pH 6.6 was used at a flow rate of 1 ml min^{-1} with a pressure of 4.4 MPa across the column. Absorption was measured at 254 nm and all peak areas were integrated. With this system, the four bases were eluted within 8 min. Adenine, cytosine, guanine, thymine, *Bacillus subtilis* DNA (A grade – protein free, RNAse treated) and *Escherichia coli* DNA (A grade) were all obtained from Calbiochem-Behring Corporation (Los Angeles, CA, USA). Salmon sperm DNA (sodium salt type III; Sigma, St. Louis, MO, USA) was a gift of R. Reeves.

Results and discussion

The morphological characteristics of *Chroococcus* N41, *Chroococcus* S24 and *Chroococcus* UTEX 123 are summarized in Table 1. Cultures of the last strain are contaminated heavily with bacteria, which attach to, and persist within, the outer sheath (Fig. 1e). Attempts to achieve axenic cultures were unsucessful and therefore only limited characterization of *Chroococcus turgidus* UTEX 123 was attempted. It is clear, nevertheless, that in a number of fundamental aspects *Chroococcus* S24 and *Chroococcus* N41 differ significantly from *Chroococcus turgidus* UTEX 123. All three strains share the property of cell-wall birefringence when observed with the polarizing microscope. This finding suggests the presence in each of regularly arranged crystalline components in the cell wall. A critical survey of the presence of cell-wall birefringence in other cyanobacteria is needed, however, before the utility of this property as a taxonomic character can be assessed. The property has been observed in other coccoid forms (Ocampo 1973).

Morphological properties

Chroococcus S24 and *Chroococcus* N41 are virtually identical in terms of size and general appearance (Fig. 1c, d). The color of the cells is particularly stable and remains unchanged when the strains are grown in MN medium under light sources with quite different spectral characteristics. While variable in culture, a feature of both strains is a pronounced granulation of the cytoplasm. Generally, division of the cells is in one plane, division in more than one plane is infrequent in culture, and division in three planes has *never* been observed.

The two strains have been maintained in axenic culture for several years and still retain the thick colorless outer sheath layer that was observed in cells growing *in situ* (Fig. 1b). Other authors have drawn attention to the persistence of sheath and surface layers of *Gloeothece* and *Synechocystis* strains maintained for many years in pure culture (Vaara 1982). Such observations tend to suggest a significant role for these structures in the growth of the strains in culture.

The presence of laminations in the sheath is a variable feature, but they are seen typically in older cells. Rupture of the outer sheath occurs spontaneously in culture and may be induced, to some extent, by suspension of cells in sterile distilled water (Fig. 1f, g). When ruptured, the sheath appears much thicker than in intact cells and a radial fibrillar pattern can be discerned (Fig. 1f). This pattern was observed also in *Chroococcus turgidus* UTEX 123. The apparent increase in thickness of the sheath after rupture suggests that the cell exerts a positive pressure on the inner surface of the intact sheath. After division, half cells are *always* hemispherical when confined by the constraints of the sheath (Fig. 1c–e) but become spherical if released through a torn outer sheath (Fig. 1h). The cells, nevertheless, remain attached to one another and still possess a thin sheath layer. If division should proceed through two planes, each cell of a tetrad is of the shape of a circle quadrant with rounded corners.

Table 1. Morphological characteristics of *Chroococcus* strains N41 and S24 and *Chroococcus turgidus* UTEX 123 as determined by light microscopy[a]

	Character	N41	S24	UTEX 123
Cell morphology	Cell shape	spherical	spherical	spherical
	Cell shape after division	hemispherical	hemispherical	hemispherical
	Cell division	1 or 2 planes	1 or 2 planes	1 or 2 planes
	Mean cell diameter	24.9 ± 3.15 µm	29.7 ± 2.39 µm	50.0 ± 3.5 µm
	Mean cell length	22.08 ± 5.82 µm	20.08 ± 7.07 µm	25.0 ± 1.0 µm
	Cell color	rejane green	rejane green	palmetto
	Motility	+	+	ND
	Baeocyte formation	−	−	−
	Sheath	colorless, thick (up to 5 µm)	colorless, thick (up to 5 µm)	colorless, thick (up to 8 µm)
	Laminations of sheath	presence variable	presence variable	present
	Cytoplasm	granular	granular	granular
	Gas vesicles	−	−	−
	Chromatoplasm boundary	smooth	smooth	smooth
	Cell wall birefringence	+	+	+
Colonial morphology	Appearance of cell suspension	(blue-green) cells do not adhere to one another	(blue-green) cells do not adhere to one another	ND cells do not adhere to one another
	Growth on agar	2 mm diameter, shiny[b], smooth[b], butyrous	2 mm diameter, shiny[b], smooth[b], butyrous	ND
Ecology	Source	cryptoendolith; Nubian sandstone, medium to coarse grain; Negev Desert, Israel hot desert rock	endolith; limestone; intertidal zone, Gulf of Elat, Israel intertidal rock	isolated by Dr. R. Starr 1952; flooded limestone quarry, Bloomington, Indiana (Dr. J. Zeikus, pers. comm.)
Generic/specific assignments possible	(1) After Geitler (1932)	*Chroococcus turgidus*	*Chroococcus turgidus*	*Chroococcus turgidus*
	(2) After Rippka et al. (1979)	*Gloeocapsa* sp.	*Gloeocapsa* sp.	*Gloeocapsa* sp.
	(3) After Drouet and Daily (1956)	*Anacystis dimidiata*	*Anacystis dimidiata*	*Anacystis dimidiata*

[a] The format of this table follows closely that outlined by Trüper and Krämer (1981) for use in the description of new species of prokaryotes
[b] See the classification scheme of Bold and Parker (1962) for colonial attributes of unicellular Chlorophyta on solid media
ND = not determined

Biochemical and genetic characterization

Optimum growth of the strains was achieved at 25°C. Vigorous agitation of cultures, as well as photon flux densities in excess of 40 µmol photons m^{-2} s^{-1} tended to kill cells.

Under different light conditions, the major pigments present in both strains were chlorophyll *a*, C-phycocyanin and allophycocyanin; phycoerythrinoid pigments were not detected.

Neither of the strains is capable of growth in the "freshwater" medium BG-11 or in BG-11 medium supplemented with NaCl only. Both strains grow in MN medium with generation times of 60 h (*Chroococcus* N41) and 72 h (*Chroococcus* S24). Optimal growth is achieved in MN medium supplemented with 0.2 molal NaCl (Ψ_o = −3,080 kPa), the generation times being 48 and 36 h, respectively (Table 2). Higher concentrations of NaCl inhibit growth, *Chroococcus* S24 generally showing a somewhat greater sensitivity than *Chroococcus* N41 (Fig. 2).

The mean generation times of the two strains are not particularly different from those of a number of other slow-growing coccoid cyanobacteria such as *Synechococcus* 7001 and *Gloeothece* 6909 (Rippka et al. 1974) but are markedly slower than those found for several other marine strains, e.g. *Agmenellum quadruplicatum* PR-6 (Van Baalen 1962) and somewhat faster than that of *Gloeobacter 7421*. Most fast-growing strains of cyanobacteria lack a prominent sheath, but further investigation of the nutritional requirements of slow-growing strains is needed before any conclusions can be drawn concerning a correlation between the possession of a thick sheath and a tendency to grow slowly. Results of experiments using lyticase and Congo Red suggest that β-1,3 linkages are absent in the sheath layers of *Chroococcus* S24 and *Chroococcus* N41.

In liquid cultures, cells grow as a thin deposit over the base of the culture vessel and are easily detached and resuspended by swirling of the flask contents. No obvious vitamin B_{12} requirement could be demonstrated for either strain, and sucrose was the only carbon source of those tested that supported photoheterotrophic growth. Addition of sucrose to cultures growing under photoautotrophic conditions lead to a change in the color of the cells from bright blue-green to grass-green, but no decrease in mean generation time.

Both strains reduce acetylene under anaerobic conditions but not in the presence of air. They cannot grow in MN_o medium (Rippka et al. 1979) under air. Under photoheterotrophic conditions, in the absence of air and presence of sucrose – the only carbon source tested found to support photoheterotrophic growth – nitrogenase activity was not detected after 24 h incubation with acetylene.

It is of interest to consider several of the data available for unicellular cyanobacteria that synthesize nitrogenase. A number of strains of *Synechococcus* (which lack a sheath) do so only anaerobically, while five strains of *Gloeothece*, which

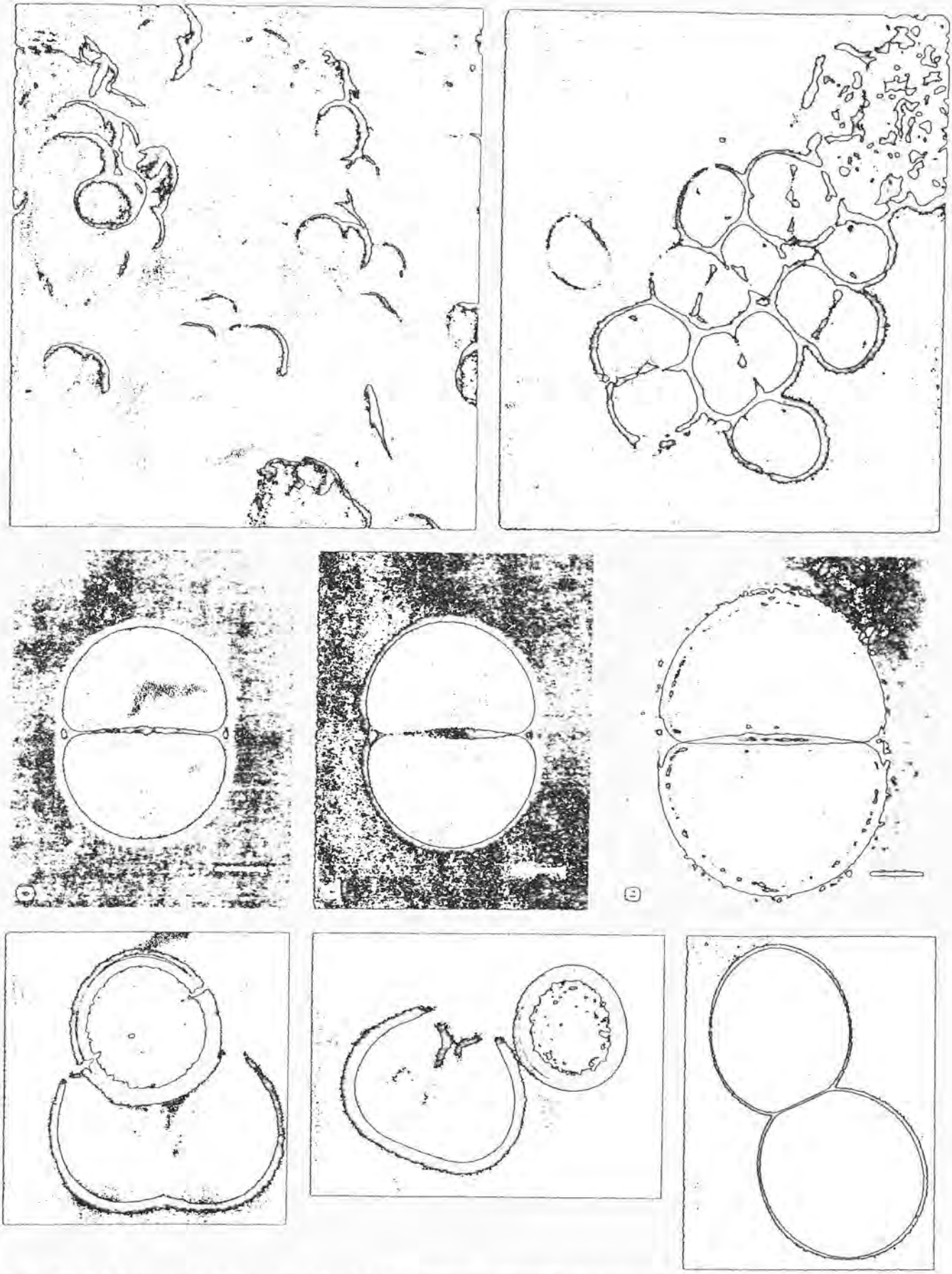

Fig. 1. a SEM of *Chroococcus* N41 in rock sample; b cells of *Chroococcus* N41 immediately after removal from rock; c *Chroococcus* N41, axenic culture; d *Chroococcus* S24, axenic culture; e *Chroococcus turgidus* UTEX 123, non-axenic; f *Chroococcus* S24, cell released from ruptured sheath (arrows indicate plane of division); g *Chroococcus* N41, cell released from ruptured sheath; h *Chroococcus* S24 "hemispherical" cells after release from sheath. Scale bar = 10 μm. Figure 1 a and b are reproduced from Friedmann (1971) with permission of the publishers

Table 2
Comparison of the mean generation time of *Chroococcus* N41 and *Chroococcus* S24 with other coccoid and filamentous cyanobacteria

Strain	Mean generation time (h)	Source
Chroococcus S24	43	present study
Chroococcus N41	36	present study
Gloeobacter 7421	73	Rippka et al. (1974)
Aphanocapsa 6714	27	Rippka et al. (1974)
Synechococcus 7001	36	Rippka et al. (1974)
Gloeothece 6909	40	Rippka et al. (1974)
Anacystis nidulans TX 20	2.0	Kratz and Myers (1955)
Agmenellum quadruplicatum PR-6	3.4	Van Baalen (1962)
Anabaena CA	4.3	Stacey et al. (1977)
Anabaena cylindrica	14	Weare and Benemann (1973)
Anabaena 7120	12	Peterson and Burris (1976)

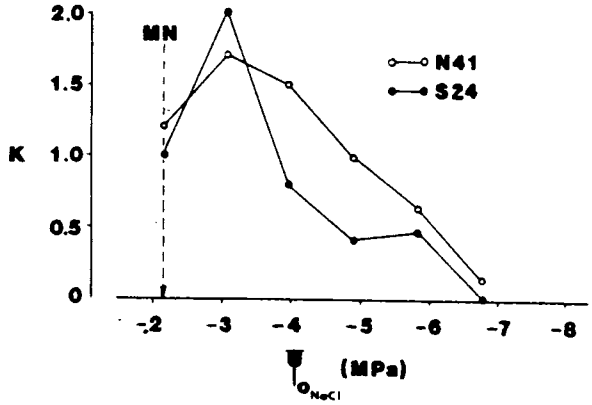

Fig. 2. Effect of osmotic water potential ($\Psi_{o_{NaCl}}$) on the specific growth constant (K) in $\log_{10}$ units per day × 10; $K = \log N - \log N_o/(0.301)t$

Table 3. The mol % GC content of DNA's from *Chroococcus* strains and standards as estimated with HPLC

Source	From T_m[a]	Literature[b]	HPLC
Bacillus subtilis	43.0	42.4	40.6
Escherichia coli	50.0	50.1	50.2
Salmon sperm	43.0	41.2[c]	41.4
Chroococcus N41	NA	NA	47.1
Chroococcus S24	NA	NA	48.9

[a, b] Marmur and Doty (1962)
[c] Includes substitution of cytosine by methylcytosine
NA = not available

possess a sheath, can also reduce acetylene aerobically (Rippka et al. 1979). *Chroococcus* N41 and *Chroococcus* S24, which have a prominent sheath, reduce acetylene only under anaerobic conditions. This finding suggests that there may be different mechanisms for protecting nitrogenase in unicellular forms grown aerobically rather than simply an ability to form a thick sheath (see Stewart 1980).

The methods used to assess antibiotic sensitivity were unsatisfactory, largely because of the very slow growth rates of the two stains.

Chroococcus N41 and *Chroococcus* S24 have identical nutritional characteristics and behave as halotolerant marine forms with elevated requirements for Na^+, Cl^-, Mg^{2+} and Ca^{2+}.

The mol % GC contents of the DNA's from *Chroococcus* S24 and *Chroococcus* N41 are very similar, 48.9 and 47.1 mol % respectively (Table 3). The HPLC method gives reproducible and apparently accurate values for the GC contents of DNA's from other sources.

While susceptible to treatment in a French Pressure Cell, *Chroococcus* S24 and *Chroococcus* N41 show a marked resistance to agents that are commonly used to lyse cells gently or weaken cell walls. The strains are both resistant to lysozyme and show negligible increase in sensitivity after preincubation in the presence of either 4% w/v SDS, at 65° C for 3 h, or pronase (250 µg ml^{-1}). Cells do not lyse when boiled for 60 s in STET buffer. They resist repeated freezing and thawing and EDTA treatment, and the cells appear intact after several washes in 0.5 M NaCl, 0.5 M sucrose, and subsequent lysozyme treatment. Lysis of the cells was achieved using alkaline hydrolysis at pH 12.4 in the presence of 4% w/v SDS, although the relative efficiency depended largely upon the age and density of the cell suspension. When cleared lysates, prepared by this method, were subjected to gel electrophoresis in the presence of ethidium bromide, a single diffuse band was observed upon irradiation of the gel with UV light. The position of the band in the gel was the same for each strain and corresponds to chromosomal DNA. Plasmid DNA was not detected using this method.

Taxonomy

Traditionally, *Chroococcus* and *Gloeocapsa* are considered the major genera of ensheathed, coccoid cyanobacteria that divide in two or three planes. In *Chroococcus*, the cells are hemispherical after division; in *Gloeocapsa* they are spherical (Geitler 1932). The shape of cells after division is influenced by the outer sheath layers and may be variable in field material as well as in culture, a fact that persuaded Rippka et al. (1979) to propose the recognition of the single genus *Gloeocapsa*, with *Chroococcus* as a later synonym. A general paucity of information on cyanobacterial sheath structure, composition, synthesis and function, and the *persistent* hemispherical post-division cell shape in culture have led us to assign the genus *Chroococcus sensu* Geitler to the strains in this study. The taxon *Chroococcus turgidus* (Kütz.) Näg. is regarded by Geitler (1932) as a Sammelspezies, i.e. a collection of polymorphs that may or may not represent different species. The use of characters such as presence or absence of lamination in the sheath, color of the sheath, and color of the cells does not permit a satisfactory discrimination to be made between other *Chroococcus* "species" such as *C. tenax*, *C.*

westii and *C. schizodermaticus*. Nevertheless, Geitler made the distinction between the "type" *C. turgidus* found in peat-bogs (generally with indistinct lamination of the sheath and bright blue-green, never violet, cells) and the form found on moist rocks (with a dull coloration and pronounced lamination)[1].

The mol % GC contents of the DNA's of the two strains in this study (47.1; 48.9 %) are greater than the upper value (46 %) reported for the cluster of four strains assigned to *Gloeocapsa* by Rippka et al. (1979). The two *Chroococcus* strains also differ from all four *Gloeocapsa* strains in one fundamental property: synthesis of nitrogenase under anaerobic conditions. Although no data are available on DNA-DNA hybridization for these strains, the present evidence suggests strongly that they are one and the same species. This species is distinct from UTEX strain 123, a freshwater form that was allocated the binomial *Chroococcus turgidus* after its isolation in 1952.

Influence of matric water stress upon the ultrastructure of Chroococcus N41

Cells inside rocks suffer no apparent shrinkage, and viable cells were recovered after 6 years of dry storage (Fig. 1a, b).

When cells were brought to equilibrium at water potentials as low as −673 MPa (*c* 12 % R.H.) the spherical shape and overall cell diameter remained the same as in cells at −2,165 kPa (control), but shrinkage of the protoplast was pronounced (Fig. 3a, b). This shrinkage appeared to be "uniform" as judged by the relatively constant distance between the sheath and protoplast perimeters and the homogeneous appearance of the sheath in low magnifications (Fig. 3b).

The ultrastructure of the cells is somewhat similar to that observed for other unicellular coccoid cyanobacteria (Drews and Weckesser 1982) with several notable exceptions. There is no obvious partition of the cell contents into peripheral chromatoplasm and central nucleoplasm (centroplasm) as seen in other coccoid cyanobacteria (Golecki and Drews 1982). The thylakoids are tightly appressed and fill the cells in a more or less random arrangement (Fig. 3a–c). Fimbriae and spinae (and other cell wall elaborations, see Vaara 1982; Lounatmaa et al. 1980) are absent. Superficially, the sheath is similar in structure and appearance to the fibrous material deposited outside, but adherent to, the outher membrane of pleurocapsalean cyanobacteria (Waterbury and Stanier 1978). The sheath is fibrous and, under normal growth conditions, appears as two distinct layers (termed S_1 and S_2) separated by an electron-dense region (Fig. 3c, d). Similar arrangements and structures have been observed in strains of *Gloeothece* (Vaara 1982), *Gloeocapsa* (Cox et al. 1981) and *Gloeobacter* (Rippka et al. 1974) and seem to correspond to laminations seen with the light microscope. In the outermost layer, S_1, fibres appear to follow, and run parallel to, the cell wall (Fig. 3c), a feature that becomes more apparent in cells maintained at lower water potentials (Fig. 3e) and that is, surprisingly, in contrast to the radial pattern seen with the light microscope (Fig. 1f). The orientation of fibrils in the S_2 layer is less distinct. This layer remains in close contact with the cell wall, even when the protoplast is shrunken, and appears as a loose matrix of interconnecting fibres (Fig. 3e, h).

The electron-dense region is most conspicuous in cells subjected to water stress, appearing irregular and nodulated: a loose arrangement of fibres runs perpendicular to it and connects it to the S_1 layer (Fig. 3f, h). Cox et al. (1981) suggested that the electron-dense lamellae observed in the sheath of a *Gloeocapsa* strain were composed of acidic mucopolysaccharides as no staining was seen in unstained sections or in those stained only with uranyl acetate.

The uniformity in the appearance and thicknesses of each of the different layers observed in the sheaths of cells maintained at low water potentials is striking (Fig. 3f). Even in desiccated cells at −673 MPa, the S_2 layer remains in close contact with the cell wall (Fig. 3e, g, h) and no clear separation was observed between the two as has been reported for other coccoid forms fixed under "normal" conditions (see Cox et al. 1981). A loose network of strands and fibrils connects S_2 with the outer membrane of the cell wall (Fig. 3g).

Overall, the major differences between cells grown at −2,156 kPa and those maintained at extreme low water potentials are the shrinkage of the protoplast and expansion of the sheath in the latter. Interestingly, even in shrunken protoplasts the cell wall, cell membrane, and major inclusions such as thylakoid membranes, carboxysomes and cyanophycin granules all retain their structural integrity without any apparent or obvious damage (Figs. 3g, h; 4g–i). A conspicuous difference between cells at −2,156 kPa and those kept below −168 MPa is, however, the appearance in the latter of regular arrangements of granules between thylakoids (Fig. 4f, h). These aggregates are, presumably, polyglucan (polyglucosyl granules; Fogg et al. 1973). In *Agmenellum quadruplicatum* PR-6, a similar marked accumulation of polysaccharide was induced when cells were grown under nitrate limitation (Stevens et al. 1981), and polyglucan granules replace cyanophycin granules abruptly at temperatures above 17° C and then decrease above 20° C in *Spirulina platensis* (Van Eykelenburg 1979). Such accumulations may be a general feature of cells exposed to stress. For *Chroococcus* N41, however, it is of greater interest to pose the following question: does the accumulation of polyglucan represent de novo synthesis during water stress, or do pre-existing granules become more conspicuous because of the removal of cell water?

Membrane structures are common in cells grown under normal conditions as well as those kept a low water potentials (Figs. 3f; 4a–e). Typically these were located toward the periphery of cells and sometimes near the sinus of a dividing protoplast (Fig. 4a), although no connection with the cell membrane could be observed (Fig. 4b–d). In all examples studied, the structures had a distinctive honeycomb appearance and were almost always associated with an accumulation of lipid droplets (Fig. 4b–e). In one case, the structure was associated with a carboxysome at the cell periphery (Fig. 4b). The structures bear no resemblance to those specialized membrane arrangements observed occasionally in cyanobacteria and referred to variously as mesosomes, lamellasomes, lamellar elaborations, or plasmalemmasomes (Allen 1972; Echlin 1964; Edwards et al. 1968; Ingram and Thurston 1970; Niklowitz and Drews 1956; Van Eykelenburg 1979; Whitton and Peat 1969).

Chroococcus N41 and *Chroococcus* S24 are two examples of coccoid cyanobacteria that can survive prolonged de-

1 Extensive detailed information on *Chroococcus* species as well as photographs of many herbarium specimens can be found in Drouet and Daily (1956)

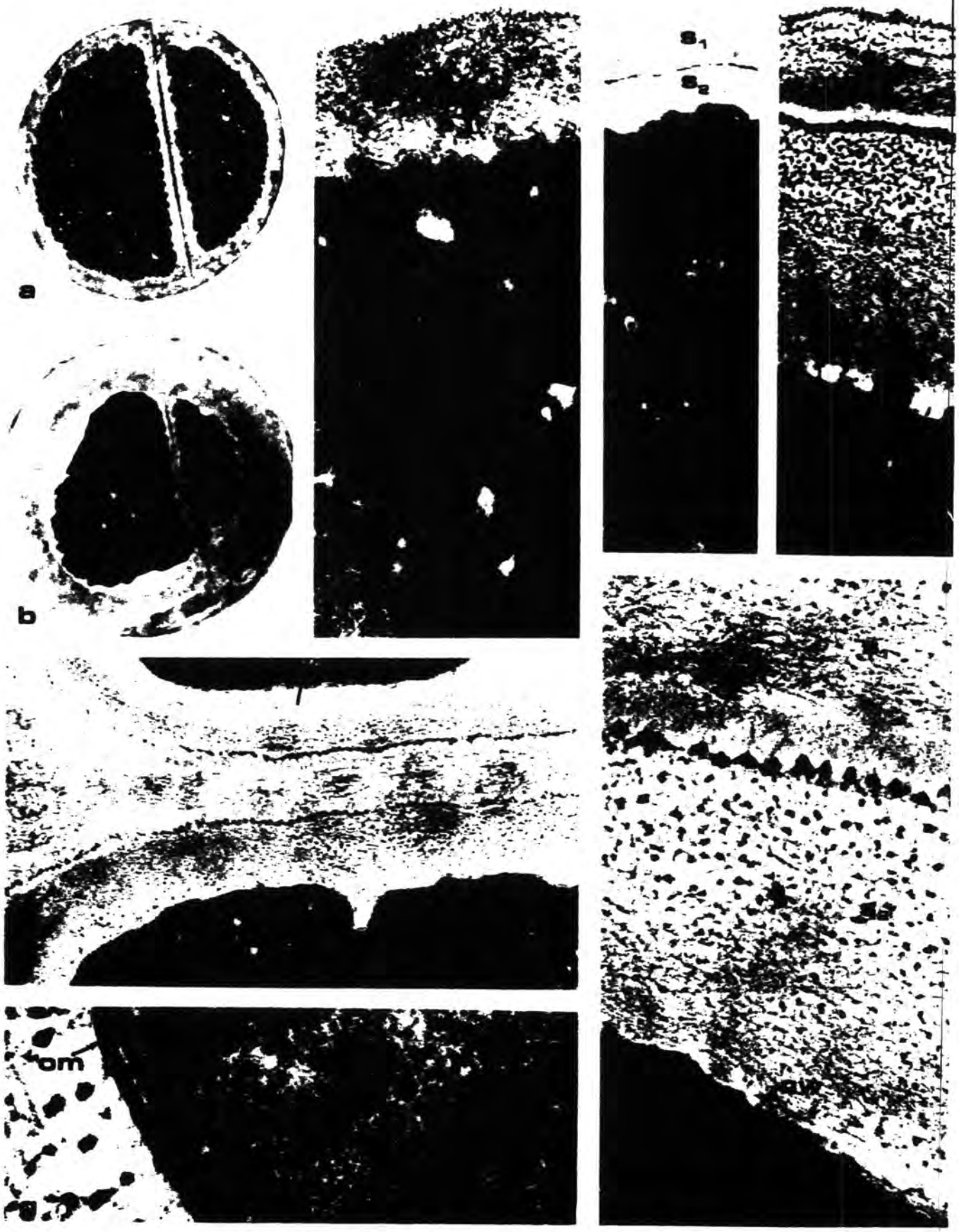

Fig. 3a–h. *Chroococcus* N41. **a** $\Psi = -2{,}156$ kPa, × 4,250; **b** $\Psi = -673$ MPa, × 4,250; **c** $\Psi = -2{,}156$ kPa, sheath layers S_1 and S_2, × 30,630; **d** $\Psi = -2{,}156$ kPa, sheath layers S_1 and S_2, × 25,525; **e** $\Psi = -206$ MPa, sheath layers S_1 and S_2, × 25,525; **f** $\Psi = -673$ MPa, sheath layers between daughter cells, arrow indicates membrane structure, × 45,706; **g** $\Psi = -206$ MPa, cell wall showing outer membrane (*om*), peptidoglycan layer (*pg*), cell membrane (*cm*); thylakoid (*t*), × 115,400; **h** $\Psi = -206$ MPa, higher magnification of **e**, cell wall (*cw*), sheath layers (S_1, S_2), vertical fibril layer between electron dense layer and S_1, × 75,000

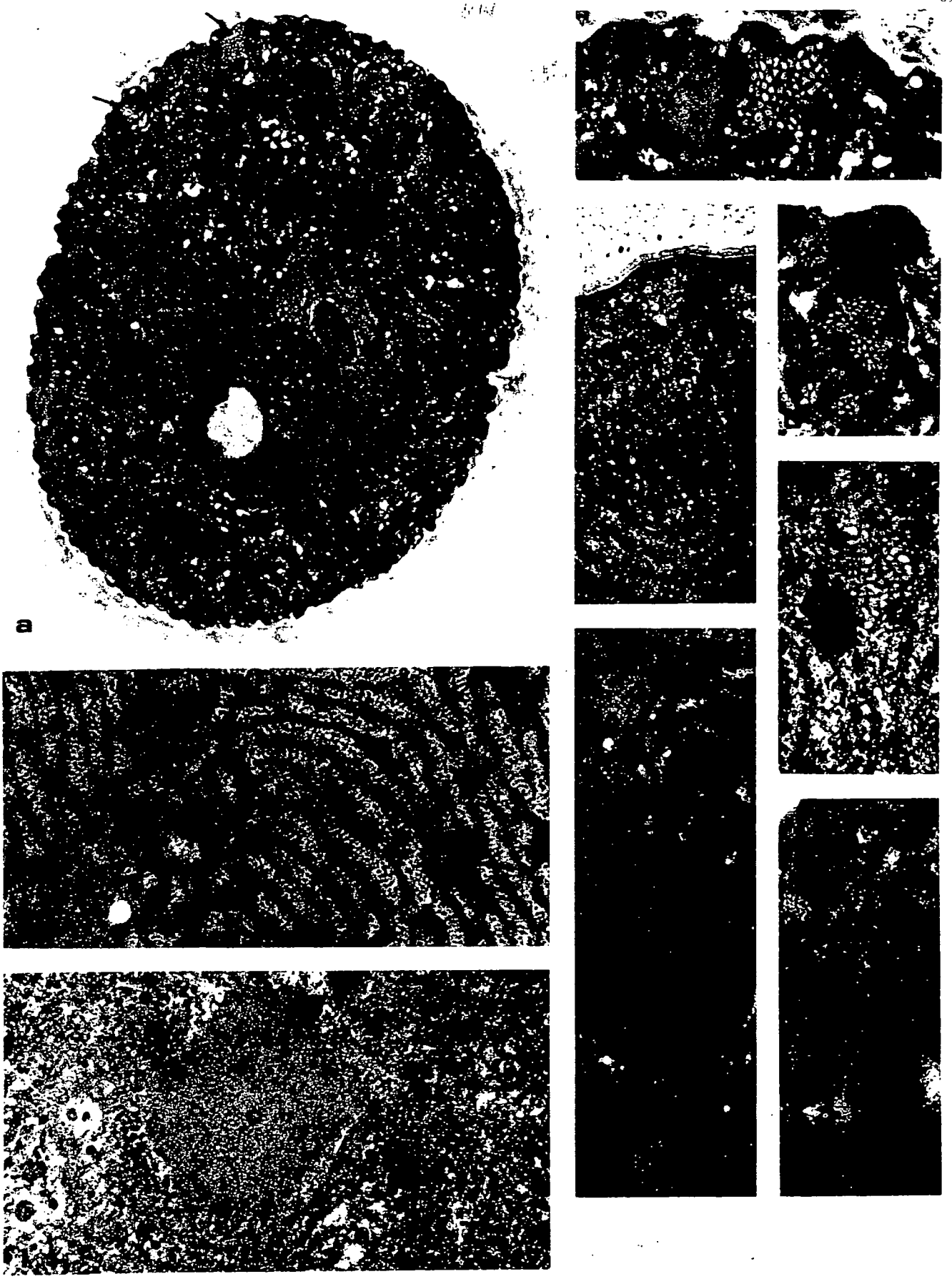

Fig. 4a–i. *Chroococcus* N41. **a** $\Psi = -2{,}156$ kPa, tangential section of dividing cell, arrows indicate membrane structures, × 10,725; **b** $\Psi = -2{,}156$ kPa, carboxysome (*c*) with associated membrane structure, × 33,100; **c** $\Psi = -673$ MPa, membrane structure, × 45,422; **d** $\Psi = -206$ MPa, membrane structure, × 33,100; **e** $\Psi = -206$ MPa, membrane structure, × 49,680; **f** $\Psi = -673$ MPa, accumulation of polyglucan granules, × 30,736; **g** $\Psi = -2{,}156$ kPa, cyanophycin granule, × 33,100; **h** $\Psi = -168$ MPa, cyanophycin granules and polyglucan granules, × 23,483; **i** $\Psi = -673$ MPa, carboxysome (*c*), thylakoid (*t*), × 63,590

siccation and that can revive when rewetted. This tolerance is particularly significant for cells at −673 MPa, a water potential where the cells have, presumably, lost more than 99 % of their cell water (Mazur 1980).

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The ultrastructure of immobilised desiccated cells of the cyanobacterium *Nostoc commune* UTEX 584

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Key words: *Nostoc commune*; Matric water stress; Anhydrous fixation; Ultrastructure

1. SUMMARY

Cells of the cyanobacterium *Nostoc commune* UTEX 584 were immobilised, subjected to acute matric water stress ($\psi_m = -128$ MPa) and then desiccated. Their ultrastructure was investigated by the use of an anhydrous fixation procedure. Although shrunken and bleached, the integrity of the vegatative cells at the ultrastructural level was apparently preserved. The ease with which certain cyanobacterial cells can recover from desiccation may be consequent upon the maintenance of cellular organisation at the ultrastructural level.

2. INTRODUCTION

Despite numerous studies there is still no consensus of opinion on the sequence of events that leads to the suppression of cell growth by water stress [1–3]. The view has been expressed that the most meaningful approach to the problem is to investigate forms which are adapted to acute water stress, and which also experience this stress in their normal habitat [4–7]. The cyanobacteria, which occupy a wide range of habitats, are often subject to wide fluctuations in environmental conditions [8,9]. Many are able to survive extended periods of desiccation and a number of studies have examined physiological and biochemical parameters under conditions of both osmotic and matric water stress [10–16]. *N. commune* shows a marked capacity for desiccation tolerance; the purpose of this study was to devise a procedure which would permit examination of the cells in the desiccated state in an attempt to identify ultrastructural features which may contribute to this trait. Additionally the technique had to be of use in subsequent immunochemical studies.

3. MATERIALS AND METHODS

3.1. Growth conditions and immobilisation of cells

Conditions of growth and immobilisation are described elsewhere [13,17] with the exception that the photon flux density was 50 μmol photons m^{-2} s^{-1}.

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3.2. Preparation for transmission electron microscopy

3.2.1. Control material

Cells, in exponential growth, were washed once in distilled water and then resuspended in 2% v/v glutaraldehyde in 50 mM phosphate buffer (pH 7.1) for 2 h, at room temperature. After washing, the cells were dehydrated rapidly through an ethanol series.

3.2.2. Desiccated cells

The nylon support with its layer of desiccated cells was immersed, for 2 h at room temperature, in an anhydrous solution of 2% (v/v) glutaraldyhyde, made from 70% w/v glutaraldehyde and anhydrous acetone. Both the acetone, and the glutaraldehyde solution in acetone, were equilibrated over an excess of Linde 4A molecular sieve pellets (modified form [18]). The cells were then washed in 4 changes of 100% ethanol to ensure removal of acetone. Both samples were embedded in LR White hard-grade resin. Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM10.

4. RESULTS AND DISCUSSION

In aseriate cells from the control material (Fig. 1) the organisation of the wall layers is similar to that of other cyanobacteria [19]. External to the outer membrane is a space which, typically, separates it from the layered fibrous sheath material. A mesosome-like structure [20] is also associated with the plasma membrane of this cell. The thylakoids, which permeate the cell, exhibit a characteristic whorled arrangement; a peripheral distribution is rarely seen. Ribosomes are present within the whorls, and also in the nucleoplasmic area of the cell. Larger granules, and electron-lucent areas of polyphosphate, similar to those observed in *Plectonema boryanum* [21], are in evi-

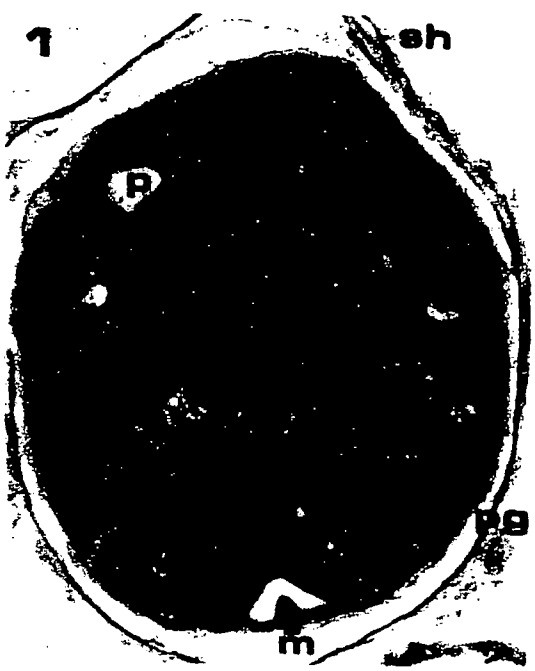

Fig. 1. Aseriate cell from control culture. ×19 250. Abbreviations: c, cyanophycin granule; ds, damaged sheath; is, incomplete septum; m, mesosome; p, polyphosphate granule; pg, polyglucoside granule; po, heterocyst pore; r, ribosome; sh, sheath; th, thylakoid; w, heterocyst wall.

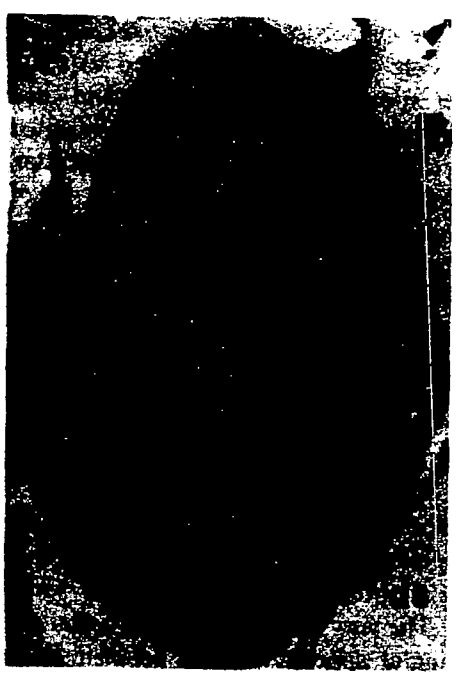

Fig. 2. Aseriate cell after acute water stress ($\psi_m = -128$ MPa), desiccation and exposure to a photon flux density of 50 μmol photons m^{-2} s^{-1} for 14 days. ×22 250. For abbreviations, see legend to Fig. 1.

Fig. 3. Seriate cell after acute water stress, desiccation and exposure to light for 14 days. An incomplete septum can be discerned. ×24 250. For abbreviations, see legend to Fig. 1.

dence. An extensive membrane system and large numbers of polyphosphate granules were observed in hypolithic cyanobacteria which are naturally exposed to high temperatures, low light intensity, and low relative humidity [22]. Deposits of polyglucoside occur in the interthylakoidal spaces (see Fig. 4). Carboxysomes and cyanophycin granules are also found, although the latter appear infrequently. Aseriate, immobilised cells are shrunken and the sheath and wall layers external to the

Fig. 4. Polyglucoside granules between thylakoids in an aseriate cell from the control culture. ×66 500. For abbreviations, see legend to Fig. 1.

Fig. 5. Desiccated aseriate material showing reduced interthylakoidal space and polyglucoside granules. ×66 500. For abbreviations, see legend to Fig. 1.

plasma membrane may be damaged (Fig. 2). This is probably an artefact consequent upon the characteristically thick and compact sheath. The integrity of both the plasma membrane and the thylakoid membrane system appears always to have been preserved, with the whorls becoming accentuated by dense central areas. Such areas may represent nucleoplasmic material, ribosomes

Fig. 6. Degenerate heterocyst from desiccated material showing pore, lack of internal organisation and distorted wall layers. ×22 250. For abbreviations, see legend to Fig. 1.

and associated polyphosphate, which have become condensed as a consequence of the removal of water. A reduction in the interthylakoidal space also serves to heighten the accentuation. Polyglucoside granules can still be discerned in this space (see Fig. 5). The wall layers in desiccated cells of the seriate stage appear intact, although there is marked separation of the peptidoglycan layer from the plasma membrane (Fig. 3). In all other respects the ultrastructural features resemble those of the aseriate stage. In a previous study with strains of *Chroococcus*, intracellular inclusions appeared intact after prolonged desiccation—this study did not, however, utilise non-aqueous fixation [23]. Despite their thickened walls heterocysts showed evidence of damage after desiccation. The wall layers, although intact, were deeply indented and the cellular contents showed no signs of internal organisation (see Fig. 6). The adjacent vegetative cell had an apparently intact thylakoid system. Heterocysts from axenic cultures of *N. commune* UTEX 584 appear to be weakened by desiccation and are susceptible to breakage under certain conditions of rewetting [17].

Our previous work demonstrated that extended desiccation of immobilised *N. commune* leads to pronounced protein degradation through light-dependent effects [13]. We suggested that photo-oxidation was responsible and this contributed in part, to the more rapid recovery of nitrogenase activity, in dark-stored cells, when they were rewetted [17]. In the present study photo-oxidative bleaching does not appear to have resulted in thylakoid degradation. Indeed, their integrity, in the vegetative cells, is remarkable in view of the severity of the stress imposed upon the cells. Photooxidation has been shown to lead to progressive loss of thylakoids in *Anacystis nidulands* and also to the evolution of significant amounts of ethane [24]. The latter is indicative of lipid and, presumably, membrane degradation. Cells of *N. commune*, exposed to a range of different water stresses, have not been shown to evolve ethane (unpublished data). The loss of chlorophyll from immobilised cells which were desiccated in the light reflects, presumably, pigment degradation. A dissociation of phycobilisomes, and loss of phycobiliprotein (predominantly light-dependent) has already been recorded for *N. commune* under these conditions [13]. Other conditions which lead to the loss of high-M_r chl-protein complexes and phycobilisomes from thylakoids include iron deficiency [25,26]. Iron induced reconstitution of such starved cells has been shown to provide a useful developmental system for studying the assumbly of the photosynthetic apparatus in the thylakoid membrane [27]. These results suggested an initial incorporation of phycobiliproteins and chlorophyll into preexisting membranes followed by synthesis of new membrane systems. A similar mechanism may operate in rewetted desiccated cells of *N. commune*. A sequential restoration of respiration, photosynthesis, then nitrogen fixation was recorded in three species of *Nostoc*, when they were rewetted following a 2-year drought period [15]. It was suggested that the availability of ATP and reduced pyridine nucleotides may be decisive for recovery. Similar sequential recoveries of the size of the intracellular ATP pool and capacity for nitrogen fixation have also been recorded [15–17,28]. Our results suggest that reserve materials are maintained intact during desiccation and, as such, they may provide the substrates required for the recovery of cell function upon rewetting. Additionally, as the heterocysts were evidently damaged by desiccation, the time taken for the recovery of nitrogen fixation may reflect the time needed for new heterocysts to be differentiated.

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Ultrastructural Analysis of the Rehydration of Desiccated *Nostoc commune* HUN (Cyanobacteria) with Particular Reference to the Immunolabelling of NifH

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Summary

Vegetative cells and heterocysts of the filamentous desiccation-tolerant cyanobacterium *Nostoc commune* HUN retain their ultrastructural organisation and the integrity of their intra- and extracellular membranes after two years of desiccation and subsequent rehydration. Immunogold-labelling of thin sections demonstrated the presence of NifH (Fe protein of nitrogenase) in vegetative cells and heterocysts within five minutes of the rehydration of dried colonies. Immunogold label accumulated in discrete areas vegetative cells within 5 minutes of the rewetting of cells, and after 30 minutes a conspicuous association of NifH protein with heterocyst ribosomes was detected. After longer periods of rehydration, the deposition of gold particles became more random within both cell types but occurred with a greater frequency in heterocysts. Up to 24 hours after the rewetting of cells, two morphologically-distinct forms of heterocyst could be discerned. NifH protein was detected through Western blotting (subunit M_r = 33,800) in protein extracts from samples of *Nostoc commune*, collected in different parts of the world and including some which had been desiccated for periods of up to 10 years. The results are discussed in relation to the sequential recovery of metabolic functions, particularly nitrogen fixation, which occurs upon the rehydration of cells after their prolonged storage in the air-dry state.

Keywords: Heterocysts; Nitrogenase; Fe protein; *nif* gene expression; Oxygen sensitivity; Sheath material.

1. Introduction

Opinions vary as to the sequence of events which leads to the suppression of plant growth by water stress as well as the nature of desiccation tolerance. It is clear, nevertheless, that the capacity of certain cells for desiccation tolerance is the reflection of a complex array of factors which interact at the ultrastructural, physiological and molecular level (Leopold 1986).

Nostoc commune is a filamentous nitrogen-fixing cyanobacterium that shows a marked capacity for desiccation tolerance (Potts and Bowman 1985). In the absence of a source of fixed nitrogen, vegetative cells of this photosynthetic prokaryote can undergo differentiation to form structurally- and biochemically-modified cells, the heterocysts, which permit *N. commune* to fix nitrogen aerobically (Carr and Whitton 1982). Although heterocysts are terminally differentiated, they continue to synthesize RNA and protein and these processes are thought to contribute to the functional lifetime of the heterocyst (Lynn and Ownby 1987). In the absence of molecular oxygen, the differentiation of heterocysts is arrested and the vegetative cells of Nostocacean cyanobacteria express the genetic information which encodes the synthesis of nitrogenase (Rippka and Stanier 1978, Helber *et al.* 1988). However, because nitrogenase enzyme shows acute sensitivity to oxygen, the heterocysts are thought to be the sole sites of nitrogenase activity in filaments of heterocystous cyanobacteria when they are grown aerobically, though heterocysts may not necessarily be the sole sites of nitrogenase synthesis under these conditions (Fleming and Haselkorn 1974, Rippka and Stanier 1978). Significantly, aerobic nitrogen fixation has been observed in certain unicellular and filamentous non-heterocystous

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cyanobacteria (KALLAS *et al.* 1983), RIPPKA *et al.* 1979, STAL and KRUMBEIN 1985).

Previous studies with pure cultures of *N. commune* UTEX 584 and field materials of *N. commune* have shown that nitrogen fixation is curtailed rapidly when cells are dried (POTTS and BOWMAN 1985; WHITTON *et al.* 1979). Upon the rewetting of dried cells, there occurs a sequential restoration of the capacities for respiration, then photosynthesis and finally nitrogen fixation (SCHERER *et al.* 1984). Rehydration induces changes in gene expression of desiccated *N. commune* UTEX 584, and the recovery of the capacity for nitrogen fixation requires *de novo* protein synthesis (ANGELONI and POTTS 1987, POTTS 1986, POTTS and BOWMAN 1985). This study analyses the changes in cell ultrastructure and distribution patterns of NifH (Fe protein of nitrogenase) which occur during the rehydration of desiccated *N. commune*.

2. Materials and Methods

2.1. Microorganisms and Growth Conditions

Nostoc commune UTEX 584, grown in a 2–1 airlift fermenter (POTTS 1985), was used as the control material. Desiccated colonies of *Nostoc commune* HUN were collected in Chi-Yang Hunan Province, People's Republic of China, on two separate occasions in 1980 and 1985 by Professor T.-W. CHEN. Desiccated colonies of *N. commune* were also collected from coastal lowland adjacent to the Ross Ice Shelf, Antarctica (in 1979), and in Reichenau, W. Germany (in 1985; provided by Dr. SIEGFRIED SCHERER). These materials are referred to as *Nostoc* strain ANT, and *Nostoc* strain REICH respectively (see POTTS *et al.* 1987).

2.2. Rewetting of Desiccated Cell Materials

Desiccated colonies were rehydrated in sterile Petri dishes with sterile distilled water, at 32 °C, under a photon flux density of 10 µmol photons $m^{-2}s^{-1}$. Samples were collected for the extraction of total proteins and for EM study at time 0 (desiccated colonies), and after 1 minutes, 5 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, 24 hours, and 72 hours of rewetting.

2.3. Antibodies

Polyvalent serum antibodies directed against the purified Fe protein of nitrogenase from *Rhodospirillum rubrum* were provided kindly by Dr. PAUL LUDDEN, University of Wisconsin—Madison. Protein-A colloidal gold particles were obtained from Janssen Life Sciences Products (distributed by SIPI supplier, PA). The gold particles were G 5 EM grade with a mean particle diameter of 5 nm.

2.4. Western Blotting

Proteins were transferred from polyacrylamide gels to nitrocellulose sheets using the technique of SYMINGTON (1984). The detection of the antigen—antibody complexes on Western blots employed protein A Horseradish Peroxidase conjugate (Biorad) as described (POTTS 1986).

2.5. Extraction and Purification of the Protein

Procedures used for the extraction of proteins from desiccated and rehydrated cells of *N. commune* are described by POTTS (1985).

2.6. Fixation and Embedding of Cells

A technique for the preparation of desiccated cells of *N. commune* for EM study has been published (PEAT and POTTS 1987). Samples of rehydrated material were fixed for 3 hours at room temperature in 1% w/v glutaraldehyde 2% w/v paraformaldehyde in 50 mM PO_4 buffer (pH 7.1). The samples were rinsed twice in distilled water, processed rapidly through an alcohol series and immersed overnight in LR White hard grade acrylic resin (The London Resin Co. Ltd., Basingstoke, England). The resin was then changed at 24-hour intervals on each of three successive days. After transfer to gelatin capsules the samples were cured for 12 hours, at 60 °C.

2.7. Identification of Cyanophycin

The presence of cyanophycin (multi-L-arginyl-poly[L-aspartic acid]) was confirmed using a modification of the Sakaguchi reaction (in THOMPSON 1966).

2.8. Immunolabelling

Thin sections were immunolabelled by using a modification of the technique described by TITUS and BECKER (1985). All procedures were performed at room temperature. Grids were floated, for 10 minutes, on 20-µl droplets of a solution of Tris-HCl (pH 7.2), 500 ml NaCl, 0.3% v/v Tween 20 (TBST) which contained 1 w/v bovine serum albumin (BSA). The grids were then transferred, without rinsing, to 20-µl droplets of antiserum (1 : 100 dilution) and incubated for 1.5 hours. After this time, the grids were transferred to TBST + BSA for 10 minutes, washed gently under a stream of TBST, blotted dry rapidly, placed on the surface of droplets of PA-gold (1 : 20 dilution) and incubated for 45 minutes. The grids were placed on the surface of droplets of TBST, left for 10 minutes, washed gently with TBST, then with water, and subsequently dried. Prior to examination the sections were stained for 15 minutes in 2% w/v ethanolic uranyl acetate and then, after a brief rinse, for 5 minutes in lead citrate solution. To check for non-specific staining samples were prepared where the incubation stage with antiserum was omitted from the above protocol.

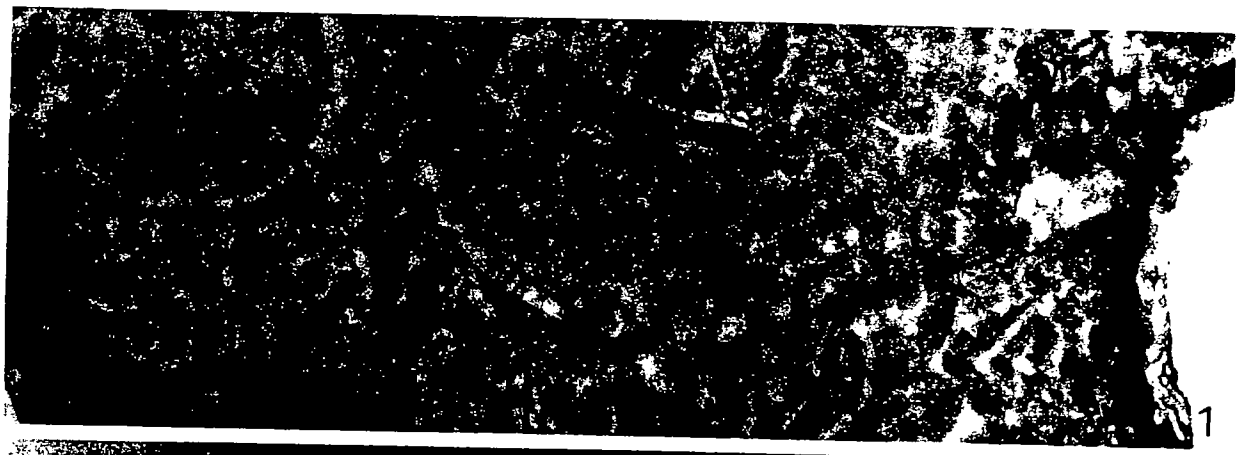
3. Results

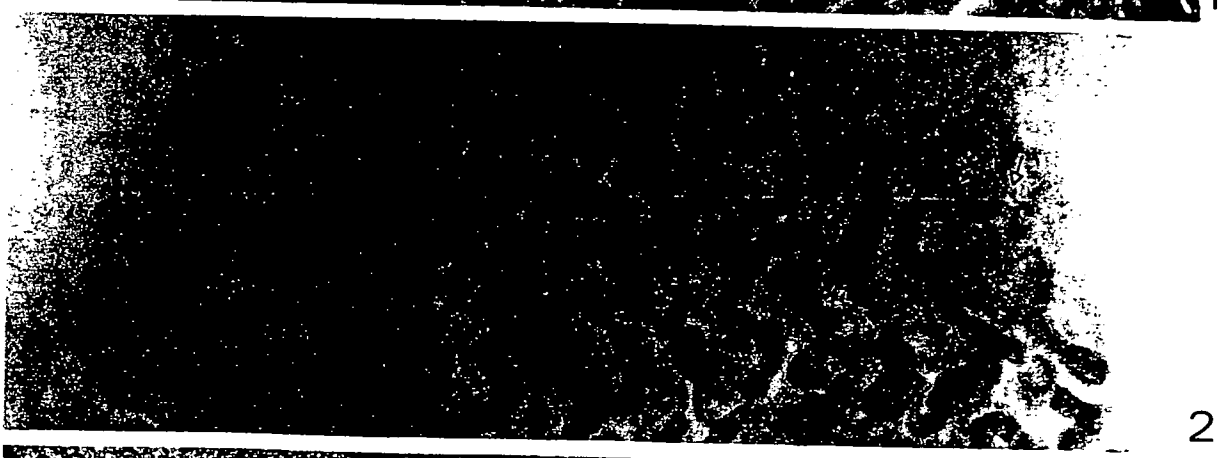
3.1. Rewetting of Desiccated Colonies

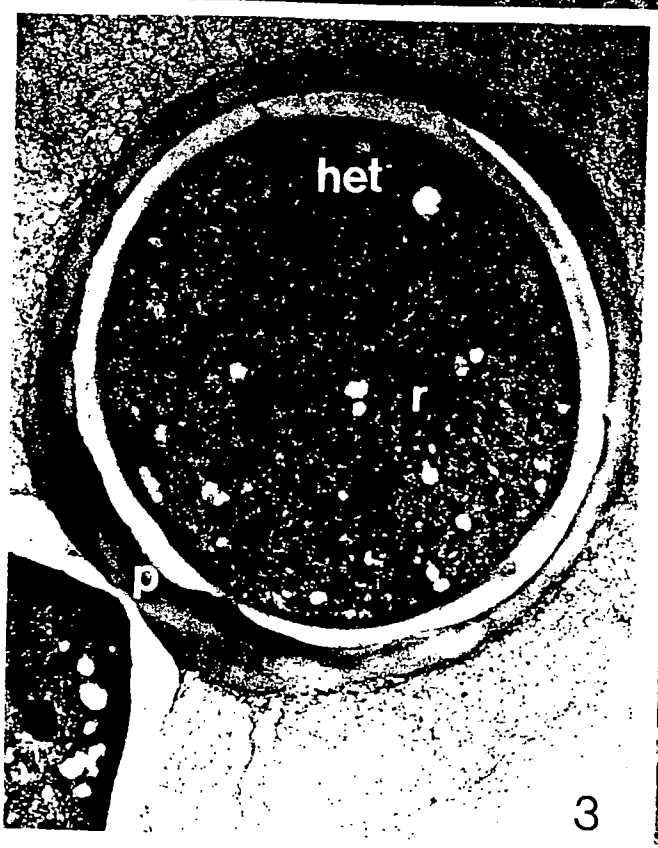
Desiccated colonies were black in color, friable and brittle. Upon contact with water the colonies began to swell and developed a consistency of soft cartilage. Individual filaments of vegetative cells and heterocysts were recognizable within seconds of rehydration (Figs. 1 and 2).

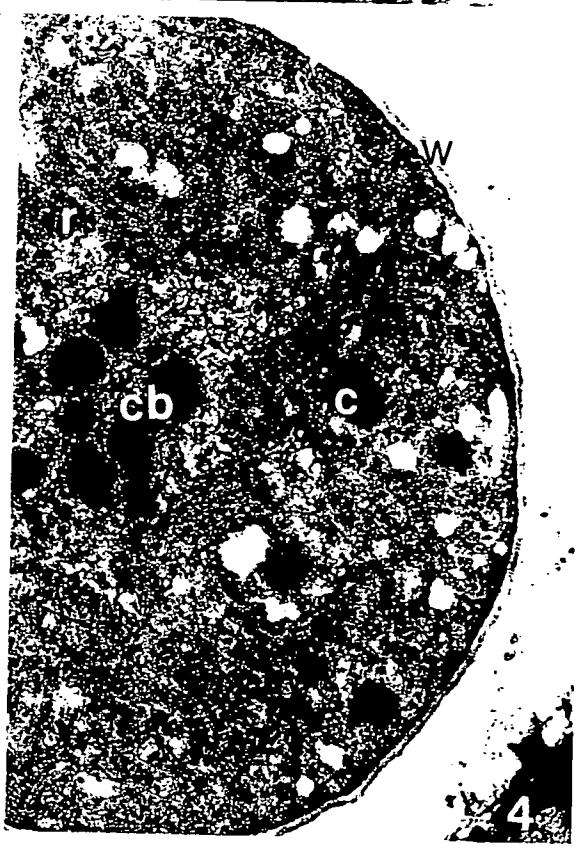
3.2. Ultrastructure of Heterocysts and Vegetative Cells

The ultrastructural appearance of both heterocysts and vegetative cells suggested that neither cell type had been damaged by two years of storage in the air-dry state. This is in contrast to the obvious damage to heterocysts









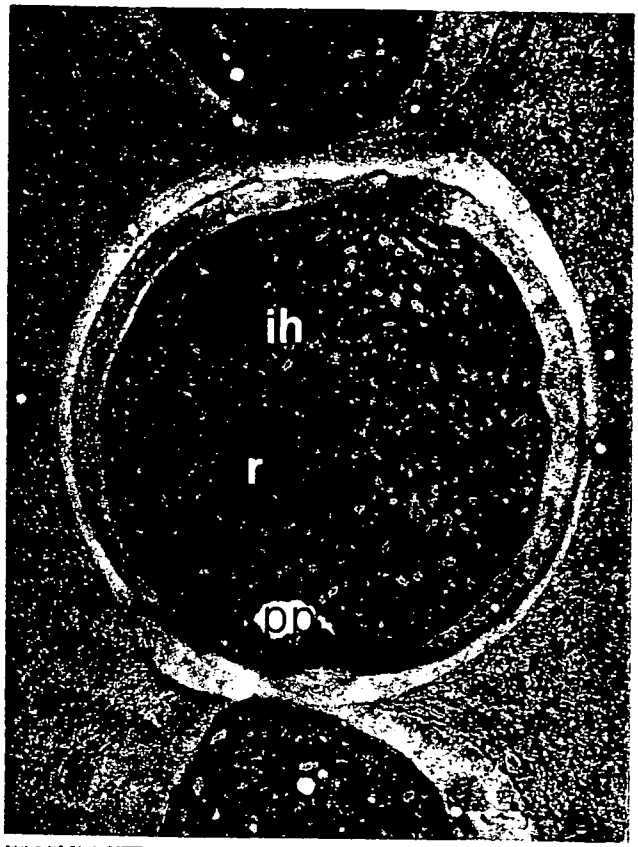
Key to figures: *c* cyanophycin granule, *cb* carboxysome, *cw* wall layers, *g* gold particles (= NifH), *het* terminal heterocyst, *ih* intercalary heterocyst, *p* pore, *pp* polar plug, *r* ribosome, *t* thylakoid, *w* wall layer

Fig. 1. Desiccated crust of *N. commune* HUN immediately following addition of water. Filaments (diam = 7 μm) are indistinct

Fig. 2. As in Fig. 1, same magnification, material rewetted for 15 sec. Filaments are visible (diam = 7 μm) and arrows indicate heterocysts

Fig. 3. Terminal heterocyst (*het*) with pore (*p*) showing dense aggregations of ribosomes (*r*) after 1 minute rewetting × 16,300

Fig. 4. Vegetative cell showing intact wall (*w*) layers, carboxysomes (*cb*), small cyanophycin (*c*) deposits and dense aggregates of ribosomes (*r*) after 1 minute rewetting × 34,200



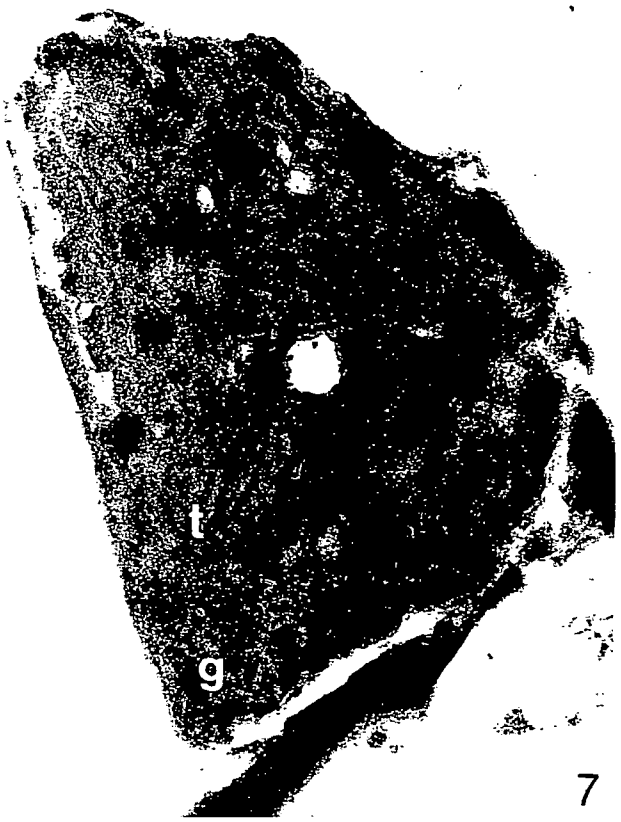
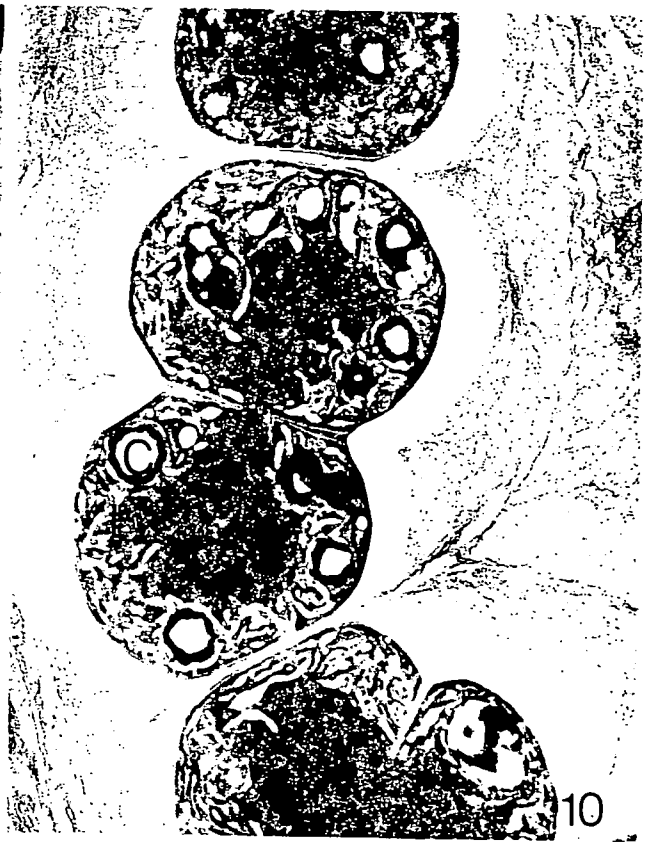


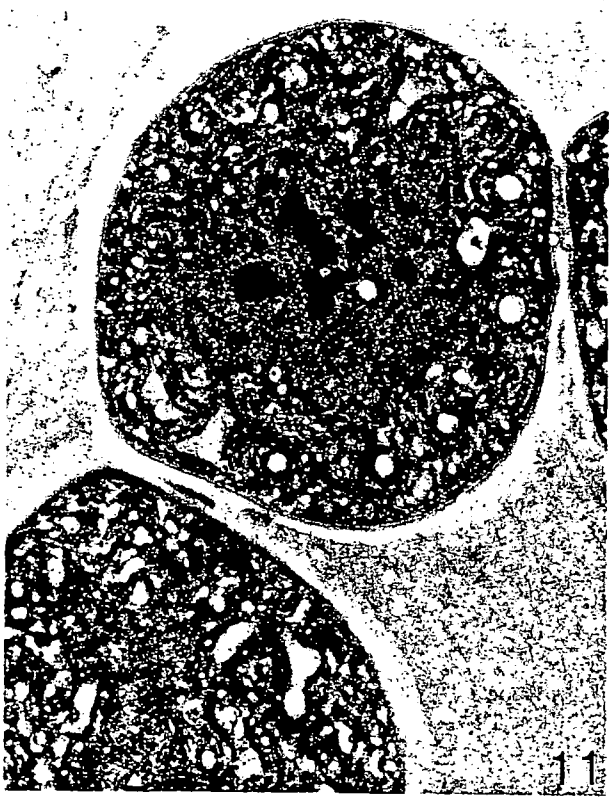
Fig. 5. Note accumulation of PA-gold NifH-antibody (*g*) in "clear" areas of vegetative cell after 5 minutes rewetting × 92,000

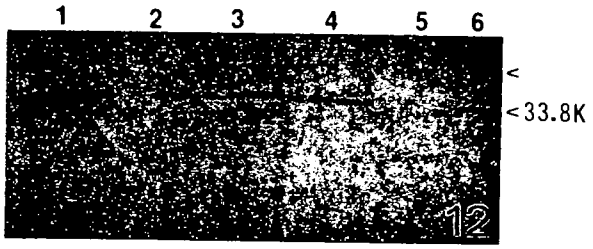
Fig. 6. Intercalary heterocyst (*ih*) with polar plug (*pp*) and dense aggregates of ribosomes (*r*) after 30 minutes rewetting × 10,900

Fig. 7. Dense heterocyst with random array of thylakoids (*t*) and PA-gold particles (*g*) after 5 minutes rewetting × 44,700

Fig. 8. PA-gold particles (NifH) localised in association with ribosomal (*r*) aggregates after 30 minutes rewetting × 92,000







Figs. 9 and 10. Vegetative cells after 24 hours rewetting showing massive accumulations of cyanophycin (*c*) and clearly defined thylakoids (*t*) × 11,500 (Fig. 9) and × 8,600 (Fig. 10)

Fig. 11. Vegetative cells after 3 days rewetting cyanophycin deposits have disappeared completely × 13,600

Fig. 12. Western blot of protein extracts from liquid culture of *N. commune* UTEX 584 (lane *1*) and desiccated colonies of *N. commune* HUN (collected in 1985, lane *2*; collected in 1980, lane *4*), *N. commune* REICH (collected in 1985, lane *3*) and *N. commune* ANT (collected in 1979, lane *6*). Molecular mass standards were loaded in lane *5* (control). Positions of the main and minor bands detected with NifH antibody are arrowed

Table 1. *Electron microscopy and immunolabelling of desiccated cells of N. commune HUN subjected to rehydration*

Time of rewetting	Appearance of vegetative cells	Appearance of heterocysts	Distribution NifH PA-gold conjugate
1 minute	Intact; cell wall layers visible; dense aggregation of ribosomes; some shrinkage; intrathylakoidal vacuolation; membranes apparent around carboxysomes; small cyanophycin granules present	Intact; cell wall layers visible; dense aggregations of ribosomes	*Small numbers* in heterocysts only
5 minutes	Cell outlines irregular; large vesicles present; carboxysomes and some cyanophycin present; no intrathylakoidal vacuoles	A second form of heterocyst with dense cytoplasm and a thick wall is evident; for dense array of thylakoids	Heavy deposition in vegetative cells particularly in open clear vesicular areas; heavy deposition in heterocysts also
30 minutes	More "normal" in appearance	Two forms of heterocyst present; dense aggregation of ribosomes in the "normal" heterocyst	Heavy deposition in heterocysts only associated specifically with ribosomes; no deposition in control samples; no gold deposition in proheterocysts
1 hour	As at 30 minutes	Sheath material thick; as at 30 minutes	Gold particles associated with both vegetative cells and heterocysts; *not* associated specifically with ribosomes
3 hours	As at 30 minutes	Two forms of heterocyst present; as at 30 minutes	As at 1 hour; particularly heavy deposition in "dense" heterocysts; serial section show identical localization of gold particles in sections through heterocyst
6 hours	As at 30 minutes; cyanophycin granules which are left have a much eroded surface	As at 30 minutes	Little evidence of gold particle deposition despite presence of both heterocysts and vegetative cells
24 hours	Cells characterised by well defined thylakoids and large cyanophycin deposits	Two forms of heterocyst present;	Gold deposition only in heterocysts
3 days	As at 24 hours but cyanophycin deposits fewer and smaller in number	Only normal type of heterocyst evident	Gold deposition in heterocysts

that occurs when axenic cultures of *N. commune* UTEX 584 are immobilized and dried for much shorter periods of time (PEAT and POTTS 1987).

The vegetative cells did not appear to have suffered from the period of desiccation. After 1 minute of rewetting, filaments of vegetative cells, together with intercalary and terminal heterocysts, were evident in the material, (Figs. 1–6). Two morphologically distinct forms of heterocyst were observed, one with characteristic wall layers and polar plugs (Fig. 6), and another much smaller form with denser cytoplasm (Fig. 7). The latter form, first detected after 5 minutes of rehydration of the cells, was not detected after 24 hours of rehydration. In all samples the filaments were encased within layers of thick dense sheath material. Vegetative cells had the appearance of being separated from this dense sheath material while heterocysts were always in close contact with it (Figs. 6 and 10). The distance of this apparent separation between vegetative cells and the sheath remained constant over the three days of rewetting. As rehydration of the cell material progressed, subtle changes were observed in cell ultrastructure and in the localisation of NifH. The most obvious changes are summarised in Table 1. Small numbers of cyanophycin granules, with very irregular outlines, were present in cells during the initial stages of rehydration (Fig. 4) and these became more obvious after 6 hours. Although cells rehydrated for 24 hours had massive accumulations of these cyanophycin inclusions (Figs. 9 and 10), the granules had virtually disappeared after 3 days of rewetting (Fig. 11). The identification of cyanophycin in all samples was confirmed by the Sakaguchi reaction.

3.3. Distribution of NifH

Depending upon the duration of rehydration, NifH was associated either exclusively with heterocysts, or with both heterocysts and vegetative cells. Non specific

staining effects were ruled out in experiments by the use of appropriate controls. When the two forms of heterocyst were present in a single sample, deposition of PA-gold NifH-antibody conjugate tended to be greatest in those with dense cytoplasm (Fig. 7). In the majority of heterocysts and vegetative cells the localisation of NifH, studied during rehydration of cells over 3 days, appeared to be random. However, no deposition of gold particles was achieved with dried materials, and a specific localisation of NifH was observed in two samples. After 5 minutes of rehydration gold particles were observed in large clear spaces within a vegetative cell (Fig. 5), and after 30 minutes of rehydration there was a definitive association of PA-gold particles with the ribosomes of heterocyst ribosomes (Fig. 8).

3.4. Western Blotting Analyses

Western blotting demonstrated the presence of NifH protein in all the dried materials tested including *N. commune* ANT which had been stored in the desiccated state for 9 years (Fig. 12). The main band, observed after detection of the antigen—antibody complexes in protein extracts from field materials, corresponded exactly in molecular mass with the main band seen in the assay of a protein extract obtained from an axenic culture of *N. commune* UTEX 584 grown under nitrogen-fixing conditions (see POTTS 1986). This band corresponded to a polypeptide with an acidic pI and an M_r value of 33,800. However, a minor band, corresponding to a higher molecular weight antigen of $M_r = \sim 45{,}000$, was more conspicuous in samples from the desiccated cell material even when the major band was not particularly intense in colour (Fig. 12).

4. Discussion

Extended desiccation and subsequent rehydration of *N. commune* HUN does not lead to any major discernable structural damage to either vegetative cells or heterocysts. The structural organisation of these cells, and the integrity of their membrane systems, appeared to be retained during the extensive fluxes in water stress imposed by drying, prolongued desiccation, and rehydration. In this respect the cells of *N. commune* HUN show a different response to water stress when compared to desiccation-tolerant plants (BEWLEY 1979). For example, it appears that many hours of rehydration are required before "normal" chloroplasts can be detected in desiccated *Borya nitida*. (FERRARI *et al.* 1984). The cells of *N. commune* HUN, immediately upon rehydration, did show some shrinkage and irregularity of outline, although the possibility that these features are preparation artifacts should not be discounted. Of the two types of heterocyst observed in the rehydrated material, the form with the dense cytoplasm, (which was absent from samples rehydrated for longer than 24 hours), may represent an incompletely rehydrated cell *i.e.*, a cell not fully "recovered" from desiccation. The seemingly denser deposits of gold particles in such cells could, therefore, result from the reduced cellular volume. It is important to note, however, that vegetative cells were of one uniform type. There is extensive protein turnover in desiccated cells of *N. commune* when they are rehydrated, and the rapid drying of cells leads to considerable proteolysis (POTTS 1985, 1986). Clearly, NifH is one protein which remains undegraded in those cells subjected to extensive (years) periods of desiccation (see Fig. 12) but from the present study it is difficult to generalise about the pattern of changes in the localisation and abundance of this protein during the rewetting period. And, it is not possible, at present, to assess whether NifH, while not degraded, remains functional. The main band identified on Western blots corresponded to a polypeptide with a subunit M_r of 33,800 and it is assumed that this represents the subunit of Fe protein (see POTTS 1986). The identity of the minor band remains unknown. The minor band may represent a reaction of the NifH antibody with the gene product of *nifH*-like DNA sequences, particularly as the minor band was present in Western analyses of *N. commune* UTEX 584, a strain which is known to contain two *nifH*-like genes in addition to *nifH* (DEFRANCESCO and POTTS 1988). However, and apparent change in the migration characteristics of the Fe protein isolated from *Anabaena* sp. strain CA was observed when the cells were transferred from microaerobic to aerobic growth conditions (SMITH *et al.* 1987). These data were interpreted as the result of a modification of Fe protein by oxygen.

Although noted on only one occasion the observation of a specific association of PA-gold with heterocyst ribosomes within 30 minutes of rehydration (with no such deposition in heterocysts, or proheterocysts, rehydrated for longer or shorter periods) suggests that some heterocysts may have the capacity for *de novo* NifH synthesis after this time of rehydration. This view is strengthened by the observation that heterocysts, when isolated free from vegetative cells, have the capacity to maintain RNA and protein synthesis although the activity is limited by the finite pool of precursors within such heterocysts (LYNN and OWNBY 1987). In the present study, the presence of NifH was not re-

stricted to heterocysts and the location within vegetative cells may be significant. It has been suggested that under aerobic conditions the heterocyst is the sole site of nitrogen fixation in Nostocacean filaments although not necessarily the sole site of nitrogenase synthesis (RIPPKA and STANIER 1978). Evidence has now accumulated for the operation of efficient aerobic nitrogen fixation in non-heterocystous cyanobacteria (KALLAS *et al.* 1983) provided efficient oxygen scouring mechanisms are available (WEISSHAAR and BOGER 1983, STAL and KRUMBEIN 1985). It may be that the vegetative cells of *N. commune* HUN were both synthesising nitrogenase as a consequence of the low oxygen tensions in the interior of a rehydrating colony, and fixing nitrogen. Such a regime could prevail if photosynthetic oxygen evolution was limited by low light penetration into the rehydrating colony. Furthermore, respiration is the first physiological process which desiccated cells recover upon rehydration. As oxygen may be limiting until its diffusion pathway is eased by complete rehydration of the colony, then the environment existing in vegetative cells at the onset of rehydration may be conductive to nitrogen fixation. Interestingly, rehydration of desiccated material in the presence of TTC (2,3,5-triphenyl-2-tetrazolium chloride) lead to the deposition of formazan crystals initially only within the central confines of both rehydrating colonies of field material (unpublished data) and immobilised cell masses of *N. commune* UTEX 584 (POTTS and BOWMAN 1985).

The most striking feature of the recovery of dried cells of *N. commune* cells from desiccation is the orderly and sequential restoration of metabolic functions (SCHERER *et al.* 1984). The available data suggest that the longer the cells are desiccated, then the longer the lag period which will ensue before physiological levels of nitrogenase can be detected and prior to the recovery of steady state levels of nitrogen fixation upon cell rehydration. For field materials it has been noted that the recovery of the capacity for nitrogen fixation was paralleled by an increase in the frequency of intercalary heterocysts, *i.e. de novo* differentiation (SCHERER *et al.* 1984). No ultrastructural survey accompanied this study. The present work indicates that the occurrence of apparently intact heterocysts should not be ignored since such cells (together with the vegetative cells) could contribute significantly to nitrogen fixation during the early stages of recovery from desiccation.

After 6 hours of rewetting cyanophycin granules were apparent in both vegetative cells and proheterocysts and these increased in size and frequency up to 24 hours. Following 3 days of rehydration the granules were fewer in number and smaller in size. In Nostocacean cyanobacteria the pattern of cyanophycin deposition in anaerobically-induced filaments, following the introduction of nitrogen to cultures, strongly suggests that vegetative cells as well as proheterocysts are able to fix nitrogen (KALLAS *et al.* 1983, HELBER *et al.* 1988). In such filaments cyanophycin granules appeared rapidly in both vegetative cells and the polar regions of proheterocysts and there was no indication of a gradient of cyanophycin accumulation in vegetative cells determined by their position relative to the proheterocysts.

Although protein turnover does occur upon rehydration, the proteolysis required to support such massive cyanophycin accumulation would likely be reflected in some obvious ultrastructural change, *e.g.*, membrane decomposition etc. As no gradient of cyanophycin deposition relative to either proheterocysts or heterocysts was evident in this study, it is possible that dinitrogen fixation is occurring in all cells in which NifH is located and the deposits of cyanophycin are witness to this event. The increase in the amounts of cyanophycin present in cells up to 24 hours following rewetting may be due to a slow growth rate accompanied by dinitrogen fixation. The subsequent decline could then reflect the increased growth rate commensurate with completed rehydration and a reduction in the nitrogen fixing capacity through loss of nitrogenase in the vegetative cells.

Although quantitative data have not been obtained for the distribution of NifH it appears that there is an increase over the first 3 hours of rewetting after which time a steady state prevails. The presence of NifH in vegetative cells and heterocysts considered together with the observation that nitrogenase activity, albeit low, can be detected in a short period after the rehydration of cells, suggests that desiccated *N. commune* may perform short term dinitrogen fixation upon rehydration without *de novo* heterocyst differentiation. Such a response would be invaluable for colonies present in environments where rainfall was transitory and the period of rewetting too short to permit heterocyst differentiation. Whether this short term nitrogen fixation upon rewetting is at the expense of functional nitrogenase present in desiccated cells or, a reflection of the translation of either stable *nif* transcripts or rapidly transcribed *nif* mRNA, is under investigation.

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Biochemistry and structure of the glycan secreted by desiccation-tolerant *Nostoc commune* (Cyanobacteria)

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Summary. Filaments of the desiccation-tolerant cyanobacterium *Nostoc commune* are embedded within, and distributed throughout, a dense glycan sheath. Analysis of the glycan of field materials and of pure cultures of *N. commune* DRH 1 through light and electron microscopy, immunogold labelling and staining with dyes, revealed changes in the pattern of differentiation in glycan micro-structure, as well as localized shifts in pH, upon rehydration of desiccated field material. A Ca, Si rich external (pellicular) layer of the glycan acts as a physical barrier to epiphytic bacteria on the surface of *N. commune* colonies. A purified fraction (> 12 kDa) of an aqueous extract of the glycan from desiccated field material contained glucose, N-acetylglucosamine, glucosamine, mannose, and galactosamine with ratios of 3.1 : 1.4 : 1 : 0.1 : 0.06, respectively. Lipid soluble extracts of *N. commune* contained trehalose and sucrose and the levels of both became undetectable following cell rehydration. Intracellular cyanobacterial trehalase was identified using immunoblotting and its synthesis was detected upon rehydration of desiccated field cultures. Elemental analysis of glycan extracts showed a flux in the concentrations of salts in the glycan matrix following rehydration of desiccated colonies. Water-stress proteins (Wsp; most abundant proteins in glycan), a water soluble UV-A, B-absorbing pigment, the lipid-soluble UV-protective pigment scytonemin (in both its oxidized and reduced forms), as well as two unidentified cyanobacterial glycoproteins (75 kDa and 110 kDa), were found within the glycan matrix. An unidentified 68 kDa protein, the second most abundant protein in aqueous extracts of the glycan, was isolated and its N-terminal sequence was determined as
AFIFGTISPNNLSGTSGNSGIVGSA.
Gene bank searches with this sequence identified significant homologies (35–45%) with various carbohydrate-modifying enzymes. The role of the glycan in the desiccation tolerance of *N. commune* is discussed with respect to structure function relationships.

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Abbreviations: EPS extracellular polysaccharides; Wsp water-stress protein; SEM scanning electron microscopy; TEM transmission electron microscopy; EDX energy dispersive X-ray analysis; FPLC fast performance liquid chromatography; SDS-PAGE sodium dodecylsulfate polyacrylamide gel electrophoresis; TLC thin layer chromatography; UV ultra-violet radiation; UTEX University of Texas Culture Collection.

Introduction

Extracellular polysaccharides (EPS) are a conspicuous feature of most bacterial cells. These investments may have the appearance of diffuse slimes, or may develop as rigid layers with a defined and a complex ultrastructure (Sutherland 1977). EPS layers are formed by the accumulation of various types of polymeric substances of high viscosity, they tend to be hygroscopic and, for aerophytic bacteria, often contain more water than the immediate environment. In view of the copious amounts of water trapped in these extracellular gels their structural analysis has proved to be difficult (Roth 1977). As a consequence while analytical data are available, structural analyses of extracellular structures and elucidations of structure function relationships are few. Functions attributed to EPS include their participation in the anchorage of the bacterial cell to its substrate, and protection against desiccation, phagocytic predation, antibody recognition, and lysis by other bacteria and viruses (Tease and Walker 1987). The EPS of *Beijerinckia* may protect the cells against oxygen damage (Barbosa and Alterthum 1992). The EPS of bacteria

represent an additional cell compartment and one that may contain the bulk of the water associated with a single cell. As such, it is widely believed that EPS provide bacterial cells with a means to survive drying, yet studies on the specific response of polysaccharide synthesis to drying are few, and the mechanisms of sensing of water deficit, with subsequent induction and regulation of polysaccharide synthesis, remain poorly understood (Ernst et al. 1987, Roberson and Firestone 1992). The presence of proteins, uronic acids, pyruvic acids and O-methyl, O-acetyl and sulfate groups in EPS emphasizes the complexity of these extracellular layers and suggests that a number of enzymes are required to degrade (and synthesize) the polysaccharide structure. The occurrence of carbonyl, carboxyl, hydroxyl and sulfate groups provide a means to attach cations. In the latter respect these EPS may scavenge metals that may be used either in physiological processes such as nitrogen fixation, or as toxins to repel predators (Tease and Walker 1987).

The extracellular polysaccharides of cyanobacteria provide some of the most complex examples of bacterial sheath structures and are well-documented in the classical and contemporary literature (Geitler 1932, Weckesser et al. 1988). For the most part cyanobacterial EPS are reminiscent of the glycocalyxes, slimes and capsules of other eubacteria (Nakagawa et al. 1987, Tease and Walker 1987). Many EPS of them have a complex composition and ultrastructure (Pritzer et al. 1989, Cardemil and Wolk 1979, Kessel and Eliff 1975) and tend to be pigmented and sometimes heavily calcified, especially in communities growing in situ (Potts and Whitton 1979).

Brittle, dried and darkened crusts of one cyanobacterium, *Nostoc commune*, are a characteristic feature of limestone (Karst) regions from the Poles to the Equator where they accumulate in shallow depressions in rock or become scattered over rocks and nutrient-depleted soils (see Whitton et al. 1979: fig. 1 a). The marked desiccation tolerance of *N. commune* reflects its ability to couple long-term structural and functional integrity in the air-dried state with a capacity to achieve instantaneous recovery of metabolic capacities upon cell rehydration (Scherer et al. 1986). The basis for such desiccation tolerance likely reflects a complex array of interactions at the structural, physiological, and molecular levels (Potts 1993). In the primary publications we have documented that colonies of *N. commune* accumulate abundant amounts of water stress proteins (Wsp). These proteins are secreted from the cells together with a water-soluble UV-A, B-absorbing pigment (Hill et al. 1994). The immediate environment of these secreted components is an extracellular glycan that constitutes the greater part of the colony bulk. The present study documents a comprehensive analysis of this peculiar extracellular glycan and provides an appraisal of its role in the desiccation tolerance of *N. commune*.

Materials and methods

Microorganisms and growth conditions

Field materials of *Nostoc commune* were collected during the past 55 years from a wide range of geographic locations and all have been maintained in the air-dry state, in the dark, since their collection (Fig. 1). Further details for some of these materials are presented in Hill et al. (1994). Colonies from rocks in a creek crossing Salt Sulfur Turnpike, Giles County, Virginia, were collected by John C. Strickland on August 9, 1939. The sample was obtained from the herbarium of the Department of Biology, University of Richmond. *Nostoc commune* strain DRH 1 is derived from field materials of *N. commune* CHEN that can be grown under laboratory conditions as two distinct forms. On inert supports such as 1.2% w·v agar supplemented with BG 11_0 (Rippka et al. 1979) medium, or on nylon membranes subjected to isopiestic control of water potential (Potts et al. 1984) the strain forms spherical colonies of 1 mm to 1 cm in diameter (Fig. 2 B). The strain forms a diffuse growth when grown in liquid BG 11_0, at 32 °C, under a photon flux density of approximately 50 µmol photons. m^{-2} s, with shaking.

Fixation, embedding, staining, and examination of cells

A technique for the preparation of desiccated cells of *Nostoc commune* for electron microscopy study has been published (Peat and Potts 1987). Samples of all materials were fixed in 2% w v glutaraldehyde 2% w v paraformaldehyde in 0.2 M sodium cacodylate buffer, pH 7.0, for 3 to 4 h. Where necessary 1% w v osmium tetroxide was employed for secondary fixation of samples. Samples were dehydrated and infiltrated with either paraffin or Lowacryl for light or electron microscopy, respectively. Resin for electron microscopy was changed at 24 h intervals on each of seven successive days. After transfer to gelatin capsules the samples were cured for four days, under UV light, at 4 °C. Samples for light microscopy were vacuum-infiltrated with paraffin and processed following a standard protocol.

Replicate paraffin sections of 15 µm thickness were used for scanning electron microscopy (SEM) and qualitative staining at the light microscopy level. Paraffin sections, adhered to glass coverslips, were cleared with xylene, critical point dried, carbon coated and placed on aluminum stubs for viewing with a Philips 505 scanning electron microscope. Sections for energy dispersive X-ray microanalysis (EDX) were placed directly on carbon stubs, processed as described above and examined with an EDX 9900 system. Calibration for EDX analysis was performed on the element calcium and all scan counts were carried out for 330 s.

All staining protocols for qualitative light microscopy followed the instructions of Pearse (1980). Alcian blue staining was performed using a standard procedure and a pH of 2.5 to permit assessment of the acidity of the stained material. Periodic acid-Schiff reagent (PAS) was used for the determination of the presence of glycoproteins. Stained and unstained sections were examined using an Olym-

pus Model BH 2-NIC microscope and photographed utilizing the PM-10ADS Olympus Automatic Photomicrographic System and Kodak Ektachrome 160-T slide emulsion.

The immunolabeling of ultra-thin sections followed the protocol described in Hill et al. (1994). To assess non-specific labelling, replicate samples were treated with pre-immune serum. A Zeiss 10CA transmission electron microscope was used. Post-staining of non-immunolabelled ultra-thin sections was performed using 2% w v ethanolic uranyl acetate for 15 min and then, after rinsing, for 5 min in a lead citrate solution.

Isolation of the extracellular glycan

The extracellular glycan of desiccated field material was obtained following grinding of the sample, in liquid nitrogen, to a fine powder. The sample was then incubated in 2% w v SDS, 1% v/v NP-40, in 100 mM Tris-HCl, pH 7.5, at 65 °C, overnight. The preparation was extracted three times with hot phenol and chloroform : isoamyl alcohol (24 : 1). The aqueous phase was collected and the glycan was recovered after precipitation with ethanol. The sample was reconstituted in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.4) treated with DNase, phenol chloroform extracted once again, re-precipitated with ethanol, lyophilized to dryness and then weighed. A 14-liter culture of *N. commune* DRH 1 was grown as described (Hill et al. 1994). The cell-free supernatant fraction was recovered following harvesting of the cells and was then concentrated using an Amicon concentrator with a YM 10 membrane (10–12 kDa cut off; Amicon Inc). The filtrate was lyophilized to dryness and weighed. The retentate was extracted as described above, taken to dryness, and weighed.

Carbohydrate analysis

Samples of purified extracellular glycan were acid hydrolyzed for neutral and amino sugars with 2 M trifluoroacetic acid at 100 °C for 4 h, dried, and reconstituted in water. Hydrolysis for sialic acids required a separate reaction using 0.1 M hydrochloric acid at 80 °C for 1 h, drying and reconstitution of the sample in water. Chromatography was performed on a Dionex BioLC using a Carbopac PA 1 anion exchange column. A Rainin Dynamax data acquisition system was used to record and integrate the data. The addition of 2-deoxy glucose was used as an internal standard. All samples were analyzed in duplicate injections.

Low molecular mass carbohydrates were obtained from desiccated colonies following extraction with 80% v v ethanol, in the dark, overnight at 4 °C. Lyophilized aliquots of the ethanol extracts were silylated using the Pierce HMDS-STOX reagent and the protocol of the supplier. Silylated sugars were separated on a Varian 3700 gas chromatograph using a 3% OV 17 Chromosorb W(HP) 80–100 mesh column. The column was calibrated using standards of trehalose, glucose, and sucrose.

The presence of amino sugars in culture supernatants of *N. commune* DRH 1 was confirmed using thin layer chromatography with silica gel plates and a solvent system of propanol : water (6 : 1) v/v followed by the staining of the plates with ninhydrin. A replicate plate was sprayed with sulfuric acid and charred at 120 °C.

Extraction and analysis of water- and lipid-soluble components in the glycan

Desiccated colonies were rehydrated with sterile distilled water, overnight, at 4 °C. The aqueous extract was then passed through a 2 µm pore size acrodisc filter. The lipid-soluble fraction of the desiccated glycan was extracted using 8 M urea, 1% v v NP-40 and 1 mM β-mercaptoethanol with gentle abrasive action employing alumina powder. The lipid soluble fraction was passed through an acrodisc filter and washed with 20 mM Tris buffer, pH 7.5 using a Centricon 10 microconcentrator (10 kDa cut off; Amicon Inc.).

The water- and lipid-soluble extracts were analyzed using fast protein liquid chromatography (FPLC) as described in the figure legends. Fractions were subjected to spectrophotometric analysis and Western blotting as described (Hill et al. 1994).

Field material was subjected to a time course of rehydration with water. After recovery of the aqueous rehydration fluid, the remaining cellular material was lyophilized and extracted with 80% v v ethanol, at 4 °C overnight. Analysis on the water utilized to rehydrate the material as well as the filtered and concentrated lipid-soluble (ethanol) extract was performed via Spectral Analysis of Elements with inductively coupled plasma spectrometry (ICP) using a Jarrell-Ash ICAP 9000. All analyses were performed in duplicate.

Preparation of antibodies and immunoanalysis

A preparation of the purified glycan was used to immunize mice (3–4 week-old C3H-HEJ: Scherer and Potts 1989). The lyophilized shock fluid from *E. coli* K 12 strain pTRE 1, overexpressing *treA*, was provided kindly by Dr. Winfried Boos. *E. coli* trehalase was purified using the procedure specified by Boos et al. (1987). The purified trehalase was used to immunize rabbits (New Zealand white) again using standard protocol (Scherer and Potts 1989).

Electrophoresis and biochemical analysis

Conditions for polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and the detection of glycoproteins in aqueous as well as total cell extracts, followed the procedures and precautions discussed in previous publications (Potts et al. 1992, Hill et al. 1994). Proteins were resolved in 15% w v gels, transferred to Immobilon P membrane, and prepared for protein sequence analysis using the procedures described by Matsudaira (1987). Automated Edman degradation was carried out using an Applied Biosystems 477A Protein Sequencer. Cysteine residues were not derivatized prior to analysis.

Results

Macroscopic and microscopic features of the glycan

Analysis of materials collected from all of the geographic locations (Fig. 1) indicated an overall consistency in their structural properties, composition and appearance. The specific data presented here are largely for materials of *N. commune* CHEN but can be considered representative of all the materials unless stated otherwise. Desiccated colonies of *N. commune* collected in situ appear black and they have the consistency of brittle parchment (Fig 2 A). In localities were the colonies adhere to soils, the lower face of the colonies, i.e., the side in contact with the soil, tends to have a greener coloration and may retain water for longer periods than the exposed surface. In thick sections a yellow coloration is apparent that lends a specific banding pattern throughout the polymer, with the bands

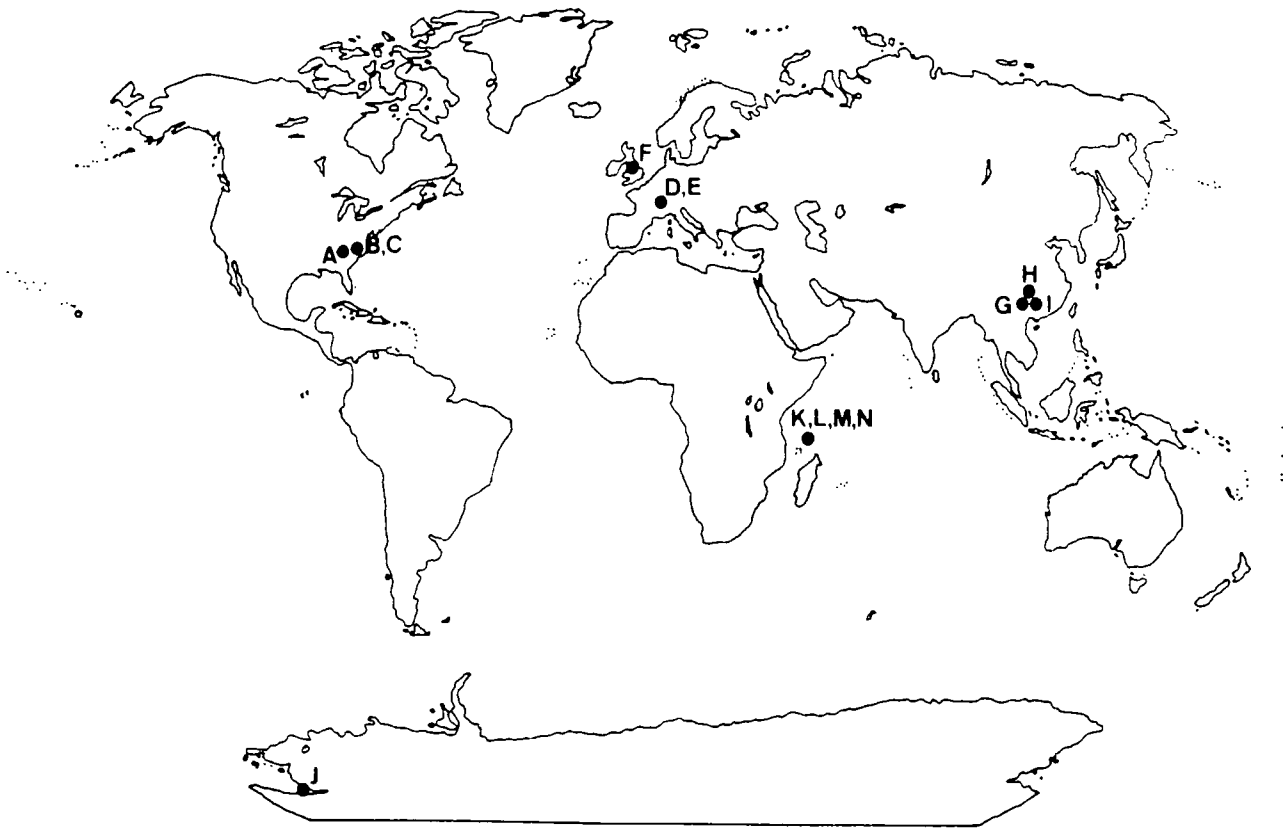

Fig. 1. *Nostoc commune* is cosmopolitan. Refer to Hill et al. (1994: table 1) for acronym descriptions. *A* TEN. *B* BBC. *C* VA 39, sample from U.S.A. Giles County, VA, 1939. *D* TAG. *E* BER. *F* MAL. *G* CHEN. *H* HUN 1. *I* HUN 2. *J* ANT. *K* 776 D. *L* 779 D. *M* 857 D. and *N* 8122

horizontal to the surface. This yellow-brown coloration is most intense at the exposed surface of the colony (Fig. 2 C). As documented below, the yellow coloration is attributed to a UV-absorbing pigment, scytonemin. A 20 µm-thick layer encompasses the outer periphery of the thallus and this layer has a microstructure that includes fibrils perpendicular to the surface of the colony (Figs. 2 D and 6 D). The filaments of *N. commune* are embedded within, and distributed throughout, a dense glycan sheath (Fig. 2 C–H). An envelope layer, with different structural and staining characteristics compared to the bulk polymer, surrounds each filament (Fig. 2 E, F, and H). PAS stain gave an intense reaction associated with heterocysts but not with the envelope layer that is constricted at heterocysts (Fig. 2 H). During rehydration, the sheath takes on a vesicular, porous and honey comb-like appearance (Fig. 2 F). After full rehydration, and then long-term incubation, numerous spherical colonies 1 mm to 1 cm in diameter, bud from the thallus (Figs. 2 B, G and 5 E). These spheres or "pearls" retain the yellow-brown coloration of the thallus, and develop conspicuous "stretch marks" if they start to dry (Fig. 2 B). Upon complete desiccation pearls form flat sheets that swell upon rewetting to restore the spherical shape of the turgid pearl (data not shown).

Ultrastructural analysis of the glycan

In the desiccated thallus the filaments are separated from one another (Fig. 2 C, E) and when examined in SEM appear to reside in narrow "tunnels" that permeate the glycan (Fig. 3). These "tunnels" have ribbed extensions when observed in transverse section in the SEM (Fig. 3 B), are clearly cylindrical when observed in a plane parallel to the tunnel (Fig. 3 D) and appear to be empty (Fig. 3 A, B, and D). In some materials these "tunnels" were less distinct or almost absent. Here, the glycan sheath at the periphery of the filaments appeared heavily pitted with a microporous structure that followed the contour of the "tunnels" observed in other materials (Fig. 3 C). Magnesium, calcium, silicon, phosphorus, and sulfur predominated the EDX spectrum of all materials that were studied (Fig. 3 E). Relative to the glycan, cells were enriched in sulfur and phosphorus. The most obvious result of these analyses

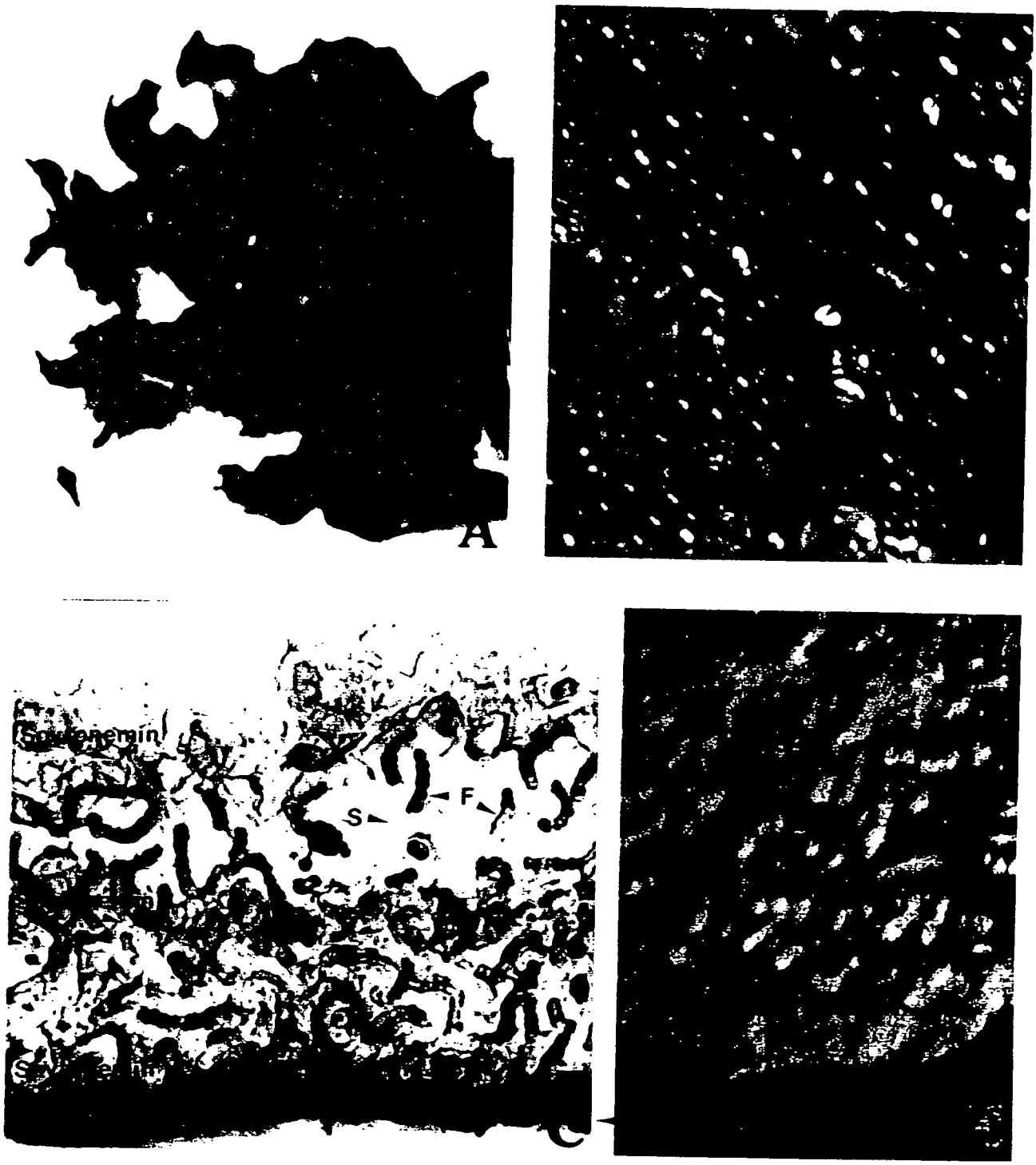

Fig. 2. Macroscopic and microscopic features of the glycan. **A** Desiccated field material *Nostoc commune* CHEN. × 2. **B** Pearls generated from rehydrated *N. commune* CHEN, arrowheads indicate "strech mark" the pearls develop if they start to dry; × 2. **C** Unstained light microscopy section of *N. commune* TEN; × 20. **D** *N. commune* TEN desiccated, stained with PAS. Nomarsky interference; × 20. **E** Desiccated *N. commune* CHEN stained with standard AB (Alcian blue) protocol; × 40. **F** *N. commune* CHEN, 5 min of rehydration. AB stained standard protocol, × 40. **G** Fully hydrated pearl preparing to "bud-off" new pearls, stained with PAS; × 40. **H** Desiccated *N. commune* 8122 stained with PAS; × 60. *F* Filament; *H* heterocyst; *S* sheath; S_i, S_j differential layers of sheath; *tH* terminal heterocyst


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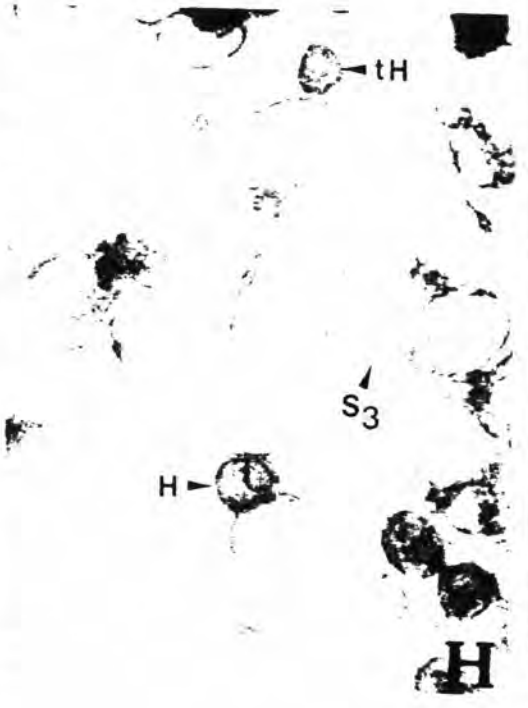
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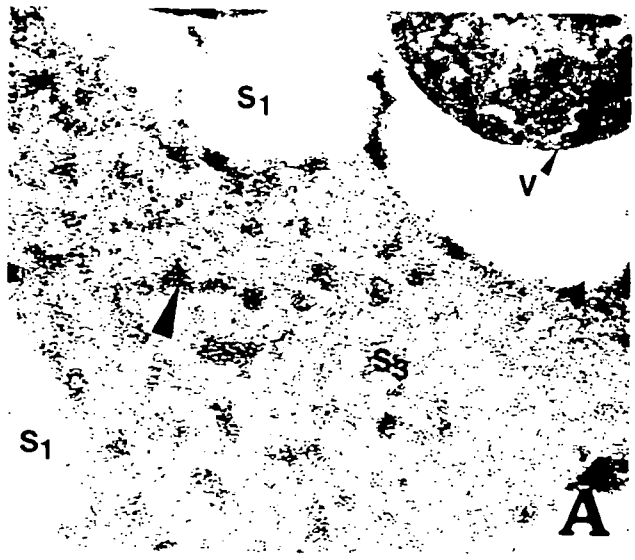
was the conspicuous accumulation of silicon and calcium in the external layer (Fig. 3 E; compare with Fig. 2 C, D).

Light microscopy and TEM resolve the immediate environment of each cell in desiccated colonies of field material as a homogenous envelope that was only weakly stained (Fig. 2 E; Fig. 4 A, B). This envelope layer, S_1, follows the contours of the individual cells and was constricted at their crosswalls (Fig. 4 A, B). The envelope layer was appressed to the glycan where a dense staining membrane-like interface layer can be discerned (Fig. 4 B, C). The glycan, but not the envelope layer, was heavily labelled with antibodies prepared using a purified extracellular carbohydrate preparation (Fig. 4 A). The pattern of labelling of the glycan suggested that the apparently homogenous fibrous structure (Fig. 4 B) was immunologically heterogeneous (Fig. 4 A). When rehydrated cells of *N. commune* CHEN pearls or *N. commune* DRH 1 were prepared under similar conditions of fixation, dehydration, infiltration, and critical point drying, the envelope layer and glycan showed a different ultrastructure in comparison to the desiccated materials (Fig. 4 F). The most apparent differences are as follows: Following short-term rehydration (30 min) portions of the envelope layer had the capacity to be post-stained (S_2). These areas appeared to consist of fibrils parallel to both the stained membrane interface layer and the contiguous fibrils of the glycan (Fig. 4 C). After longer periods of rehydration (60 min), the developing layer, S_2, became more reticulate and stained with a greater intensity (Fig. 4 D). In fully rehydrated material an envelope layer was either absent, or it was hardly discernible (Fig. 4 E). Here, the glycan appeared to make contact with the cells (Fig. 4 E, inset). The glycan sheath of pearls was more heterogeneous than that of desiccated materials (Fig. 4 F). Immunogold-labelling of the glycan of pearls revealed conspicuous accumulations of electron dense cross-reactive material while the bulk of the sheath had no accumulations of gold particles (Fig. 4 F). A carbohydrate analysis was performed on the purified glycan that was used to generate the sheath antibody, the following sugars were present in pmoles (μg): glucosamine 22.9 (5.04), galactosamine 7.5 (1.65), glucose 110 (19.8), galactose 43.5 (7.8), and mannose 9.5 (1.7), which yields a ratio of 3 : 1 : 12 : 5 : 1 of the sugars, respectively.

The Ca/Si-rich external layer was conspicuous in cross sections of pearls (Fig. 5 A–C; compare Figs. 2 D and 5 B). Sheath layers compared to those associated with cells of desiccated field material were difficult to discern (Fig. 5 D). Although the "tunnel"-like structures (described above) were absent, Fig. 5 D shows clearly that filaments are encased in a layer that has different structural properties to the bulk glycan. Analysis through light microscopy also indicated different staining properties of this layer. Filaments, such as those with the appearance of the one in Fig. 5 D, tend to occupy the outer portions of the colony where they form smaller pearls and packets (Figs. 2 G and 5 E), while non-encased filaments are present in the central portions of the pearl (Fig. 5 F). During growth, minute pearls bud-off from the parent pearl at the periphery of the latter (Figs. 2 G and 5 E). These buds originate as encased filaments immediately below the surface of the pearl and, at this stage, an external layer is already apparent at the periphery of these small packets of filaments (Fig. 2 G and 5 E). The staining characteristics of the material in and surrounding these packets differ from those of the parent glycan (sheath) matrix (Figs. 2 G and 5 D). For example, with Alcian blue at pH 2.5 the small packets stain an intense dark blue in comparison to the light blue-green color of the surrounding glycan (data not shown).

The general features of the ultrastructure of the cells in desiccated colonies and pearls are similar and the data presented in Fig. 6 for pearls can be considered representative of both materials (with the qualifications discussed below). In pearls, short filaments are often observed (e.g., Fig. 6 F), and in some cases may consist only of one vegetative cell and a single heterocyst (data not shown). Heterocysts may be apical (Fig. 6 A) or intercalary (Fig. 5 F). Honeycomb configurations were noted at the poles of mature heterocysts (Fig. 6 B) and both proheterocysts and mature heterocysts had a non-staining layer characteristic of these differentiated cells (Fig. 6 B, C). Filaments present in the central regions of pearls had a vesiculate nature in comparison to those present towards the periphery of the colonies (Fig. 6 E, F). A feature of desiccated materials collected in situ, and of pearls grown under laboratory conditions, is that the interior of the colony is devoid of any other microorganism save for *N. commune* (Fig. 6 D). In addition, we have never observed cyanophages or other bacteriophage-like structures within the colonies. As such, there is a considerable layer of glycan that separates cells from the atmosphere (Fig. 6 D), and this constitutes an apparent barrier that appears to remain unbroken even during the budding process described above (Fig. 5 E). In this context it can be noted that the exterior surface of the colonies is colonized by populations of microorganisms that vary in magnitude, and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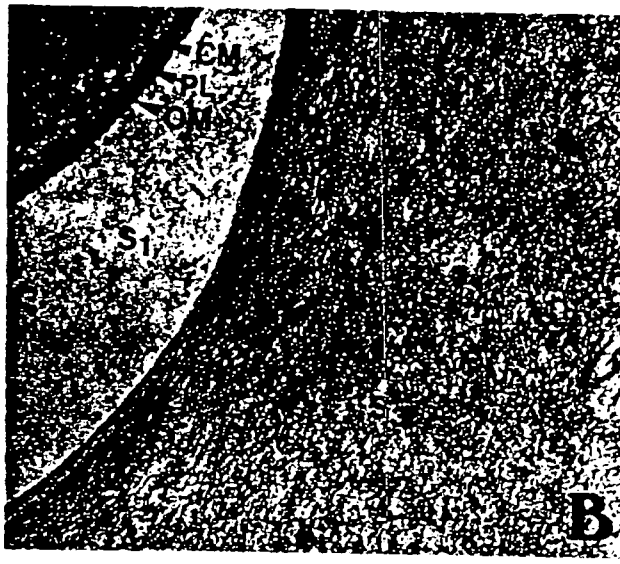
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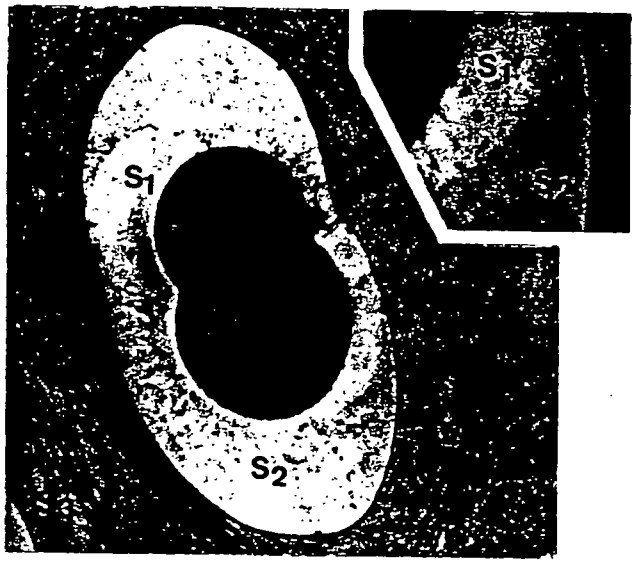
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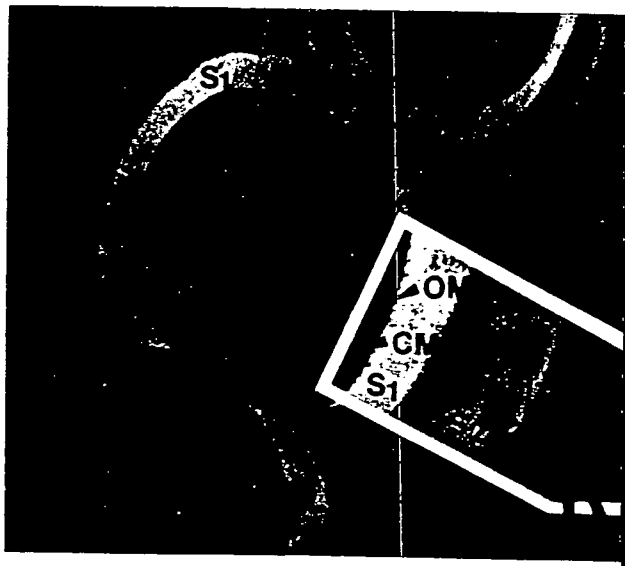
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diversity, depending on the materials studied (Fig. 6 G). Nevertheless, these organisms never penetrate the barrier of the silicon-rich external layer (Fig. 6 D; note fibrillar structure of this layer and compare with Fig. 2 D).

Features of N. commune strains UTEX 584 and DRH 1 in liquid culture

The life cycle of *N. commune* strain UTEX 584 (Hill and Potts in prep.) includes one stage that results from the encasement of growing filaments in an envelope layer (Fig. 7 A). The heterocysts in the filaments from which these structures arise are unable to divide and remain between the packets formed by the encased filaments where the envelope is constricted (Fig. 2 H). The filaments inside these packets do, however, contain newly differentiated heterocysts. These structures are virtually identical to those observed in field materials of *N. commune* (compare Figs. 7 A and 2 E, H). A thickened envelope layer also surrounds single filaments of *N. commune* strain DRH 1 (Fig. 7 B) and these ultimately generate small packets wherein the filaments are encased (Fig. 7 C).

Chemical and elemental analysis of the glycan

The glycan of field material has a firm, rubbery consistency when wet, and is brittle when dry. In liquid culture of *N. commune* DRH 1 the glycan is dispersed and lends a viscous property to the cell-free liquid medium. In order of abundance the sugars detected in the purified hydrolysate of the glycan were glucose, N-acetylglucosamine, glucosamine, mannose, galactosamine and galactose (Fig. 8). Fucose was not detected in any sample. The quantitative amounts of the sugars in sample C and D (the amount of material used in the extract of C was approximately twice that of D) illustrates the heterogeneity of the field material. However, the ratio of the sugars with respect to each other is what is important to note. The ratios for these sugars in the glycan of desiccated field materials were the same for both samples, 3.1 : 1.4 : 1 : 0.1 : 0.06 : 0. Galactose was detected only in the small molecular weight (less than 12 kDa) fraction of the glycan from *N. commune* strain DRH 1 and the ratio of sugars respectively was 1.3 : 0.5 : 1 : 0.2 : 0.3 : 0.1. The concentrations of glucosamine and N-acetylgalactosamine in this low molecular mass fraction were almost two orders of magnitude less than in the fraction that was retained by a 12 kDa cut-off membrane. The ratio of sugars for the large molecular weight (greater than 12 kDa) fraction was 1.7 : 1.5 : 1 : 0.01 : 0 : 0, respectively. The concentrations of galactosamine and mannose were higher in the desiccated glycan than in the samples from liquid grown *N. commune* strain DRH 1. In each sample a hydrolysis product, identified as sialic acid, was resolved during the carbohydrate analysis. Amounts of this compound in the field material were approximately 15 times greater than those found in the high molecular weight fraction of the glycan from *N. commune* strain DRH 1 (2 pmoles versus 0.15 pmoles per microgram dry weight, respectively). Only trace amounts (< 0.03 pmoles) of sialic acid were detected in the small molecular weight fraction of the glycan from *N. commune* strain DRH 1. Whether this compound is sialic acid and whether it is a true component of the glycan or attributable to the epiphytes on the surface of the colonies is still under investigation. Positive identification of this component must await GC-MS analysis. In order of abundance trehalose, sucrose and glucose were detected in the cells following solvent extraction. The concentrations of each were greatest in desiccated material and decreased with time of cell rehydration. Initial measurements of trehalose of a desiccated sample were approximately 1 mg trehalose/g dry weight of sample. After 30 min of rehydration the level of trehalose had dropped to 0.3 mg/g and by 6 h the level was only one-tenth that present in the desiccated sample (this level remained constant through a further 48 h of rehydration). The levels of sucrose followed the same

Fig. 4. Transmission electron microscopy of *Nostoc commune* CHEN field material. Specimens viewed in **A** and **F** were not exposed to secondary fixation with 2% w/v osmium tetroxide or post-stained with uranyl acetate/lead citrate, all other specimens in figure were treated with these steps **A** Desiccated field material immunolabeled with sheath antibody (5 nm gold particles), large arrowhead indicates dense labeling areas within the sheath; × 14,500. **B** Desiccated field material; × 14,500. **C** Field material after 30 min of rehydration; × 4,300; inset, × 12,000. **D** Field material after 60 min rehydration; × 8,200; inset, × 18,300. **E** Field material after 12 h of rehydration, large arrowhead indicates an apparent junction between sheath and vegetative cell; × 6,000; inset, × 17,500. **F** Section of pearl generated through rehydrated field material and immunolabeled with sheath antibody, arrowhead indicates dense labeling areas within the sheat; × 10,800. *C* Carboxysome; *CG* cyanophycin granule; *CM* cell membrane; *IL* interface layer; *OM* outer membrane; *PL* peptidoglycan layer; *PM* polymorphic body; *PP* polyphosphate granule; *S* sheath; S_1, S_2, S_3 differential layers of sheath; *V* vegetative cell

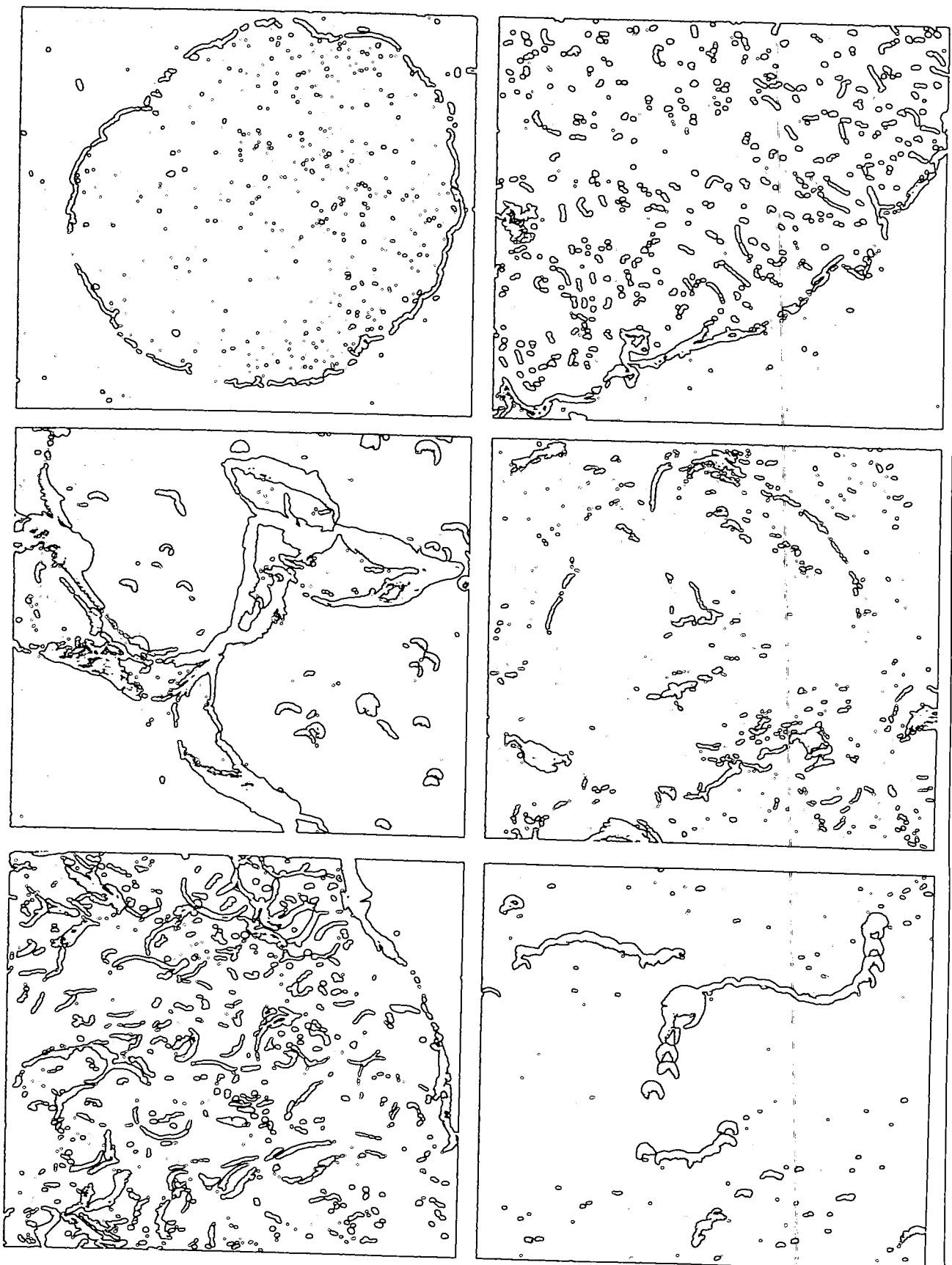


Fig. 5. Scanning electron microscopy of pearl. **A** Cross section view through a pearl approximately 2 mm in diameter; × 40. **B** View of filaments within the pearl; × 150. **C** Outer sheath edge of pearl; × 1,000. **D** Individual filament located towards the periphery of a pearl. large arrowhead indicates differentiation of the sheath; × 1,000. **E** Pearl buds on the outer periphery of a pearl; × 200. **F** Individual filaments located in the central region of a pearl; × 1,200. *F* Filament; *H* heterocyst; *P* pearl bud; *S* sheath

trend as that described for trehalose. The initial concentration of sucrose was approximately 0.8 mg/g dry weight of sample. After 30 min the level was < 0.3 mg/g and at 6 h the level was less than one-tenth the original quantity (once again this level remained constant through 48 h of rehydration). The levels of glucose gradually increased during the time the levels of the disaccharides trehalose and sucrose decreased. Initial levels of glucose in the desiccated sample were less than one-fortieth the concentration of sucrose and trehalose (approximately 0.025 mg glucose/g dry weight of sample extracted). After 30 min of rehydration the levels of glucose had risen four fold (highest concentration detected for glucose levels), then decreased during subsequent incubation and was barely detectable after 12 h. Aqueous extracts of field material were obtained, following different periods of rehydration, in order to gain some insight into the fluxes of dissolved salts that may occur in situ in response to wetting and drying. Potassium and calcium constituted the most abundant elements in aqueous extracts of *N. commune* CHEN obtained during a 6 h period of wetting (Fig. 9 A). Following the 6 h period of aqueous extraction the cells were then recovered and extracted with 80% v/v ethanol for 60 min, a process that lead to leakage of cell contents but without disruption of the colony. The concentrations of elements in this case were reduced relative to those determined for the aqueous extract (Fig. 9 B). The elemental analysis of rehydration fluids of materials, from various geographic locations, were compared following 24 h of extraction and were strikingly similar (Fig. 10). The extracts were substantially increased in K, Ca, Mg, and Na relative to the quantities measured at 6 h of wetting (compare Fig. 9 A).

Pigment composition of the glycan

Desiccated materials of *N. commune* TEN that contained conspicuous amounts of a yellow pigment (Fig. 2 C) were subjected to extraction using either aqueous or lipid-soluble solvent systems. The aqueous extract contained a water soluble UV-A/B-absorbing pigment and the water stress protein, Wsp, as observed for *N. commune* CHEN (compare Fig. 11 A, B with Hill et al. 1994: figs. 4 and 8). Inclusion of NP-40 in the extraction buffer lead to a release of the yellow pigment from the colonies as well as increased amounts of protein, notably phycobiliproteins that serve as markers of cell lysis (Fig. 12). The spectral scan of the detergent extract was enhanced in the region where the UV-absorbing pigment scytonemin absorbs (435 and 484 nm) (Fig. 12 A). Further analysis of the fractions that contained this pigment identified yellow (absorption max. 435 nm) and pink (absorption max. 435 nm and 493 nm) components. Through the use of TLC and spectrophotometric analysis, these pigments were identified as the oxidized (435 nm) and reduced (493 nm) forms of scytonemin, respectively (data not presented).

Protein composition of the glycan

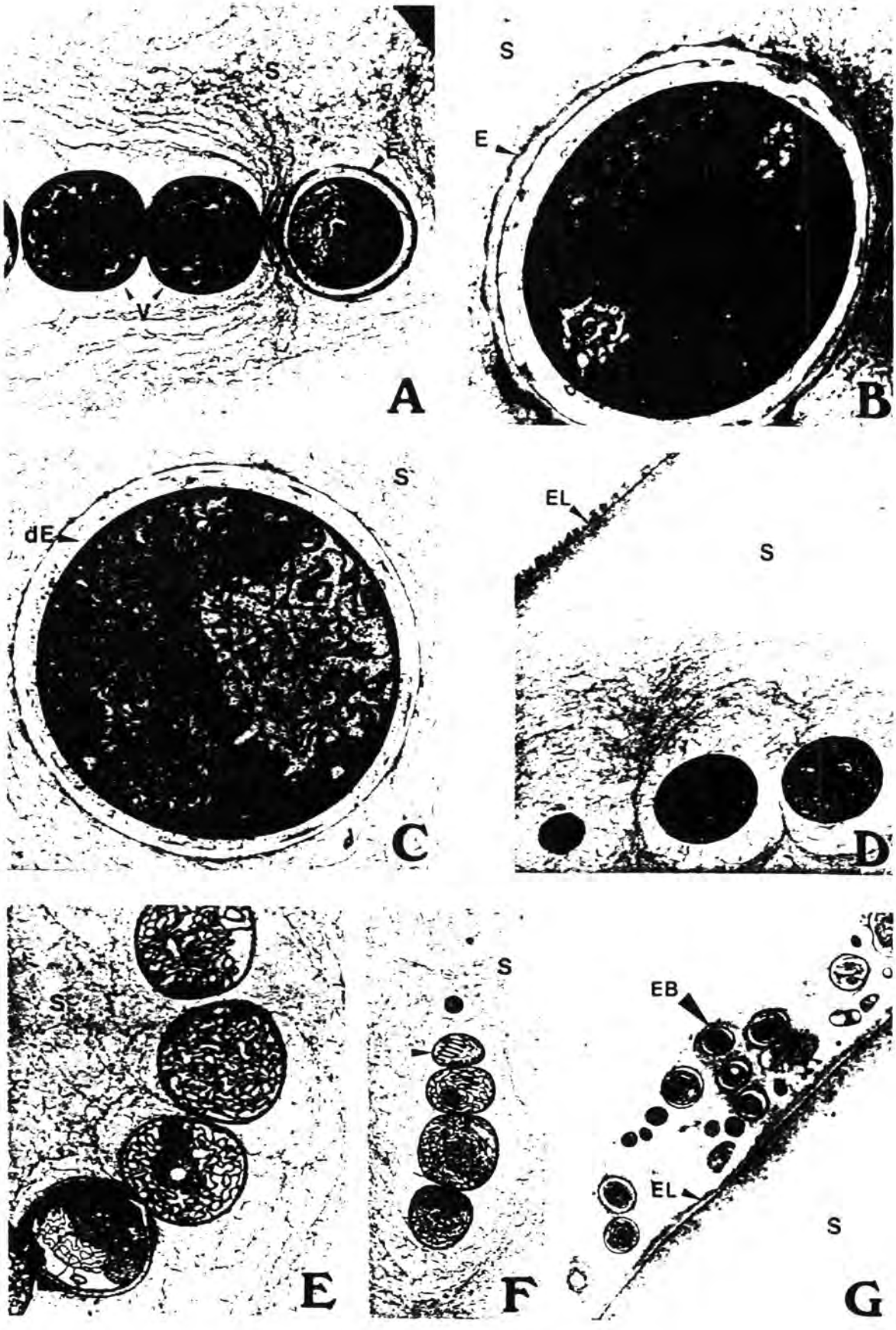
Wsp was extracted with both aqueous and non-polar solvent systems (Fig. 11, lanes 1 and 2), and was present in all materials studied save for two samples from Aldabra Atoll (Fig. 1). If present, Wsp represented the most abundant soluble protein. The abundance of Wsp in aqueous extracts of materials, which contained the protein, was estimated as 65 to 85% of the total protein in the aqueous extract (Fig. 13). The second most abundant protein in aqueous extracts of *N. commune* CHEN was a protein with a molecular mass of 68 kDa. The N-terminal sequence of this protein was AFIFGTISPNNLSGTSGNSGIVGSA.

No obvious match of this N-terminal sequence was obtained following searches of the Swiss-Prot data base. The highest scores in the search (with homology values of between 36% and 44%) were with carbohydrate-modifying enzymes encluding: fructose-1, 6-bisphosphatase, glucan endo-1, 3-β-glucosidase, mannose 6-phosphate isomerase, UDP-N-acetylglucosamine-dolichol-phosphate transferase, cyclomaltodextrin glucanotransferase, and alpha-amylase.

Experiments using light microscopy identified a conspicuous reaction of heterocysts in desiccated material with PAS, a stain used in glycoprotein detection (see Fig. 2 H). In fully hydrated pearls a more general and conspicuous reaction with PAS was observed (Fig. 2 G). Two polypeptides, that showed a positive glycan reaction, were detected in aqueous extracts of desiccated samples from six geographic locations (Fig. 14). The reactions with the polypeptides were apparent within 5 min of the initiation of the color reaction which was terminated after this time. The estimated molecular masses of the polypeptides were 75 kDa and 110 kDa, respectively. The most conspicuous reaction was associated with the 75 kDa polypeptide.

Cyanobacterial trehalase

In view of the abundance of Wsp in aqueous extracts, its role as a carbohydrate modifying enzyme, and the immunoreactivity of Wsp serum to other carbohydrate-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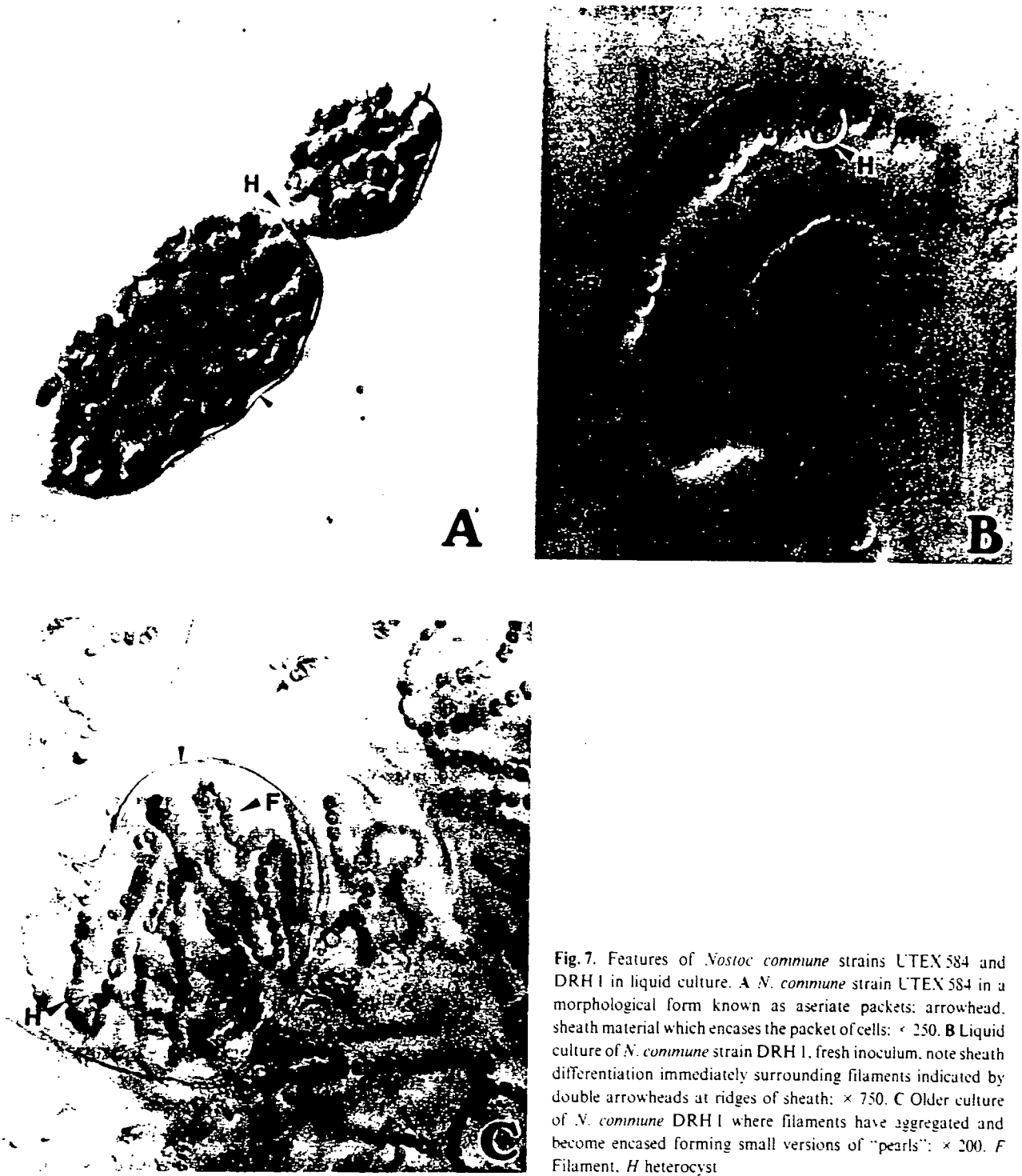


Fig. 7. Features of *Nostoc commune* strains UTEX 584 and DRH 1 in liquid culture. **A** *N. commune* strain UTEX 584 in a morphological form known as aseriate packets; arrowhead, sheath material which encases the packet of cells; × 250. **B** Liquid culture of *N. commune* strain DRH 1, fresh inoculum, note sheath differentiation immediately surrounding filaments indicated by double arrowheads at ridges of sheath; × 750. **C** Older culture of *N. commune* DRH 1 where filaments have aggregated and become encased forming small versions of "pearls"; × 200. *F* Filament, *H* heterocyst

Fig. 6. Transmission electron micrographs of pearls. **A** Filament with a terminal heterocyst located towards the periphery of the pearl; × 4,600. **B** Mature heterocyst, the fibrous structure of the outer layers of the heterocyst envelope indicated; × 10,000. **C** Immature or developing heterocyst; × 10,000. **D** External layer of the pearls appears striated; × 6,000. **E** Filament located towards the center of the pearl, note the well-defined and prolific thylakoid membranes present in cells; × 4,200. **F** Filament present in the center of a pearl, note unusual configuration of thylakoid membranes; × 2,400. **G** External layer of the pearl prevents the intrusion of epiphytic bacteria (*EB*) present on some areas of the pearl's surface; × 5,700. *dE* Developing envelope; *E* envelope; *EL* external layer; *H* heterocyst; *hc* honeycomb configuration; *S* sheath; *V* vegetative cell

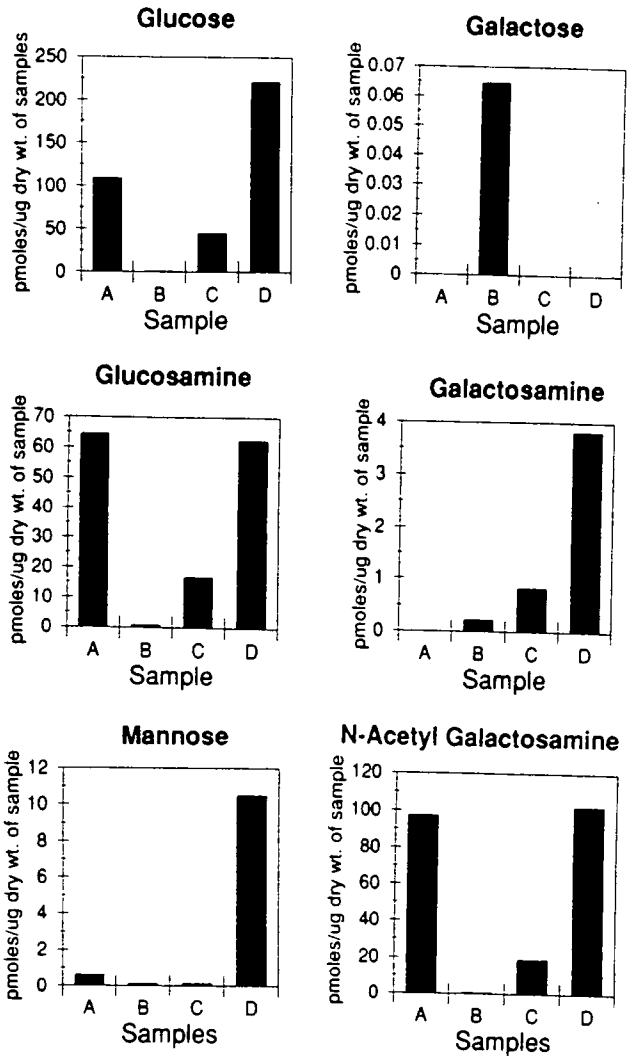


Fig. 8. Carbohydrate analysis of glycan fractions. *A* Large molecular weight fraction (> 12 kDa) of glycan extract from *N. commune* DRH 1 media; *B* small molecular weight fraction of *N. commune* DRH 1 (< 12 kDa). *C* and *D* Purified glycan preparation of *N. commune* CHEN. All sugar quantities are reported as pmoles of individual sugars per microgram dry weight of sample extract of media or field material

modifying enzymes we tested whether trehalase antibodies showed any non-specific cross-reaction with Wsp. No such cross-reactions were observed (Fig. 15 A). Two polypeptides in total cell extracts from *N. commune* CHEN, with molecular masses of 70 kDa and 106 kDa, cross-reacted with the *E. coli* trehalase antibodies (Fig. 15 B). The reactions with the 70 kDa protein became stronger as the time of rewetting of the cells prior to protein extraction was increased. The 106 kDa protein was first noticeable following 12 h of rehydration and was most obvious after 48 h of rewetting. Cyanobacterial trehalase was first noticeable in cell extracts following 30 min of rehydration, a time during which the sugar trehalose decreased in concentration in ethanol extracts of the rehydrated sheath, and increased in abundance with increased time of rehydration.

Trehalase antibodies did not cross react with any proteins present in the aqueous extracts (data not presented). Therefore, while the glycoproteins and the trehalase cross-reactive proteins are of very similar size they cannot be the same protein.

Discussion

Form and structure of the glycan

The extracellular glycan of *N. commune* is abundant, it is both structurally- and chemically-complex, it effectively isolates the cells within the colony from their immediate environment, and it undergoes physical and biochemical changes some of which are marked and others subtle, in response to the principal environmental variable of water availability. What is the nature of this glycan and its components? The structures present in the immediate environment of each filament of *N. commune*, that is the S_l (envelope) layer, requires a critical appraisal. Extracellular polysaccharides, such as capsules, tend to have a very low affinity for various dyes, and work using electron microscopy has established that in general they are less electron dense than the cell wall and the cytoplasm (Roth 1977). It is widely stated, largely on the basis of what seems to be a good deal of anecdotal evidence, that certain structures observed in the electron microscope are artifacts and the result of the "collapse" of cellular material during fixation and embedding. Particular controversy has surrounded the interpretation of the capsular polysaccharide (M antigen, colanic acid) of *Escherichia coli* (Roth 1977). The data of Schmidt (1981), however, clearly document that in dehydrated and fixed cells the capsule is readily observable with the light microscope but when using the very same sections the capsule cannot be imaged with the electron microscope. The staining properties of the S_l envelope layer of *N. commune* suggest that it lacks both lipid and protein. In desiccated colonies the contents of the envelope are removed during the preparation of sections for examination by SEM but not during their preparation for analysis with light microscopy or TEM. Since the only major difference in these procedures is that SEM requires a critical point drying step these data suggest that the envelope layer contains a volatile material, most likely one or more carbohydrates, and it is acidic in nature as determined through Alcian blue staining. The basis of the staining reaction of Alcian blue is debated. What

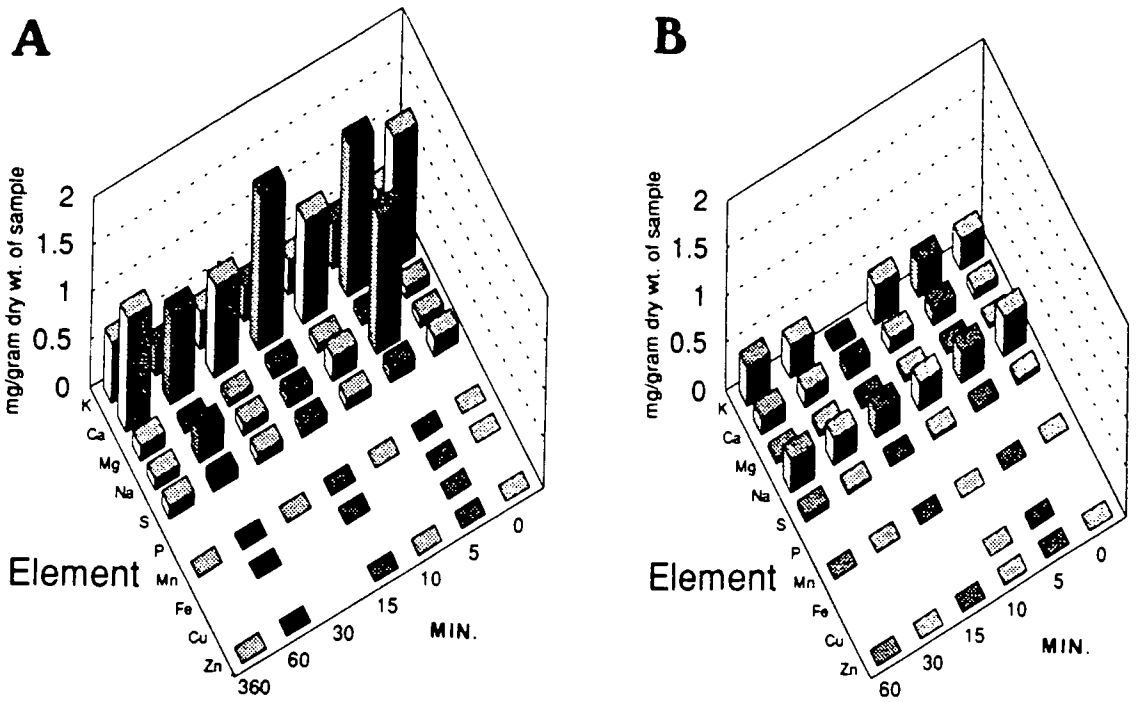


Fig. 9. Elemental analysis of *Nostoc commune* CHEN. **A** Analysis of aqueous extracts during a time course of rehydration from totally desiccated to 6 h. **B** Ethanolic extract of material in **A** which remained after aqueous extraction. Elemental quantities reported as milligram of element per gram dry weight of desiccated material used in extract

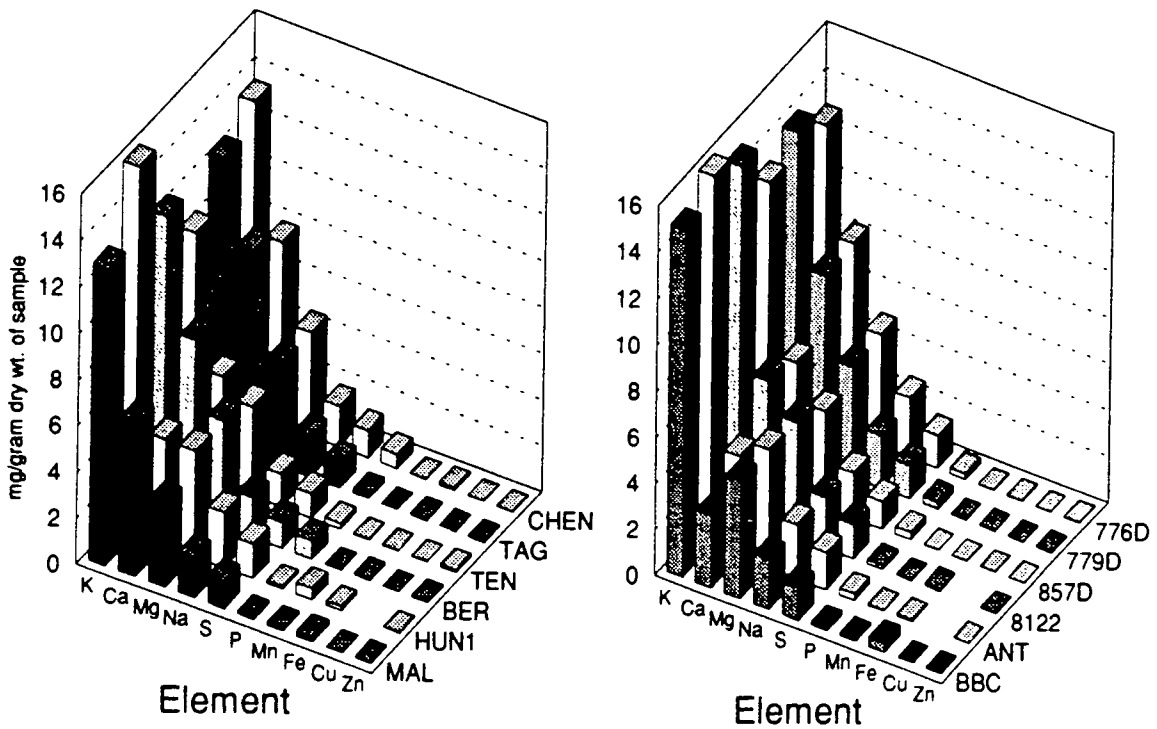
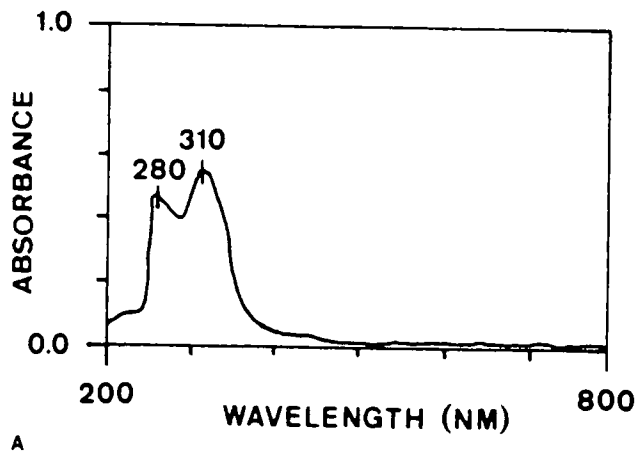
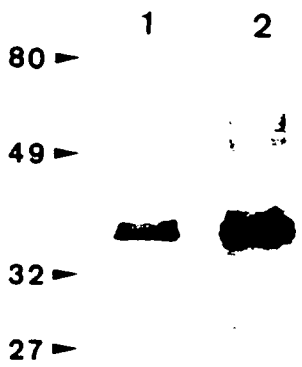


Fig. 10. Elemental analysis of aqueous extracts of *Nostoc commune* from various geographic locations. All quantities reported as in Fig. 9





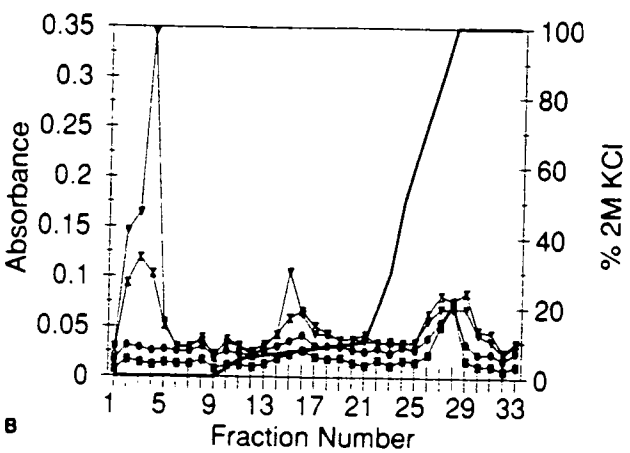


Fig. 11. Aqueous extract of *Nostoc commune* TEN contains Wsp and a UV-absorbing complex. **A** UV-visible spectrum of aqueous extract and Western blot of aqueous extract in lane *2* and non-aqueous extract in lane *1*, using Wsp antibody. **B** Elution profile of 2 ml of rehydration fluid (approximately 0.3 g dry weight of desiccated material) from a Mono Q HR 5 5 column equilibrated in 20 mM Tris-HCl, pH 7.5 (buffer A) with a flow rate of 1 ml min. Profile was developed with a gradient buffer B (buffer A, 2 M KCl; thick line). Absorbance of each fraction was determined simultaneously on a Shimadzu spectrophotometer at 280 nm (X), 310 nm (▼), 435 nm (O), and 493 nm (■). Aliquots from 1 ml fractions were subjected to Western blotting using the Wsp antibody (data not shown)

is known is that staining with Alcian blue is blocked by methylation, the stain carries 2 or 4 positive charges that bind to polyanionic mucosubstances and, the more acidic the target, the less intense is the staining. The envelope contents were either not immunogenic in mice, and thus may be of low molecular mass, or they were not present in the antigen complex that was used to generate the "sheath" antibodies. There appears to be a turnover of trehalose and sucrose upon rehydration of the cells. However, trehalose and sucrose were not detected in the glycan aqueous extraction nor were they present in the antigen complex that was used to generate the "sheath" antibodies. It is conceivable, however, that the envelope space contains free sugars that are soluble only in a non-polar solvent.

Desiccated colonies contain only 4–5% water, yet during all the time of our studies of *N. commune* we have never observed shrunken cells or cells which show any degree of structural aberration or damage. Could the envelope layer perhaps be a glass? The glassy state arises through interactions between water and different solutes in response to temperature. Glasses are metastable, they are viscous, and they are either supercooled or supersaturated (Burke 1986). Because glasses are viscous, they should impede or stop all chemical reactions that require molecular diffusion and thus they may contribute to dormancy and stability over time (Mackenzie 1977). The significance of glasses is that, in principal, the complete dehydration of bacterial cells may be avoided at temperatures below the melting point of the glass. Glasses are expected to have lower water vapor pressures than the corresponding crystalline solid and therefore they may add resistance to further dehydration of the system. The glass transition temperatures and phase relations for several saccharide-water systems have been reported (Green and Angell 1988), however, the distribution and form of glasses in complex biological systems, such as bacterial cells, remain poorly understood. In reference to our previous speculation, it can be noted that both trehalose and sucrose have been identified as glass-forming sugars. The enhanced staining and fibrous appearance of the envelope layer following different periods of rehydration (e.g., Figs. 4 C, D and 6 D) can be interpreted in

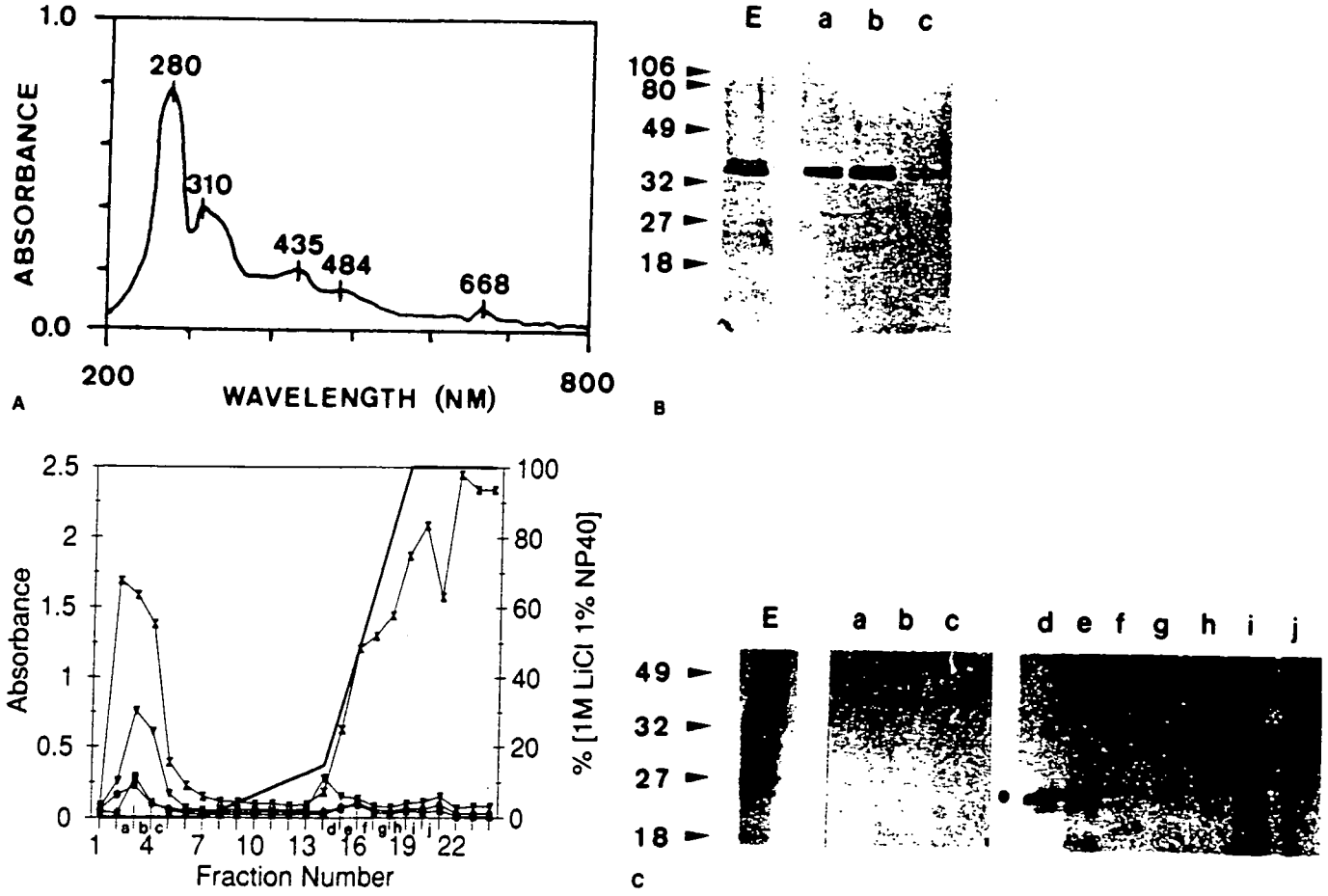


Fig. 12. Urea NP-40 extract of desiccated *Nostoc commune* TEN contains Wsp, UV-absorbing complex, and the reduced and oxidized forms of scytonemin. **A** Spectral scan of urea NP-40 extract indicating presence of protein (280 nm), UV-absorbing complex (310 nm), oxidized form of scytonemin (435 nm), reduced form of scytonemin (484 nm), and phycobiliproteins (668 nm). **B** Western blot of aliquots from extract (*E*) and fractions (*a*, *b*, and *c*) from Mono Q profile. **C** Silver stained SDS-PAGE gels of aliquots from Mono Q profile. ● Position of phycobiliproteins

two ways. During rehydration either the contents of the envelope undergo some degree of polymerization or there is some encroachment of the glycan into the envelope. The latter could result either from de novo synthesis of the glycan or through a swelling of shrunken and compacted layers at the periphery of the envelope (see Fig. 4). In the TEM the latter appears as a dark layer upon post-staining with gross appearance of a membrane (Fig. 4 C). A previous study documented that this layer is associated with a discrete localization of Wsp polypeptides (Hill et al. 1994: fig. 1 E). In fully rehydrated cells there is either no obvious envelope (interface) layer or only a very thin one (Fig. 4 E). However, in rehydrated cells a transition from loosely dispersed fibrils to densely aggregated fibrils marks a boundary that corresponds, in its dimensions, to the perimeter of the envelope layer of desiccated cells (see Figs. 5 D and 6 D). Our observations indicate that the conspicuous envelope layer, present in desiccated cells, develops during the drying of colonies, a process that may take several days to reach completion (Scherer et al. 1984).

In desiccated cells the bulk glycan is constricted at the cell cross walls but does not come into physical contact with the cells or filaments (Fig. 4 A, B). In contrast, the glycan is closely appressed to the envelope layer. The envelope layer thus represents the last refuge of *N. commune* from its air-dried environment and it is the ultimate transducer of those forces which are caused by the shrinkage and/or swelling of the bulk glycan in response to water availability. Never have we observed either a retraction of the glycan from the envelope layer or a retraction of the envelope layer from the cell outer membrane (Fig. 4). These observations suggest that the envelope layer, the bulk glycan, and the ribs of glycan that appear in a regular pattern around the inner sur-

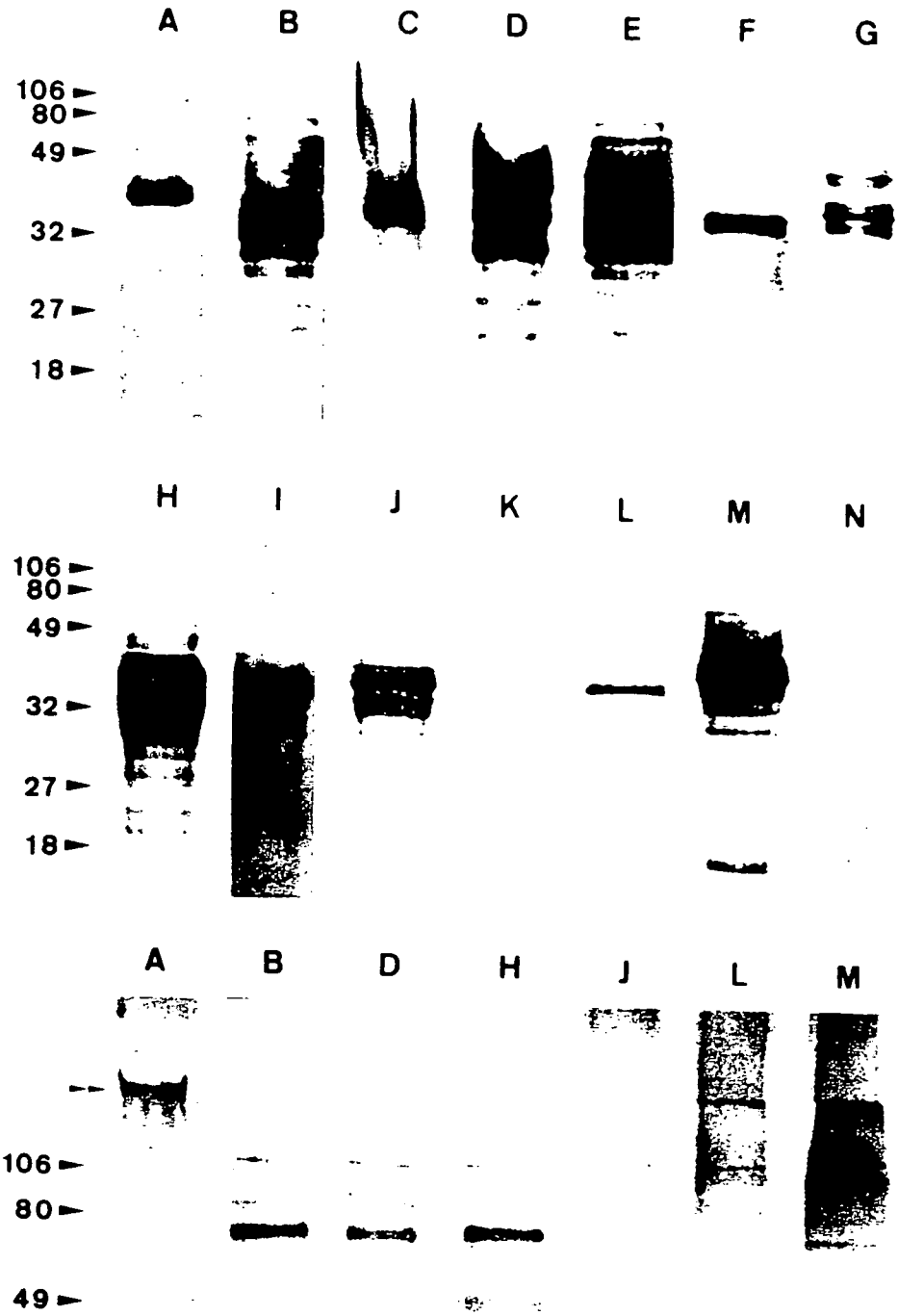


Fig. 13. Wsp in aqueous extracts of *Nostoc commune* from various geographic locations. Labeling of lanes corresponds to nomenclature used in Fig. 1. Western blot of aliquots of aqueous extracts, using the Wsp antibody. Approximately 1.5 µg of protein from the aqueous extract is present in each lane

Fig. 14. Two glycoproteins are secreted by *Nostoc commune*. Glycan stain of selected locations, using same amount of protein as used in Western blot of Fig. 13. ● Position of the glycoproteins

face of the "tunnels" and which are visualized in SEM (Fig. 3 B) are components of a stress-bearing structure. The structure makes it possible to retain the spatial organization of the filaments throughout the glycan during air-drying and prevents cell separation and collapse of filaments in the air-dried glycan. As such, the glycan makes it possible to exercise a degree of control and uniformity over the rate of shrinkage and swelling of the glycan, and thus the extent to which pressure and shear forces are imposed upon *N. commune*, in response to water availability. The pattern of ribs on the surface of each "tunnel" resembles the structures of sheath materials which have been described for free-living cyanobacteria and described in the classical phy-

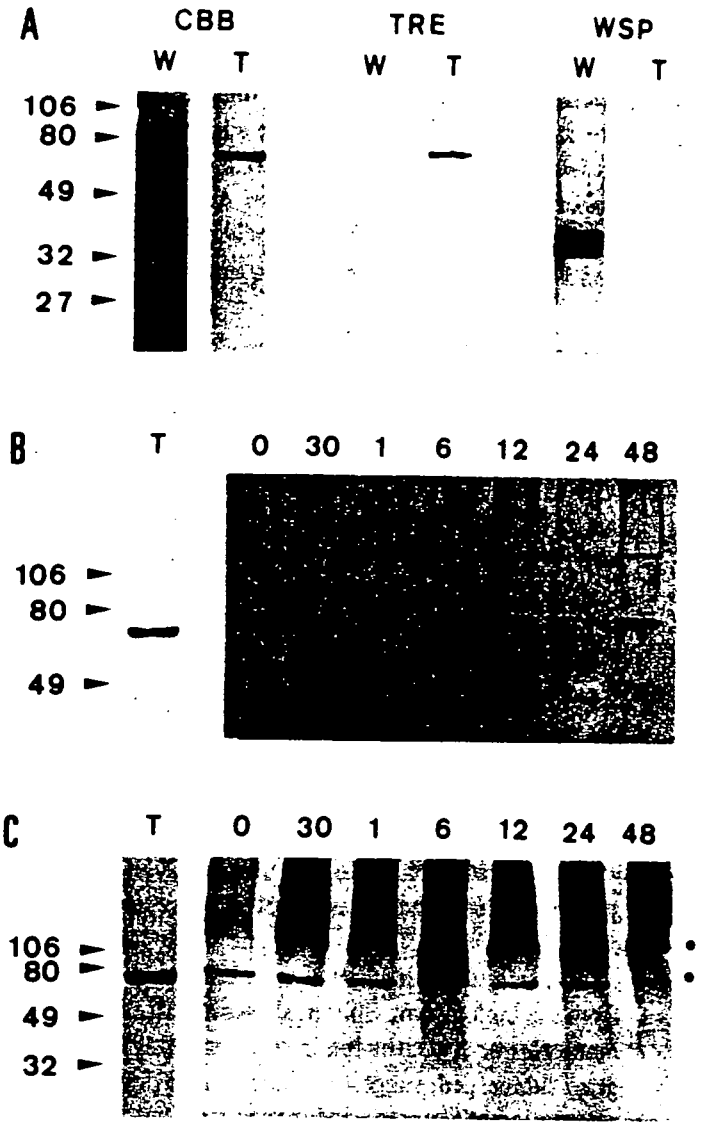


Fig. 15. Detection of a cyanobacterial trehalase in *Nostoc commune*. **A** Corresponding CBB stained SDS-PAGE gels and Western blots using the trehalase (*TRE*) and Wsp antibodies. *W* Aqueous extract from CHEN, *T* purified trehalase. **B** Western blot using trehalase antibodies of total cellular extracts (3 µg of protein per lane) from a time course of rehydration (*0* total desiccated; *30* 30 min rehydration; *1* through *48* 1 to 48 h of rehydration). *T* Purified trehalase. **C** Glycan stain (*0* through *48*) of identical samples used for trehalase blot in **B**. ● Position of the two glycoproteins

cological literature (see, e.g., Geitler 1932: figs. 507 and 527 h). Also, the apparent constriction of the tunnels, i.e., their absence at heterocysts, forms structures (Fig. 6 A) which are identical to the growth forms that arise during the life cycle of *N. commune* strain UTEX 584 in liquid culture (Fig. 7 A) (Potts and Bowman 1985, Hill and Potts in prep.). Furthermore, the envelope layer of *N. commune* cells is similar in its position, staining characteristics in TEM and in thickness, to other non-staining layers observed in vegetative cells, akinetes (Dauerzellen or resting stages) and mature heterocysts of other cyanobacteria (Sutherland et al. 1979, Bergman et al. 1985, Cox et al. 1981, Caiola et al. 1993). Differences in the ultrastructural appearance of the extracellular investments of *N. commune* have been commented by Bazzichelli et al. (1985, 1986, 1989) and have been used to define the different stages in the life cycle of this cyanobacterium. Two components of the investment were recognized from the perspective that the extracellular investment is a colloid system with fluid and fibrillar components. The terms "slime" and "sheath" were considered to represent, respectively, states of sol and gel with their clear connotations in structural analysis. The slime was defined as a structure with the characteristics of a corpuscular colloid in the state of sol, without structure and at unlimited dilution, and the sheath as a reticular colloid in the state of gel and at limited swelling.

Components of the glycan

A colorless, water-soluble pigment with an absorption maximum at 312 nm, and a yellow lipid-soluble pigment with an absorption maximum at 435 nm, represented the two major classes of UV-absorbing components in the glycan. Both have been described in *N.*

commune and in other cyanobacteria (Garcia-Pichel et al. 1991, Scherer et al. 1988, Proteau et al. 1993). The latter has the spectral properties of scytonemin, a 544 molecular weight dimeric molecule of indolic and phenolic subunits, known only from the extracellular sheath materials of certain cyanobacteria (Proteau et al. 1993). The former is a complex mixture consisting of two chromophores linked to galactose, glucose, xylose, and glucosamine (Böhm et al. in prep.). The 335 and 312 chromophores are 1,3-di-amino-cyclohexan and 3-amino-cyclohexan-1-on derivatives, respectively. Either alone, or in combination, scytonemin and mycosporines afford protection from incident solar irradiation (Garcia-Pichel and Castenholz 1991, 1993; Garcia-Pichel et al. 1993). The unique property of the *N. commune* mycosporine derivatives is that their chromophore are linked to carbohydrate (Böhm et al. in prep.). It is difficult to assess the degree of cross linking, if any, between the UV-absorbing pigments and the glycan, and this question is presently under investigation. The mycosporine compounds may constitute up to 10% by dry weight of desiccated colonies and their release upon rehydration constitutes a significant loss of cellular carbon and nitrogen. In contrast, scytonemin is not lost upon aqueous extraction. The banding pattern of scytonemin noted in colonies may represent the vestiges of the old surface of the colony as the colonies have grown, or some phenomenon associated with swelling and shrinkage of the colony. The strategy of synthesizing both water-soluble and lipid-soluble components is clear. The former can saturate the glycan compartment rapidly upon rehydration but at the expense of a high loss of the pigment, where scytonemin provides a more localized screening with the advantage that the pigment is retained by the colonies. Scytonemin may likely have a much more important role than the aqueous UV-absorbing pigments during the protection of cells upon dispersal of colony fragments.

Sucrose and trehalose both accumulate in anhydrobiotic cells and are employed as compatible solutes by a range of different organisms, including cyanobacteria, as a means to adjust intracellular water deficit (Hershkovitz et al. 1991). Sucrose and trehalose were both present in the non-polar extracts of desiccated field materials of *N. commune*, and were present, albeit at much reduced levels, in desiccated pearls (data not shown). The time of disappearance of trehalose following rehydration of desiccated colonies matched the time at which a putative trehalase was detected in cell extracts using Western blotting. No trace of the protein trehalase or the sugar trehalose was found in any of the aqueous extracts. This is consistent with the fac that the enzyme trehalase is an intracellular enzym (Boos et al. 1987). It is clear that there is a correlatio between the disappearance of the sugar trehalose an the appearance of the enzyme trehalase upon rehydration of desiccated field material of *N. commune*, however, the significance of this to desiccation tolerance i cyanobacteria is unknown at this time.

Functions of the glycan

One principal function of the glycan is that it provide a repository for water. The glycan represents a mixed system, where water and the polysaccharide tend to mix as thoroughly as they can for thermodynamic reasons (Wiggins 1990). Work is required to remove water from the gel and this can be lost through the application of pressure or temperature, and through evaporation. This provides one explanation for the striking form of the colonies of *N. commune* DRH 1. A sphere represents the minimum surface area for a given volume which would clearly provide a reduction, and uniformity, in the net rate of evaporation of water. Of course for the same considerations the spherical glycan surface provides a reduced capacity for gas uptake. Cyanobacterial sheath materials have been noted to retard gas exchange (Chang 1980). As such the outer layer of the colonies may act as a membrane which is under pressure from the bulk glycan. Spherical colonies are formed by many *Nostoc* spp. growing in situ, including those that grow submerged, suggesting functions in addition to a retardation of water loss (Martinez and Querijero 1986, Dodds and Castenholz 1987).

Previous studies of cyanobacterial envelopes and sheath structures have suggested that these may serve to concentrate metals and thus may aid in excluding the colonies from predation by gastropods, insects, etc. (Tease and Walker 1987). Although microorganisms are certainly present at the surfaces of the *N. commune* colonies, the outer silicon-rich layer represents an impenetrable barrier for them. The silicon-rich layer must be made through physico-chemical precipitation as it is hard to account for a concerted synthesis of this layer on behalf of the cells. More likely the layer is the product of some oxygen drying dependent effect on the peripheral sheath, although in liquid cultures of *N. commune* strain DRH 1 a discrete pellicular structure is also seen.

The glycan represents the bulk of the colony and constitutes a considerable diversion of the carbon and nitrogen budget (Ernst et al. 1987). The UV pigments

represent another sizeable fraction of the dry weight of a desiccated colony. In addition, Wsp is the most abundant protein in the sheath. These two components are secreted from the cells and Wsp and UV-pigment synthesis may be related (Hill et al. 1994). It remains to be determined whether *N. commune* has scavenging mechanisms for any or all of these extracellular components or their degradation products. A proteolytic activity that was specific for the 39 kDa form of Wsp has been identified although the suggestions are that this activity is intracellular in origin (Hill et al. 1994). One other protein isolated and characterized in the present study (68 kDa) is secreted, abundant in aqueous extracts of the glycan, and like Wsp, appears to be related to carbohydrate-modifying enzymes.

There is striking uniformity in the glycan isolated from materials collected from a range of different climatic environments. These are characterized by extended periods of desiccation and often rapid, and intermittent, periods of rehydration. The extracellular glycan appears to represent a buffer zone between the atmosphere and the cells. The prodigious investments made in sheath synthesis and those components found within the sheath, and our interpretation of structure and composition reported here suggest a principal role for the glycan. It is a central component of the mechanisms used by *N. commune* to tolerate desiccation. These mechanisms will be uncovered through understanding the mode of synthesis of the glycan and of those components, such as Wsp and UV-absorbing pigments, present within it.

Acknowledgements

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Purification and Biochemical Analysis of the Cytoplasmic Membrane from the Desiccation-Tolerant Cyanobacterium *Nostoc commune* UTEX 584

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The cytoplasmic membrane of the heterocystous cyanobacterium *Nostoc commune* UTEX 584 was isolated free of thylakoids and phycobiliprotein-membrane complexes by flotation centrifugation. Purified membranes had a buoyant density of 1.07 g cm^{-3} and were bright orange. Twelve major proteins were detected in the membrane, and of these, the most abundant had molecular masses of 83, 71, 68, 51, and 46 kilodaltons. The ester-linked fatty acids of the methanol fraction contained 16:0, 18:0, 18:1ω9c, 20:0, and 20:3ω3 with no traces of hydroxy fatty acids. Compound 20:3ω3 represented 56.8% of the total fatty acid methyl esters, a feature which distinguishes the cell membrane of *N. commune* UTEX 584 from those of all other cyanobacteria which have been characterized to date. Fatty acid 18:3 was not detected. Carotenoids were analyzed by high-performance liquid chromatography. The cytoplasmic membrane contained β-carotene and echinenone as the dominant carotenoids and lacked chlorophyll *a* and pheophytin *a*. Whole cells contained β-carotene and echinenone, and lesser amounts of zeaxanthin and (3*R*)-cryptoxanthin.

The cytoplasmic membrane (plasmalemma) of cyanobacteria has the structure of a unit membrane, and the current opinion is that it arises independently from the intracytoplasmic (thylakoid) membrane system (4). Data on cell membrane processes are lacking, and information on the biochemical composition of purified membranes from two unicellular forms has, only recently, become available (15, 16). The bouyant density of the cell membrane from *Synechocystis* strain PCC 6714 was 1.08 g cm^{-3}, slightly lower than the value reported for the cell membrane of *Anacystis nidulans* (1.11 g cm^{-3} [16]). A temperature-dependent change in the absorption spectrum of the cell membrane was detected with membrane preparations from *A. nidulans* but not with those from *Synechocystis* strain PCC 6714. Caloxanthin and nostoxanthin, which accounted for 36% of the total membrane carotenoids present in *A. nidulans*, were absent in the cell membrane of *Synechocystis* strain PCC 6714. It has since been shown that the outer membrane of *Synechocystis* strain PCC 6714 also contains carotenoids (9). A comparative study of three membrane fractions from this strain showed that the carotenoid content of the cytoplasmic membrane was higher than that of either the cell walls or thylakoids (16).

Nostoc commune UTEX 584 is a filamentous, heterocystous cyanobacterium that shows a marked capacity to withstand acute water stress (18, 19). The drying of cells and rehydration of desiccated material lead to rapid and significant changes in volume and turgor pressure which must impose considerable stresses upon the cells. Our studies indicate that the cell membrane is maintained intact during these changes and led us to question whether the cell wall of *N. commune* UTEX 584 possesses any unusual structural or biochemical features. This communication describes the isolation of the cytoplasmic membrane from this cyanobacterium. A biochemical characterization of the membrane is presented.

MATERIALS AND METHODS

Microorganism and growth conditions. A culture of *N. commune* UTEX 584 was obtained from R. Starr, the University of Texas Culture Collection, Austin. Clonal axenic isolates were obtained with the use of methods described by Vaara et al. (23).

Cells were grown in BG-11_0 (this medium lacks a source of combined nitrogen; 20) at 32°C under a continuous photon flux density of 300 μmol of photons m^{-2} s^{-1}. Cells were harvested during the exponential phase of growth from the 1.8-liter reactor vessel of an airlift-fermentation system (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The cell material was either used immediately or was stored at −70°C.

Isolation and purification of the cytoplasmic membrane. Cells of *N. commune* UTEX 584 show a pronounced resistance to the effects of lysozyme, detergents, and many techniques for physical disruption. It was not possible to isolate the cell envelope or cytoplasmic membrane with methods described by Murata et al. (14) or Omata and Murata (15, 16) for two unicellular strains of cyanobacteria. The method used to isolate the cell membrane from *N. commune* is a modified form of that described for *A. nidulans* by Omata and Murata (15).

Typically, the yield of membrane was highest when cells had been frozen prior to the extraction procedure. This cell material also showed a greater susceptibility to the effects of lysozyme. On a number of occasions, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) was substituted for TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] in several of the buffer solutions which are described below. This had no discernible effect on the outcome of the different fractionations.

Approximately 20 g (wet weight) of cells was vortexed at

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4°C, in 50 ml of 30 mM phosphate buffer (pH 7.0). The cells were collected by centrifugation at 5,000 × *g*, washed once in the same volume of 600 mM sucrose–30 mM phosphate buffer (pH 6.8), and then resuspended in 50 ml of 600 mM sucrose–2 mM EDTA–30 mM phosphate buffer (pH 6.8). Solid lysozyme (10 mg; Sigma Chemical Co., St. Louis, Mo.) was added to the suspension, and incubation was continued at 30°C for 2 h. Although lysozyme had little effect on the integrity of fresh cell material, its addition to the buffer medium enhanced ultimate recovery of the membrane, presumably through the release of the membrane from peptidoglycan complexes.

Cells were collected by low-speed centrifugation, washed once in a buffer of 600 mM sucrose, 10 mM NaCl, and 10 mM TES-NaOH (pH 7.0), and resuspended in 50 ml of the same buffer. This suspension was passed once through a French pressure cell at 100 MPa, at 4°C. The brie was collected and incubated at 4°C in the presence of DNase (50 μg ml^{-1}, final concentration) for 10 min, and then it was centrifuged at 5,000 × *g* for 10 min to remove unbroken cells. The supernatant was then centrifuged at 20,000 × *g* for 60 min (Sorvall SS-34 rotor; Ivan Sorvall, Inc., Norwall, Conn.) at 4°C, and 5 ml of the supernatant was transferred to a 10-ml polycarbonate ultracentrifuge tube (Oak Ridge type). The supernatant was overlaid with 1.5 to 2 ml of a buffer that contained 1 mM NaN_3, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaCl, 5 mM EDTA, and 10 mM TES-NaOH (NFNET, pH 7.2) and centrifuged at 180,000 × *g* at 4°C for 3 h (50 Ti rotor; Beckman Instruments, Inc., Fullerton, Calif.; k = 96.3, where k is the clearing factor [maximum rated rpm/actual run rpm] × $k_{max\ rpm}$). The upper membrane layer was removed with a Pasteur pipette, mixed with 1 volume of the overlay buffer, and then transferred to the surface of a 29-ml sucrose density gradient which was prepared with the following steps: 35, 28, 20, 14, and 7% (wt/vol) sucrose; 7.5, 7.5, 5, 5, and 4 ml, respectively (in NFNET buffer). This gradient was centrifuged at 112,000 × *g* at 4°C for 15 h (SW27 rotor, Beckman; k = 335).

Layers that contained the cytoplasmic membrane fraction were removed from gradients with sterile Pasteur pipettes and were then mixed with one or more volumes of NFNET buffer at 4°C to dilute the concentration of sucrose. The membranes were then collected as pellets after centrifugation at 180,000 × *g* (Beckman 50 Ti rotor) at 4°C for 2 h. This procedure was repeated several times.

Biochemical analyses. (i) Membrane proteins. Purified membrane fractions were solubilized in sodium dodecyl sulfate buffer (2% [wt/vol] sodium dodecyl sulfate, 1 mM β-mercaptoethanol, 15% [wt/vol] glycerol, 0.01% [wt/vol] bromphenol blue, 0.2 M Tris; pH 8.8) at room temperature for 30 min. Samples were analyzed on either 10 or 15% (wt/vol) acrylamide gels which were supported on gel-bond (FMC Corp., Rockland, Maine). Electrophoresis was performed with the buffer system of Laemmli (13). Gels were stained with either Coomassie brilliant blue R or silver stain (Bio-Rad Laboratories, Richmond, Calif.). After drying, protein bands in the gels were analyzed further with a Beckman microzone densitometer model R-110.

(ii) Lipid analysis. Lipid extraction of the purified membrane fraction, fatty acid purification, and analysis by capillary gas chromatography were performed as described in detail by Guckert et al. (5). Briefly, a modified Bligh and Dyer (2) chloroform-methanol lipid extraction was used. Total extractable lipids were fractionated with chloroform, acetone, and methanol by silicic acid column chromatography. The fatty acid esters linked to the phospholipids were methylated by mild alkaline methanolysis of the methanol fraction. The fatty acid methyl esters and hydroxy fatty acid methyl esters were purified by thin-layer chromatography before gas chromatography analysis. Tenative peak indications were based on coelution with standards which were obtained from either Supelco, Inc., (Bellefonte, Pa.) and Alltech Associates, Inc., Applied Science Div. (State College, Pa.) or laboratory standards which had been identified previously. Samples were analyzed on a dual column gas chromatograph equipped with a 50-m nonpolar, cross-linked methyl silicone-fused silica column (Hewlett Packard Co., Palo Alto, Calif.), a 60-m SP-2340 polar column (Supelco), and a common injector.

(iii) Extraction and analysis of carotenoids and chlorophylls. All extractions and manipulations were performed with degassed solvents, in a darkroom, under the reduced illumination of a safe light and in an atmosphere of nitrogen (in a glove bag). A subsample of the purified membrane fraction was suspended in 10 ml of ice-cold methanol and sonicated in five 30-s bursts, with 30-s intermissions on ice (Branson Sonifier cell disrupter; Branson Sonic Power Co., Danbury, Conn.). After 6 h of extraction in methanol at 4°C, 10 ml each of chloroform and phosphate buffer from the lipid extraction was added, and the phases were allowed to partition at 4°C overnight. After 24 h, the chloroform fraction was filtered through a 2V filter (Whatman, Inc., Clifton, N.J.), and the fraction was evaporated to dryness under a stream of nitrogen. The residue was dissolved in ethyl acetate and passed through a column phase which contained a C_{18} Sep-PAK cartridge (Waters Associates, Inc., Milford, Mass.) to protect the high-performance liquid chromatography (HPLC) column. The eluates were evaporated under a stream of nitrogen, redissolved in 0.5 ml of ethyl acetate, and filtered through a centrifugal-filter system (Rainin; 0.2-μm-pore-size Nylon-66 membrane filter). Portions of 100 μl were injected into a Waters HPLC system which consisted of two pumps (models 45 and 6000), a model 720 solvent programmer, a Rheodyne sample valve, and a C_{18} column (Chrompak, 25 cm by 4.5 mm, 8-μm pore size). The column was protected by an in-line filter and a guard column (46 by 4.5 mm) which was packed with 11-μm C_{18}-coated glass beads (Partisil, Whatman). Pigments were eluted within 20 min (flow rate, 2 ml min^{-1}) with a linear solvent gradient of 100% acetonitrile-water (90:10 vol/vol) to 75% ethyl acetate. After 20 min, the final solvent ratio was maintained for 1 min before the initial solvent composition was allowed to reestalish over a period of 5 min. The system was allowed to equilibrate for a futher 4 min before the next sample was injected. The pigments were detected through continuous measurement of absorbances at 7 wavelengths (280, 420, 450, 480, 640, 660, and 770 nm; Hewlett Packard model 8450A diode array spectrophotometer and Helma flowcell of 15 μl in volume). Complete spectra (200 to 800 nm) were recorded from peaks of absorbance as they appeared on the video display of a Hewlett Packard model 85 computer. The spectra, as data, were stored on disks for reproduction with a Hewlett Packard model 8HP7470 graphics plotter.

(iv) Identification of carotenoids. Carotenoids were purified from whole cells to confirm the identities of carotenoids in the purified cell membrane. Cells, grown in liquid culture (19), were subjected to extraction procedures which were identical to those used for the purified cell membrane. Pigments were identified by their absorbance spectra, retention times, and after coelution of the standards [β-carotene, chlorophyll *a*, canthaxanthin, zeaxanthin, and (3*R*)-cryptoxanthin].

Chemicals. Acetonitrile was purchased from Mallinckrodt, Inc., St. Louis, Mo. (Chromarquality), ethyl acetate and chloroform were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. (HPLC and Resiquality), and water was purchased from E. M. Science (Omnisolve). Chlorophyll *a* and α- and β-carotene were purchased from Sigma. (3*R*,3′*R*)-Zeaxanthin, canthaxanthin, and (3*R*)-cryptoxanthin were donated generously by Hoffmann-La Roche, Basel, Switzerland.

Nomenclature. Fatty acids are designated with the total number of carbon atoms:number of double bonds closest to the aliphatic (ω) portion of the molecule. The letters c and t indicate *cis* and *trans* geometry, respectively.

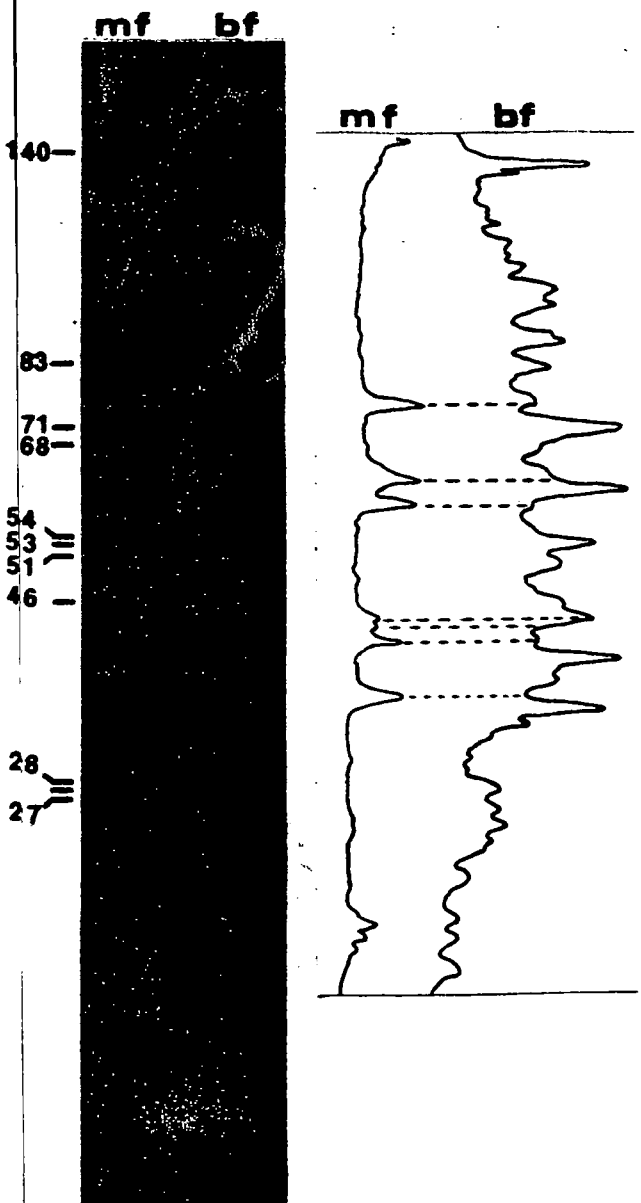

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the cytoplasmic membrane fraction (mf) and the phycobiliprotein-thylakoid fraction (bf) over which the membrane was collected in the initial stages of purification. Proteins were visualized through the use of silver stain. Molecular masses (in kilodaltons) of the individual proteins were determined with high and low range markers (Sigma products SDS-611 and SDS-7).

TABLE 1. Pigments from whole cells of *N. commune* UTEX 584

Retention time(s)	Spectral maxima (nm)	Peak ratio identity (%)
666	425, 449, 475	31–52 Zeaxanthin
831	378, 424, 616, 660	1.6 Chlorophyll *a*
847	422, 451, 478	58 β-Cryptoxanthin[a]
891	377, 392, 426, 613, 661	1.6 Chlorophyll *a*
930	454	Echinenone[a]
1,100	422, 450, 476	13 β-Carotene[a]

[a] Dominant carotenoids in the cell membrane.

RESULTS

Physical analysis and criteria for purity. Cells of *N. commune* UTEX 584 in exponential growth show a marked resistance to treatment with lysozyme. The sensitivity was increased by freezing the cells at −70°C, and then thawing them at room temperature prior to treatment with lysozyme. It was not possible to isolate the cell envelope of *N. commune* (14), despite numerous attempts with either fresh or prefrozen cells, different critical breakage pressures in the French pressure cell, and a wide range of different designs for sucrose density gradients.

The cytoplasmic membrane was isolated free of the thylakoid membranes by flotation centrifugation. After centrifugation the membrane aggregated as an orange-yellow to brown film and adhered to the inner wall of the centrifuge tube at the meniscus of the gradient. If a high ratio of cell material to buffer was maintained after treatment in the French pressure cell, the amount of this orange fraction was reduced considerably, and the ultimate yield of the cytoplasmic membrane was low. In the initial extraction, the orange fraction merged with the lower, dark-blue, layer of phycobiliproteins but was free of thylakoid membranes. In the final purification step, the orange-yellow fraction was separated from the blue-pink layer of phycobiliproteins. Phycobiliproteins and traces of thylakoid membranes could be eliminated in one step if, after the initial extraction, only the surface portion of the orange layer was removed for further centrifugation. In several isolations, two quite distinct orange-yellow fractions were observed in the final gradient, a surface fraction (the one usually obtained) with a bouyant density of 1.04 to 1.06 g cm^{-3} and a zone deeper in the gradient with a bouyant density of 1.07 to 1.09 g cm^{-3}.

The absorption spectrum of the orange-yellow membrane fraction was quite different from the spectrum obtained for the purified thylakoids (data not shown). The spectrum of the cytoplasmic membrane showed no peaks in A_{650} (the dominant peak in the spectrum of the thylakoid membrane) or A_{654} (allophycocyanin). A lack of chlorophyll *a* or pheophytin *a* in the purified membrane was confirmed by the absence of phytolalcohols in membrane analyses (see below). A comparison of the banding pattern of the denatured cytoplasmic membrane proteins and those of the biliprotein fraction over which the membrane aggregated in initial extractions confirmed the purity of the orange-yellow membrane fraction (Fig. 1).

Protein composition. The dark orange pellet, which was obtained after ultracentrifugation of the purified fraction, was solubilized readily at room temperature in sodium dodecyl sulfate cracking buffer. Some 12 bands were detected on gels with silver stain and following sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (Fig. 1). Of these, the most obvious corresponded to polypeptides with molecular masses of 83, 71, 68, 51, and 46 kilodaltons.

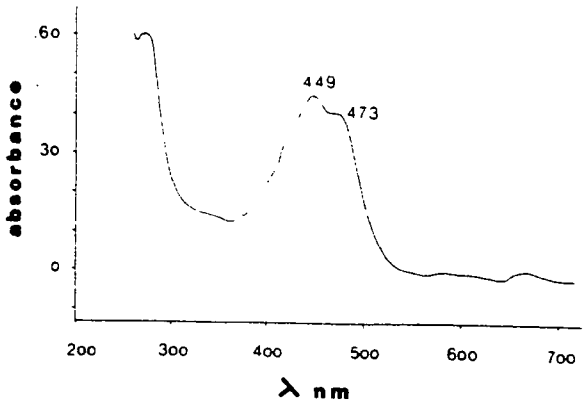

FIG. 2. Absorption spectrum of the purified cytoplasmic membrane.

Lipid composition. The ester-linked fatty acids of the methanol fraction contained 16:0 (23.1% of fatty acid methyl esters), 18:0 (9.5%), 18:1ω9c (7.7%), 20:0 (3.0%), and 20:3ω3 (56.8%) compounds but no traces of hydroxy fatty acids. Compound 20:3ω3 represented, at 56.8%, the major component of the membrane fatty acids.

Pigments. The procedures of sonication and extraction (6 to 8 h), followed by overnight partition, did not produce pheopigments. After extraction, the remains of the glass fiber filters were stained blue purple by the phycobiliproteins, which were not detected in the wavelength spectra of the methanol-water phase (data not shown).

Two major and two minor carotenoids were detected in extracts of whole cells (Table 1). The first major carotenoid eluted at 930 s with one absorbance peak at 454 nm. Through analysis of this spectrum and its elution after chlorophyll *a* (25), this carotenoid is identified tentatively as echinenone. The second major carotenoid coeluted with β-carotene at 1,100 s with three identical absorbance maxima at 422*, 450, and 476 nm (* indicates a shoulder in the spectrum; peak III to peak II).

The major peaks and shoulders on the spectrum of the membrane were present at 424, 449, and 473 nm (Fig. 2). HPLC analysis of the membrane fraction showed two major peaks (Fig. 3) which upon purification were identified as echinenone and β-carotene.

DISCUSSION

The bright-orange cell membrane isolated from *N. commune* UTEX 584 has similar properties to those described for *Synechocystis* strain PCC 6714 and *A. nidulans* (15, 16) with several exceptions. The low bouyant density of the membrane from *N. commune* reflects, presumably, its high lipid content. The value of 1.07 g cm^{-3} is somewhat lower than the values reported for the two unicellular cyanobacteria and is significantly lower than the values (mean = c1.22 g cm^{-3}) reported for other phototrophic and eubacterial strains (3, 17, 21, 24). The polypeptide profiles of the cell membranes from *N. commune* and the two unicellular strains are quite different, although a protein with an apparent molecular mass of 83 kilodaltons is a major component of all three types (Fig. 3a in reference 16; Fig. 7 in reference 15; Fig. 1 in this study).

Previous reports on the fatty acid compositions of cyanobacteria, including strains of *Anabaena* and *Nostoc*, have been obtained from whole cells (10, 11). In many strains, the composition of fatty acids is similar to that of the higher-plant chloroplast (with a high proportion of polyunsaturated C_{18} fatty acids, especially linolenic acid [18:3]) as either the α or the γ isomer (4). In *N. muscorum*, fatty acid 18:3 accounted for 20% of the total (8). It is significant, therefore, that neither the cell membranes of *N. commune* or *A. nidulans* nor the cytoplasmic and outer membranes of *Synechocystis* strain PCC 6714 contain 18:3 fatty acid (9, 15; this study). Palmitic acid (16:0), which was detected in high amounts in the cytoplasmic membrane of *N. commune*, was reported also in high concentrations in the cytoplasmic membrane of *A. nidulans* and in the inner and outer membrane of *Scynechocystis* strain PCC 6714. The absence of hydroxy fatty acids in the hydroxy ester-linked fatty acid fraction of the cytoplasmic membrane of *N. commune* UTEX 584 is in agreement with findings for the cytoplasmic membranes of the other cyanobacterial strains (14–16). However, the presence of a very high concentration of 20:3ω3 fatty acid is a significant finding and it is assumed that the membrane of *N. commune* is significantly more fluid than any of the cyanobacterial cell membranes which have been described to date. In addition, the presence of large amounts of the 20:3ω3 acid must cause a decrease in molecular stacking within the membrane. Both of these features may lend important properties to the membrane and may be significant in situations where water molecules are removed

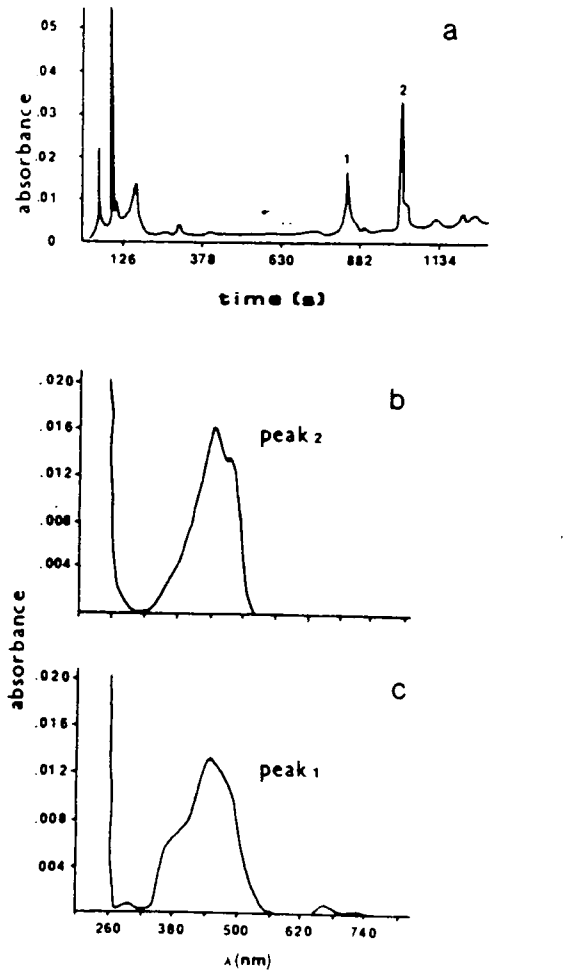

FIG. 3. (a) HPLC analysis of the pigments present in the cytoplasmic membrane. (b and c) Absorption spectra of the purified pigments.

from the membrane, or when dessicated membranes are rehydrated.

The carotenoids β-carotene, zeaxanthin, caloxanthin, nostoxanthin, myxoxanthophyll, echinenone, cantha-xanthin, and 4-hydroxy-4′-keto-β-carotene have been reported previously in *Nostoc* strains (7, 22). Myxoxanthophyll, caloxanthin, nostoxanthin, and 4-hydroxy-4′-keto-β-carotene were not detected, while β-carotene, zeaxanthin, and (3*R*)-cryptoxanthin have been confirmed by coelution of the standard with identical spectra. Zeaxanthin, which was the dominant carotenoid in the cell membrane of *A. nidulans* (15) and second in abundance in the membrane of *Synechocystis* strain PCC 6714, was detected in the cell membrane of *N. commune* only in small amounts. In a comparative study of the different membranes from *Synechocystis* strain PCC 6714, the carotenoid content was found to be higher in the cytoplasmic membrane and lower in the cell walls, relative to the thylakoid membranes (16). The cytoplasmic membrane of this strain contained β-carotene, echinenone, cryptoxanthin, zeaxanthin, xanthophyll-1, and xanthophyll-2. For the same strain, Jürgens and Weckesser (9) demonstrated the presence of carotenoids in the outer membrane and suggested that this may be the site of their synthesis through analogy with the chloroplast of higher plants. Carotenoids are thought to protect cyanobacteria and other bacteria (1) against photooxidative radiation (6, 12). While we have shown that photo-oxidative damage may be significant during desiccation of *N. commune* (18, 19), it is not possible at this time to assess the potential role of carotenoids in protection of *N. commune* against photo-oxidation.

In summary, we have described the purification of the cytoplasmic membrane from the filamentous, desiccation-tolerant cyanobacterium *N. commune* UTEX 584. Despite numerous attempts, using a range of modifications of published methods, we were unable to isolate cell envelopes from *N. commune*. This may indicate some subtle structural feature of the cell wall of this filamentous cyanobacterium. As such, the procedures described here may prove to be useful for the isolation and purification of cell membranes from other lysozyme-resistant strains of cyanobacteria. Although somewhat similar in its physical and biochemical properties to membranes which have been isolated from unicellular cyanobacteria, the presence of 20:3ω3 fatty acid in the cell membrane of *N. commune* UTEX 584, at more than 50% of the total membrane fatty acids, distinguishes the membrane from those of other strains and may be one feature which contributes to the resistance of the cells to rapid fluxes in water availability.

ACKNOWLEDGMENTS

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Novel Water Stress Protein from a Desiccation-tolerant Cyanobacterium

PURIFICATION AND PARTIAL CHARACTERIZATION*

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A desiccation-tolerant cyanobacterium *Nostoc commune* accumulates a novel group of acidic proteins when colonies are subjected to repeated cycles of drying and rehydration. The proteins occur in high concentrations; they have isoelectric points between 4.3 and 4.8 and apparent molecular masses between 30 and 39 kDa. The purification of three of these proteins with molecular masses of 33, 37, and 39 kDa is described. The amino-terminal sequence of the 39-kDa protein is Ala-Leu-Tyr-Gly-Tyr-Thr-Ile-Gly-Glu. Peptide mapping of the 39- and the 33-kDa proteins, using different proteases, gave similar patterns of digestion fragments. The amino acid compositions of the proteins isolated were similar, and each cross-reacted with a polyclonal antibody raised against the largest (39-kDa) protein. The results indicate that the microheterogeneity observed was generated by *in vivo* proteolysis of the 39-kDa protein. It is suggested that this protein is a water stress protein with a protective function on a structural level.

Desiccation tolerance is a widespread and long known phenomenon, occurring over a wide range of taxa including bacteria, plants, and animals (Crowe and Clegg, 1978). However, as Leopold (1986) stated, "until recently, the question of how organisms can tolerate desiccation has been almost a cryptic one, potentially interesting but undeciphered." Important progress toward the elucidation of the mechanisms of desiccation tolerance was made by demonstrating that both phospholipid bilayers and proteins can be stabilized during water stress by sugars, especially by trehalose (for review, see Crowe *et al.*, 1987). Trehalose is found in a variety of microorganisms including cyanobacteria when they are subjected to drying (matric water stress) or osmotic stress (*e.g.* Reed *et al.*, 1984; McBride and Ensign, 1987).

Less is known about the possible effects of desiccation on the synthesis of novel proteins. The desiccation-tolerant moss *Tortula ruralis*, when rehydrated, synthesizes "rehydration proteins" (Oliver and Bewley 1984b), which apparently allow this plant to survive the rehydration process. In desiccation-intolerant mosses, rehydration rather than desiccation seems to be the fatal event (Bewley, 1979; Oliver and Bewley, 1984a). The synthesis of specific proteins in response to desiccation, however, has been reported only recently for seeds of maize (Gomez *et al.*, 1988) and rice (Mundy and Chua, 1988). The function(s) of these proteins remains unknown.

The cosmopolitan terrestrial cyanobacterium *Nostoc commune* is able to tolerate acute water stress and can survive in the air-dry state for many years. Under natural conditions, the cells are embedded and immobilized in a water-absorbing sheath composed of carbohydrates. Growth results in the formation of macroscopic colonies (0.5–3 mm thick) which cover areas of several square centimeters. Desiccation tolerance in *N. commune* has been studied on the structural (Potts and Bowman, 1985; Peat and Potts, 1987), physiological (Coxson and Kershaw, 1983; Scherer *et al.*, 1984; Potts and Bowman, 1985), and biochemical levels (Olie and Potts, 1986; Potts and Morrison, 1986; Scherer *et al.*, 1986). Due to its procaryotic cell organization and capacity for the higher plant type of photosynthesis, *N. commune* lends itself as a suitable model system for the study of desiccation tolerance at the molecular level. As a first step in this direction, we describe the isolation and partial characterization of novel water stress protein(s) (Wsp)[1] from *N. commune*.

EXPERIMENTAL PROCEDURES

Organisms

N. commune var. *commune* Vauch. was collected in China in 1981 (Hunan province, Henyong district), August 1986 (Wuhan), September 1987 (Heibei province, Yu county), and June 1988 (Konstanz, Federal Republic of Germany). Desiccated colonies were kept at 22–25 °C in plastic bags in the dark until needed. Extended desiccation, storage, and subsequent rehydration of field material does not lead to any major discernible structural damage to either vegetative cells or heterocysts (Peat *et al.*, 1988). The functional integrity of cells is also maintained during prolonged desiccation (Scherer *et al.*, 1984, 1986). Field material was washed several times in distilled water and then subsequently dried and rewetted with $BG11_0$ (Rippka *et al.*, 1979) 6–10 times at room temperature. After washing, the field material of *Nostoc* retained only very few bacterial contaminants at the outer surface of colonies. The interior of colonies was found to be axenic (Jäger and Potts, 1988a, 1988b; Peat *et al.*, 1988). Desiccation of colonies in air (approximately 50% relative humidity, equivalent to −95.2 megapascals) was achieved after 6–12 h, depending on the thickness of the colony. Field material of *Nostoc* was cultivated in the laboratory as macroscopic spherical colonies of 2–10-mm diameter as described (Scherer *et al.*, 1988). *N. commune* UTEX 584 was grown in axenic culture in $BG11_0$, at 32 °C in an airlift fermentor as described (Potts, 1985).

Methods

Extraction of Soluble Protein—Dry *Nostoc* was frozen in liquid nitrogen and ground under liquid nitrogen to a very fine powder. In a typical experiment, 15 g of this powder was suspended in 150 ml of

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[1] The abbreviations used are: Wsp, water stress protein(s); IEF, isoelectric focusing; SDS, sodium dodecyl sulfate; PAGE; polyacrylamide gel electrophoresis; UTEX, University of Texas Culture Collection.

ice-cold cracking buffer (50 mM Tris-Hcl, pH 7.8, 10 mM $MgCl_2$, 20 mM KCl, 1 mM NaN_3, 1 mM phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol) and passed immediately through a precooled French pressure cell at 130 megapascals. After the addition of 50–100 ml of cracking buffer, the brie was passed through the French press two more times. For *analytical purposes*, the suspension was centrifuged for 20 min at 12,000 × *g*. The supernatant fraction was precipitated by trichloroacetic acid (10% (w/v) final concentration), washed twice in 5% (w/v) trichloroacetic acid, and washed once each with 50% (v/v) acetone and ether. After evaporation of the ether, the proteins were solubilized in IEF sample buffer and analyzed by two-dimensional electrophoresis (see Fig. 1, *A*, *B*, and *E* and Potts, 1986). For *preparative purposes* (compare Fig. 1, *C* and *D*, as well as the following experiments), unbroken cells, cell debris, membranes, and macromolecular carbohydrate complexes originating from the sheath material were removed by ultracentrifugation (4 °C, 2 h, 70,000 rpm, Beckman Ti-70 rotor). The supernatant fraction was subjected to ammonium sulfate fractionation, the 35–60% precipitate resuspended in 20 mM Tris-HCl, pH 7.8, dialyzed exhaustively against this buffer, and centrifuged at 100,000 × *g* for 1 h at 4 °C.

Column Chromatography—This was performed with fast performance liquid chromatography (Pharmacia LKB Biotechnology Inc.) at 22–25 °C. After separation, fractions were kept on ice. For analytical separations, a Mono Q HR 5/5 anion exchanger, a Mono S HR 5/5 cation exchanger, a Mono P 5/20 chromatofocusing column, a Phenylsuperose HR 5/5 hydrophobic interaction column, or a Superose 12 HR 10/30 gel exclusion chromatography column was used. Preparative chromatography was performed on a Mono Q HR 10/10 anion exchanger. The urea used for gel exclusion chromatography was obtained from Sigma and was recrystallized from ethanol prior to use.

Ultrafiltration—Ultrafiltration was performed at 4 °C using an ultrafiltration cell (Amicon Corp., Danvers, MA) operated under 4–5 megapascals of nitrogen with a membrane YM-30 (exclusion limit M_r = 30,000). Desalting and buffer changes were performed with PD-10 columns (Pharmacia).

Electrophoresis—Analytical SDS-PAGE (Laemmli, 1970) was performed in 1.5- or 0.75-mm minigels, using either a self-built device or a Mighty Small II SE 250 chamber (Hoefer Scientific Instruments, San Francisco). For preparative SDS-PAGE, 1.5 × 160 × 200-mm gels (12% (w/v) acrylamide) were developed in a Protean II cell (Bio-Rad). Nondenaturing PAGE was performed according to Bryan (1977) and Davis (1964) using a series of gels between 5 and 10% (w/v) acrylamide, following the protocol given in the 1986 Sigma technical bulletin MKR-137. Calibration was performed with Sigma MW-ND-500 molecular weight markers. Two-dimensional electrophoresis was accomplished using the procedure described by O'Farrel (1975), either using 110 × 2.5-mm IEF tube gels and 1.5 × 150 × 200-mm SDS slab gels (Potts, 1986) or 1.5 × 65-mm IEF tube gels and 1.5 × 55 × 80-mm SDS slab gels in the Mighty Small II chamber. Biolytes (5% (w/v), Bio-Rad) were used as described in the figure legends.

Electroelution—Bands of protein were excised from Coomassie-stained preparative SDS gels and electroeluted using the electroelution chamber of the Isco model 1750 electrophoretic concentrator (Isco, Inc., Lincoln, NE). Gels used for this procedure were stained 5 min and destained 30 min. Electroelution was performed for 3–5 h in 192 mM glycine and 25 mM Tris-HCl, pH 8.3, at a constant current of 2 A. Proteins were eluted in volumes of 200 μl and were dialyzed exhaustively against water.

Peptide Mapping—Electroeluted proteins were lyophilized, resolubilized in 125 mM Tris-HCl, pH 6.8, 0.5% (w/v) SDS, 10% (w/v) glycerol, 0.001% (w/v) bromphenol blue to a final concentration of 0.5–0.7 mg ml^{-1}, and then boiled for 2 min. After cooling on ice, either chymotrypsin (Sigma, type II), papain (Sigma, type IV) or *Staphylococcus aureus* protease (Sigma, type XVII-S) was added to a concentration of 0.1 mg ml^{-1}, and the solutions were incubated for 5 min at 32 °C. After adding β-mercaptoethanol and SDS to final concentrations of 5% (w/v) and 2% (w/v), respectively (Cleveland *et al.*, 1977), cleavage products were resolved by SDS-PAGE, using 21% (w/v) acrylamide gels, and analyzed after staining with Coomassie Blue as well as silver stain (Oakley *et al.*, 1980).

Carbohydrate Analysis—Purified proteins (2 μg) were dot blotted using nitrocellulose and assayed for concanavalin A-binding carbohydrates according to Clegg (1982). The staining of glycoproteins (separated by SDS-PAGE) with periodic acid-Schiff reagent or alcian blue was performed following the protocols of Zacharias *et al.* (1969) and Kunicki *et al.* (1981), respectively. Fluorometric carbohydrate analysis of proteins electroeluted from SDS-PAGE gels was performed according to Perrini and Peters (1982). Neuraminidase treatment was carried out as described by Chen *et al.* (1985) using neuraminidase type X isolated from *Clostridium perfringens* (Sigma).

Amino Acid Composition—After acid hydrolysis (20 h, 110 °C, HCl atmosphere), the amino acids were quantified using a Waters Picotech amino acid analyzer system (Millipore, Waters Chromatographic Division, Bedford, MA). Assays were run in duplicate. The amino acid composition divergence *D* was estimated according to Harris and Teller (1973) as

$$D = [|\text{mol } \%(i_A) - \text{mol } \%(i_B)|^2]^{1/2}$$

with $M\%(i_A)$ and $M\%(i_B)$ being the molar fraction of the *i*th amino acid in protein A or B, respectively.

Amino-terminal Sequence Analysis—The 39-kDa form of Wsp was excised from an SDS-PAGE gel, electroeluted as described above, and dot blotted to a polyvinylidene difluoride membrane (Immobilon P, Millipore) which was then washed extensively in distilled water. Edman degradation of the protein bound to the membrane (Matsudaira, 1987) was performed in a gas-phase protein sequenator (Applied Biosystems, model 470A) according to Hewick *et al.* (1981). The experiment was performed twice and gave identical results.

Generation of Antibodies—After electroelution from SDS-PAGE gels and extensive dialysis against distilled water, a solution of purified proteins was made 40 mM with phosphate buffer, pH 7.5, mixed 1:1 with complete Freund's adjuvant, and injected into 3-4-week-old C3H-HEJ mice (approximately 10 μg of protein/mouse in 0.2 ml). After 2 weeks, a booster with the same amount of protein mixed 1:1 with incomplete Freund's adjuvant was given, and the mice were bled after another 10 days. In addition, antibodies directed against the *complete* water stress protein fraction were raised in rabbits. A sample of 0.1 mg was dissolved in 0.5 ml of phosphate buffer, mixed with 0.5 ml of complete Freund's adjuvant, and injected subcutaneously at different sites. After 2 and 4 weeks, the immune response was stimulated by injecting 0.1 mg of protein dissolved in incomplete Freund's adjuvant and phosphate buffer. After precipitation of the blood clot, the serum was used without further treatment. Preimmune serum was collected before injection.

Western Blotting—After separation on SDS-PAGE, proteins were transferred (24 V, 1 A, 1 h) to nitrocellulose (Bio-Rad) using 25 mM Tris, 192 mM glycine, 20% (v/v) methanol. Immunodetection was performed according to standard techniques, using mouse antiserum diluted 1:500 and an alkaline phosphatase-conjugated sheep anti-mouse IgG antibody (Sigma) diluted 1:1,000, following the protocol described in the Promega Biotec technical manual (Madison, WI). Rabbit antiserum was diluted 1:400, and bound antibodies were visualized using the protein A-horseradish peroxidase Immunoblot kit (Bio-Rad), following the manufacturer's instructions.

RESULTS

Presence of Water Stress Proteins in Desiccated Cells—The two-dimensional protein index of field-grown *N. commune* collected in China is shown in Fig. 1*A* for a sample that has been kept in the dry state for 1 year and in Fig. 1*B* for a sample that had been desiccated for 5 years. A conspicuous cluster of acidic proteins with isoelectric points of 4.5–5 and molecular masses between 30 and 40 kDa, designated Wsp, was found in the desiccated field materials but not in the liquid-grown laboratory cultures (Fig. 1*E*). Colonies from the field usually contained much less phycobiliproteins (*Pbp*) compared with laboratory-grown colonies (desiccation of liquid-grown *N. commune* UTEX 584 caused phycobilisomes to dissociate from thylakoids and induced light-dependent phycobiliprotein degradation (Potts, 1985)). After prolonged desiccation, Wsp appear to be more persistent than other soluble proteins (compare Fig. 1, *A* and *B*). Protein extracts of colonies collected from different geographic regions (Europe, Antarctica, North America, China, Aldabra Atoll) all contain the Wsp. Although present in varying relative amounts in colonies collected from these different locations, Wsp accounts always for a considerable fraction of soluble protein.

Wsp were nearly absent in laboratory-grown liquid cultures that were derived from desiccated field material (compare Fig. 1, *C* and *D*). Laboratory-grown colonies of field material

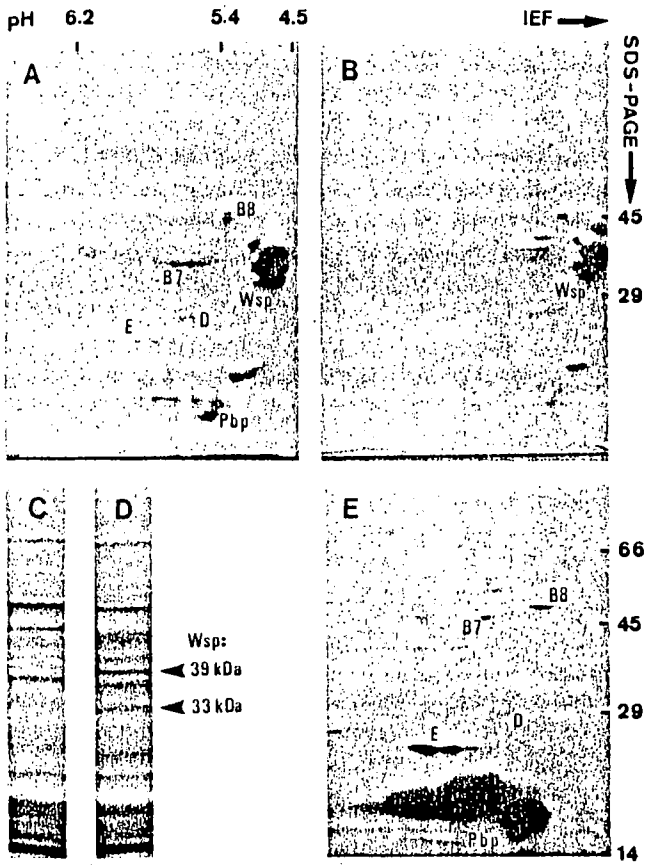

FIG. 1. **Water stress proteins are present only in desiccated *N. commune* cells.** *A*, soluble proteins of a sample collected at Wuhan, after 1 year of desiccation; *B*, soluble proteins of a sample collected at Hunan, after 5 years of desiccation; *C*, soluble proteins of a laboratory-grown culture derived from the Heibei sample, which had not been subjected to desiccation; *D*, soluble proteins of desiccated field-grown *Nostoc* collected at Heibei; *E*, soluble proteins of *N. commune* UTEX 584 grown in liquid culture. *A–D*, Coomassie stain; *E*, Coomassie and silver stains. *Pbp*, phycobiliproteins. *B*, *D*, and *E*, protein constellations used for identification (Potts, 1986). For IEF, Bio-Lyte 5–7 (2%, w/v), 6–8 (2%, w/v), and 3–10 (1% w/v) were used. Note that different preparations of proteins were used for the experiments shown in *A*, *B*, and *E* compared with *C* and *D* (for details, see "Methods").

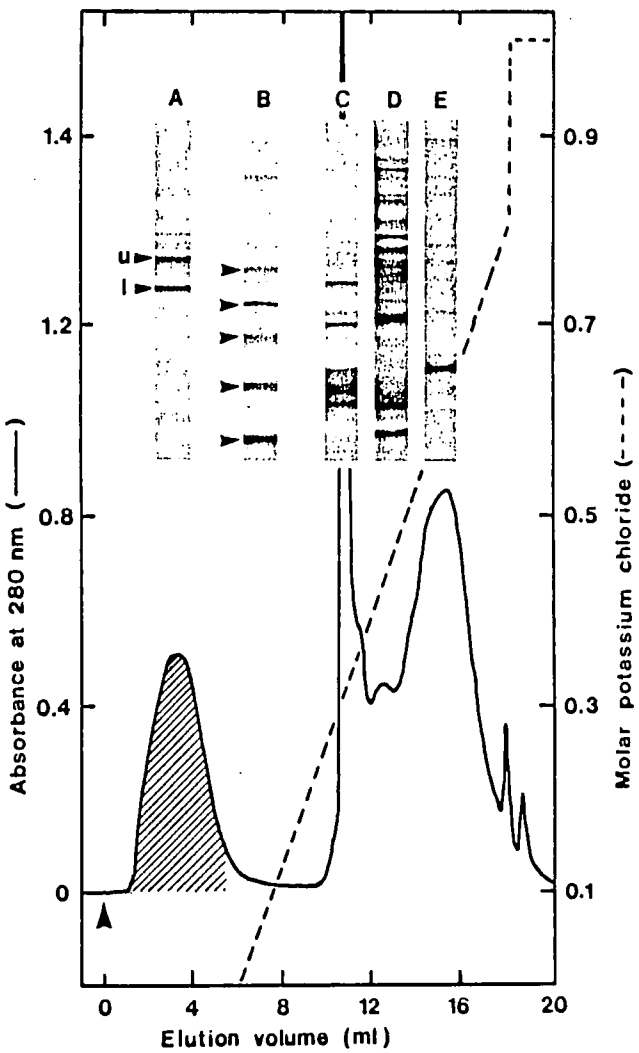

FIG. 2. **Water stress proteins do not bind to an anion exchange resin.** A typical elution profile from a Mono Q HR 5/5 column, operated in 20 mM Tris-HCl, pH 7.8, with a flow rate of 1 ml/min is shown. *Inset*, Coomassie-stained SDS-PAGE (12% (w/v) acrylamide) of fractions A (flow-through, containing Wsp; *u*, upper band; *l*, lower band) and C and D as well as molecular mass markers (*B*, *arrows* indicate 67-, 36-, 29-, 24-, 20-, and 14-kDa markers, Pharmacia). Fractions A and C–E were concentrated prior to electrophoresis, which did not permit estimation of the relative amount of single proteins.

subjected to one cycle of drying contain the Wsp but in lower concentrations than in desiccated field material (compare with Fig. 7).

Isolation of Water Stress Proteins—Although SDS-PAGE (Fig. 1, *C* and *D*) revealed several differences between desiccated and nondesiccated cells, further efforts were focused on the purification of the Wsp cluster with apparent M_r values of 30,000–40,000. For this purpose, field-grown colonies collected in the desiccated state in Heibei province, China (stored for 5 months) were used. An ultracentrifugation step after breakage of the cells was essential in order to remove high molecular weight carbohydrate complexes that otherwise reduced column efficiency. Unfortunately, this centrifugation step also removed a considerable fraction of the water stress proteins (compare Fig. 1, *A* and *D*). After ammonium sulfate fractionation and extensive dialysis, the highly viscous extract was diluted when necessary and applied to a Mono Q anion exchange column (Fig. 2). The flow-through contained the Wsp. Although the phycobiliprotein content of field material was not as high as that of liquid cultures, the major components of fractions C and D were the biliproteins C-phycocyanin and R-phycoerythrin, respectively. Reliable protein determinations were not possible at this stage since all fractions still contained high amounts of carbohydrate which also caused the comparatively low resolution on the Mono Q column when developed with the KCl gradient.

The flow-through of the anion exchange column was concentrated by ultrafiltration, which resulted in a further purification of Wsp since an abundant UV-A/B protecting pigment (Scherer *et al.*, 1988), carbohydrates, and small proteins all passed the ultrafiltration membrane. The concentrated Wsp fraction was then loaded onto a gel filtration column in the presence of 5 M urea (Fig. 3*A*). The bulk of Wsp eluted from the column in the first fraction at a high apparent molecular weight. SDS-PAGE revealed two major bands at M_r = 33,000 (*lower band, l*), M_r = 39,000 (*upper band, u*), and a minor band at M_r = 37,000 (*middle band, m*). In fraction II, the same Wsp were found, enriched with the band at M_r = 33,000, and were contaminated with several low molecular mass proteins. By raising the urea concentration to 8 M, most of the Wsp were eluted in fraction II_8 (Fig. 3*B*) but were still contaminated with low molecular weight proteins. By calibrating the column in the presence of 8 M urea with molecular

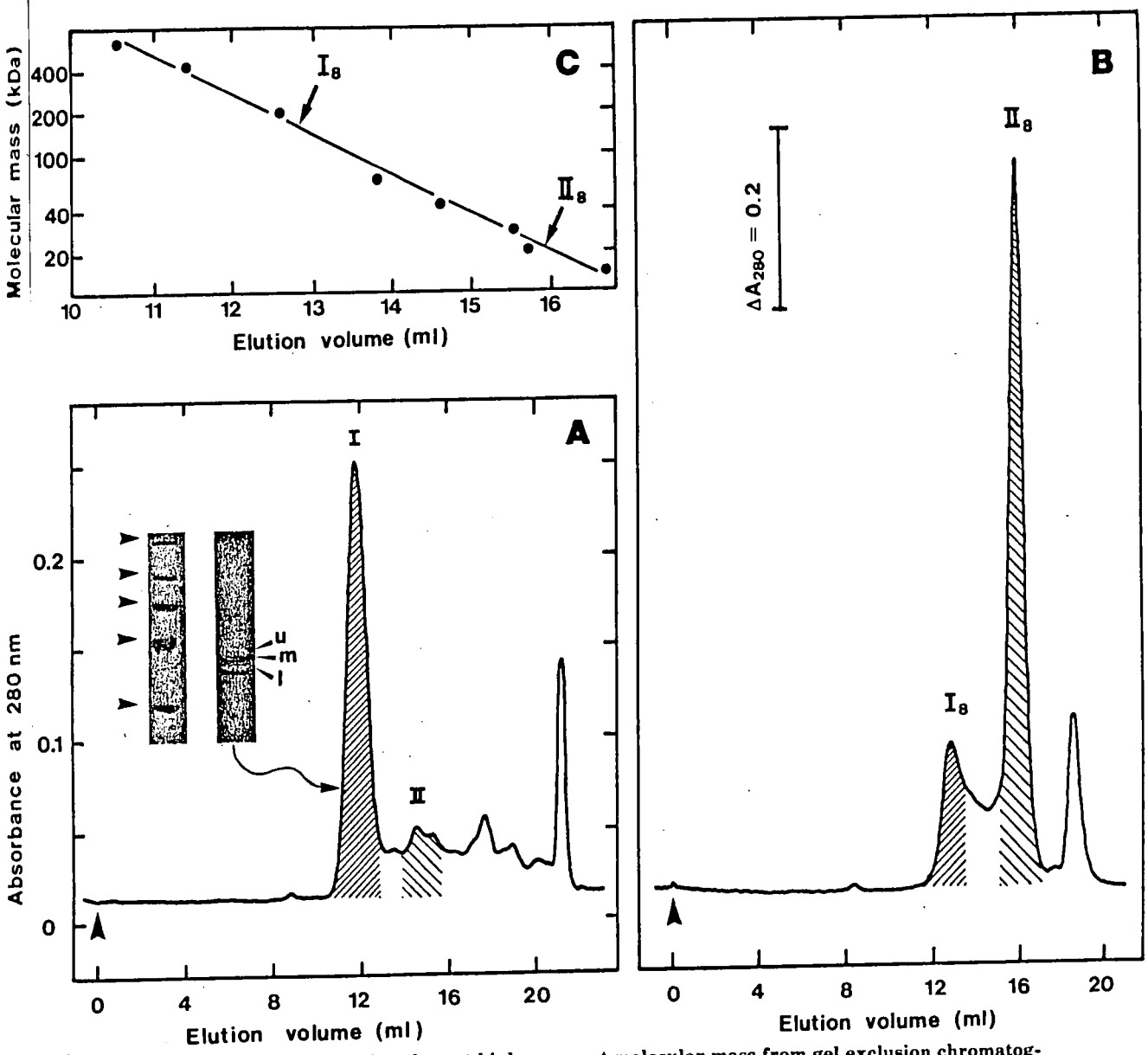

FIG. 3. **Water stress proteins elute at high apparent molecular mass from gel exclusion chromatography.** *A*, a typical elution profile from a Superose 12 HR 10/30 column operated in 20 mM Tris, pH 7.8, and 5 M urea at a flow rate of 0.3 ml/min is shown. Fraction A shown in Fig. 2 was applied to the column after concentration. *Inset*, Coomassie-stained SDS-PAGE (15% (w/v) acrylamide) of molecular mass markers (*arrows* indicate 200-, 97-, 66-, 43-, and 26-kDa marker proteins) and the main Wsp fraction. *u*, upper band; *m*, middle band; *l*, lower band. *B*, gel exclusion chromatography of another Wsp preparation in the presence of 8 M urea. The apparent molecular masses of fractions I_s and II_s were determined from the calibration slope depicted in Fig. 3*C*, using molecular mass markers of 669, 443, 200, 66, 45, 29, 20, and 14 kDa, operating the column in the presence of 8 M urea.

weight markers (Fig. 3*C*), apparent molecular masses of 130–170 kDa for fraction I_s and 20–30 kDa for fraction II_s were determined. The optimum purification of Wsp, as judged from SDS-PAGE, was thus achieved by collecting fraction I (Fig. 3*A*) from gel exclusion chromatography in the presence of 5 M urea. This fraction was desalted and transferred into distilled water by using PD-10 columns, lyophilized subsequently, and stored desiccated at −20 °C. This preparation was used for the further characterization of Wsp.

Efforts to separate the three bands in the Wsp fraction, either using Phenylsuperose (hydrophobic interaction chromatography) or chromatofocusing on Mono P, were not successful (data not shown). Therefore, single proteins were isolated by preparative SDS-PAGE and subsequent electroelution of the bands excised from gels stained with Coomassie Blue (see above).

Characterization of Water Stress Proteins—The apparent molecular masses of Wsp were estimated by using nondenaturing PAGE. Interestingly, the Wsp hardly entered the gel prior to treatment with urea, using the fraction derived from anion exchange. After purification, however, the bulk of Wsp migrated as a single major band (Fig. 4*C*) with a very small satellite band. However, as revealed by subsequent SDS-PAGE, both bands were composed of similar proteins, although being present in different ratios. For each of the bands, the slope of the plots of relative mobility *versus* acrylamide concentration (Fig. 4*A*), and thus the values for apparent molecular weights of proteins in the bands, were identical, yielding an apparent M_r value between 30,000 and 40,000 (Fig. 4*B*).

As illustrated in Fig. 4*C*, *lane b*, the Wsp fraction exhibits a certain degree of microheterogeneity with respect to the apparent molecular weight. By analyzing the fraction by two-dimensional electrophoresis, this microheterogeneity became

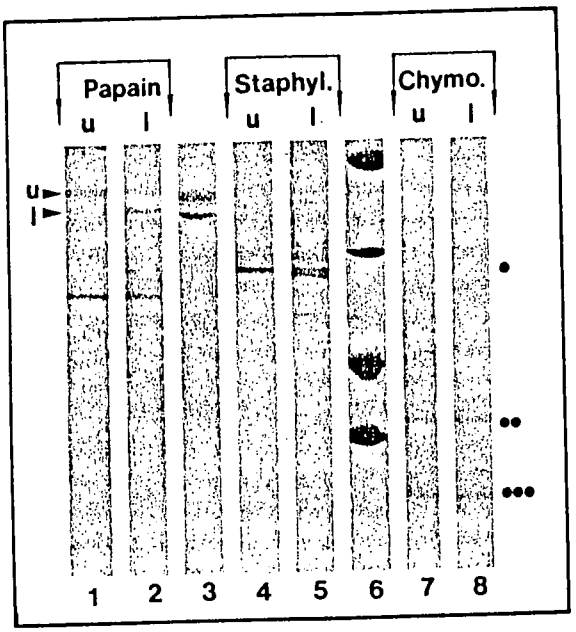

FIG. 6. **Water stress proteins with different molecular masses yield similar fragments after limited proteolytic degradation.** The position of single Wsp isolated by electroelution is shown by *arrows* (*left side* of *panel*; *u* and *l* stand for upper and lower bands, respectively; compare with Fig. 2, *inset*, *lane A*). For comparison, the sample prior to electroelution is shown in *lane 3*. *Filled circles* (*right side* of the *panel*) identify bands that are stained by silver staining (*three circles*, heavily stained; *one circle*, weakly stained). *Lane 6* shows molecular mass markers of 43, 26, 18, and 14 kDa. *Staphyl.*, *S. aureus* protease; *Chymo.*, chymotrypsin.

TABLE II

Amino acid compositions of different Wsp are similar

Figures are based on amino acids asparagine/aspartic acid through phenylalanine. Tyrosine and lysine are present only in low concentrations and could not be quantified reliably with the small amounts of protein available. Isoleucine is present but could not be quantified since its signal was partly superimposed by a signal probably originating from carbohydrate. Methionine is present but partly destroyed by hydrolysis. Tryptophan and cysteine are partly destroyed by hydrolysis and were not detectable (ND).

	Isoform		
	39 kDa (upper band)	37 kDa (middle band)	33 kDa (lower band)
	mol %		
Asx	12.1	11.9	11.5
Glx	8.0	8.4	6.2
Ser	9.4	9.0	7.0
Gly	13.2	13.8	15.8
His	0	0	0
Arg	8.1	8.7	5.5
Thr	11.2	10.3	10.9
Ala	10.0	10.2	9.1
Pro	6.0	6.1	6.0
Val	4.8	5.9	6.2
Leu	21.9	22.8	19.2
Phe	2.9	3.1	3.0
Tyr	+	+	+
Lys	+	+	+
Ile	+	+	+
Met	+	+	+
Trp	ND	ND	ND
Cys	ND	ND	ND

ther supported by immunological evidence. A polyclonal antibody raised against the upper band cross-reacted strongly with both the middle and lower bands after blotting proteins from SDS-PAGE gels to nitrocellulose and probing the primary antibody with phosphatase-conjugated sheep anti-

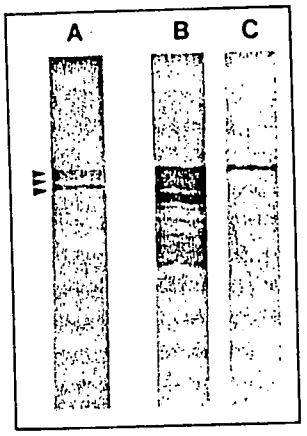

FIG. 7. **Isoforms of Wsp cross-react immunologically and are subjected to proteolysis *in vivo*.** *A*, 60 ng of the Wsp were separated by 15% (w/v) SDS-PAGE and transferred to nitrocellulose, which was probed with an antibody raised in a mouse against the upper band. *B*, dry powder of *N. commune* collected in Wuhan was extracted rapidly (6 mg in 0.1 ml) without previous rewetting in 8% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM Tris-HCl, pH 6.8, and 0.001% bromphenol blue by boiling and freezing the sample five times. After separation on a 15% (w/v) polyacrylamide-SDS gel and transfer to nitrocellulose, proteins were detected using antibodies raised in rabbits and directed against the complete Wsp fraction. *C*, a laboratory-grown colony derived from field material collected in Heibei province was dried once and treated as described for *lane B*. Experiments with preimmune serum gave no color reactions.

mouse antibodies (Fig. 7, *lane A*). A population of antibodies directed against the mixture of Wsp (all three bands) was used to probe the Wsp in a field-grown colony (Fig. 7, *lane B*). A variety of bands was detected, but the apparent M_r values of all of them were below 40,000. In contrast, when laboratory-grown colonies derived from field material were dried once and probed with the antibodies, only one band with an M_r value of 39,000 was found. Occasionally a fainter second band with an M_r of 24,000 was detected. Protein extracts from cells grown in liquid culture in a fermentor gave no signals with the antibody.

DISCUSSION

The Proteins Isolated Are Water Stress Proteins—Although the function(s) of the Wsp remains unknown, their involvement in the water stress response of cells is supported by the following findings. (i) Wsp are present in cells subjected to drying and desiccation but not in cells grown without water stress (Fig. 1). Laboratory-grown colonies, when subjected to drying, synthesized the 39,000-kDa band of Wsp. (ii) The Wsp protein fraction withstands prolonged desiccation more than other soluble proteins (compare Fig. 1, *A* and *B*) despite the extensive proteolysis that occurs after prolonged desiccation of *N. commune* UTEX 584 (Potts 1985, 1986). In this respect, it might be significant that the Wsp show only low levels of tyrosine, methionine, and lysine (Table II), amino acids that are sensitive to oxidative damage or amino group modification. (iii) Wsp form a substantial fraction of the cellular proteins of *Nostoc*, often more than the phycobiliproteins of photosystem II[2] (Fig. 1).

Since the Wsp were isolated from desiccated field material with an essentially unknown history, it is worth considering the possibility that the proteins studied in this work might have been partly damaged by (photo)oxidation or nonspecific proteolysis during desiccation and storage. This, however, seems to be unlikely for the following reasons. (i) Colonies

[2] S. Scherer and M. Potts, unpublished data.

grown in the laboratory and desiccated once contained the protein with the same maximum molecular mass (39 kDa) as found in the field material (Fig. 7). (ii) The pattern of Wsp on Western blots (see Fig. 7) was found to be very similar for materials collected over a period of 14 years in China, Europe, North America, Aldabra Atoll (Indian Ocean), and Antarctica (data not shown).

Molecular Weight of Wsp—With an isoelectric point of approximately 4.5, the Wsp should bind to an anion exchange column at pH 7.8; but as is shown in Fig. 2, this is not the case, indicating that the charged groups are shielded. The Wsp elute from gel filtration columns at urea concentrations below 5 M with apparent molecular masses of 130–170 kDa (Fig. 3). Interestingly, the elution volume changed considerably when comparing different preparations, which prohibited the elucidation of a certain molecular weight. Furthermore, Wsp hardly entered native PAGE gels unless treated with urea concentrations higher than 4 M. On the other hand, SDS-PAGE as well as native PAGE analysis (Fig. 4) of purified Wsp indicated approximate apparent molecular masses between 30 and 40 kDa. High urea concentrations led to an elution of Wsp from gel filtration columns with approximate molecular masses of 20–30 kDa, apparently splitting the Wsp fraction into smaller products. From these data, we conclude that Wsp occur *in vivo*, as high molecular weight complexes composed of microheterogeneous smaller units. A certain stoichiometry, however, cannot yet be established.

Microheterogeneity of Wsp—The occurrence of Wsp in microheterogeneous forms is evident from the different molecular masses (Fig. 4*C*) and isoelectric points (Figs. 1 and 5). The different forms appear to be very similar as judged from peptide mapping (Fig. 6) and cross-reaction of monospecific antibodies (Fig. 7*A*). The composition divergence values as derived from the very similar amino acid compositions (Table II) demonstrate the close relatedness of the Wsp (compare Harris and Teller (1973) with Cornish-Bowden (1983)) but also clearly show that they are different.

Glycoproteins are known to occur in different isoforms (*e.g.* Butler and Bond, 1988; Ziltner *et al.*, 1988). A major carbohydrate content of Wsp, however, can be excluded by the data reported. If the different forms had been generated by differential glycosylation, the amino acid composition should be identical, which is clearly not the case. Also, contrary to the data obtained (Fig. 6), peptide mapping should yield identical patterns, except if carbohydrate is bound to Wsp at the cleavage sites of all of the proteases tested, which seems rather unlikely. Since neuraminidase treatment did not change the isoelectric point of Wsp, it is assumed that sialic acid is not present.

Alternatively, the microheterogeneity can be explained by either a proteolytic digestion of Wsp by cellular proteases *in vivo*, by proteolysis during preparation, or may reflect highly homologous genes encoding different Wsp forms. Multigene families have been reported occasionally for procaryotes (*e.g.* Golden *et al.*, 1986; White *et al.*, 1988); and in *N. commune* UTEX 584, multiple *nifH* sequences are present (Defrancesco and Potts, 1988). However, Western blots of total cellular extracts of heterogenous field material revealed a variety of proteins with molecular masses below, but not higher than, 39 kDa reacting with the antibodies against Wsp. In contrast, in water-stressed laboratory cultures, a Wsp at 39 kDa, but not at apparent molecular masses of 37 and 33 kDa, was present (Fig. 7). Since the microheterogeneity in molecular weight was also found in field material boiled without previous rewetting in buffer containing high concentrations of SDS, mercaptoethanol, and phenylmethylsulfonyl fluoride (Fig. 7*B*), proteolysis during preparation appears to be unlikely. We suggest, therefore, that the microheterogeneity of Wsp most probably is due to *in vivo* proteolytic cleavage. The largest fragments, which are still similar in size and ionic properties, copurified during the preparation procedure.

According to the amino-terminal sequence of the 39-kDa form, an amino-terminal methionine is missing. It has been reported that the distribution of amino termini of proteins from microorganisms is highly nonrandom. The presence of, among others, alanine in the second position (Table I) does allow the post-translational removal of methionine (Tsunasawa *et al.*, 1985). Furthermore, highly expressed genes most frequently contain GCU (alanine) in the second position (Gold and Stormo, 1987). Since a Wsp with a molecular mass higher than 39 kDa was not detected, we assume that methionine is removed by proteolytic cleavage during maturation of the Wsp.

Protective Function of Wsp?—Liquid-grown cells of *N. commune* UTEX 584 maintain the protein synthesis machinery intact after subjection to drying (Angeloni and Potts, 1986); but under the conditions of rapid desiccation applied in these studies, the mRNA pool did not change significantly (Jäger and Potts, 1988a, 1988b), and no novel proteins accumulated as shown by ^{35}S pulse labeling (Potts, 1986). It has been reported frequently that rapid drying does not allow sufficient time for the expression of desiccation tolerance (Bewley, 1979; Leopold, 1986; Dhindsa, 1987). The presence of high amounts of Wsp in field material after numerous slow desiccation events and the smaller amounts produced after one desiccation event in laboratory-grown cells may suggest that Wsp accumulate during the growth of a colony in order to protect the cellular structure. Interestingly, fast dried *N. commune* UTEX 584 filaments do not preserve structurally intact heterocysts (Peat and Potts, 1987), in contrast to field material that does and that contains the Wsp in high amounts (Peat *et al.*, 1988).

The Wsp isolated from *N. commune* differ markedly from the proteins synthesized in seeds of wheat, which contain 69.4% glycine and which are formed during preparation of the embryo for dormancy (Gomez *et al.*, 1988). Similarities of *N. commune* Wsp to the proteins synthesized in rice embryos are evident only in that the latter are found as microheterogenous forms in both molecular weight and isoelectric point. However, the rice proteins are basic and, as found for the wheat proteins, are rich in glycine (Mundy and Chua, 1988). It was noted that the proteins found in dry rice embryos disappeared quickly after germination and exhibited some similarity to DNA-binding proteins such as histones (Mundy and Chua, 1988). In contrast, it has been suggested that the "E_m-protein" isolated from wheat embryos is a water-binding protein, maintaining a certain level of hydration in the cells (McCubbin *et al.*, 1985). The high amounts of Wsp in *N. commune* (up to 20% of cellular protein) may suggest a structural function. Since cyanobacteria are readily accessible to genetic manipulation, the isolation of Wsp from *N. commune* provides a promising tool for the investigation of both the action of water on gene expression and the possible protective nature of Wsp in photosynthetic cells.

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Water Stress Proteins of *Nostoc commune* (Cyanobacteria) Are Secreted with UV-A/B-absorbing Pigments and Associate with 1,4-β-D-Xylanxylanohydrolase Activity*

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Acidic water stress polypeptides (Wsp) with molecular masses of 33, 37 and 39 kDa are the most abundant soluble proteins in the cyanobacterium *Nostoc commune*. Wsp polypeptides and UV-A/B-absorbing pigments are secreted by cells, accumulate in the extracellular glycan sheath, and are released from desiccated colonies upon rehydration. No evidence was obtained for either glycosylation, phosphorylation, or acylation of Wsp polypeptides. NH_2-terminal amino acid sequences of the 33-, 37-, and 39-kDa polypeptides were identical: Ala-Leu-Tyr-Gly-Tyr-Thr-Ile-Gly-Glu-Gln-*X*-Ile-Gln-Asn-Pro-Ser-Asn-Pro-Ser-Asn-Gly-Lys-Gln. This consensus NH_2-terminal sequence and an internal sequence (Glu-Ala-Arg-Val-Thr-Gly-Pro-Thr-Thr-Pro-Ile-Asp) showed homologies with the sequences of carbohydrate-modifying enzymes. Purified Wsp polypeptides associate with a 1,4-β-D-xylanxylanohydrolase activity that was inhibited specifically by Wsp antiserum. In the absence of salt, Wsp polypeptides, and the water-soluble UV-A/B-absorbing pigments, form multimeric complexes through strong ionic interactions. A possible role is suggested for Wsp polypeptides in the synthesis and/or modification of a xylose-containing UV-A/B-absorbing pigment.

The removal of water from cells, the storage of cells in the air-dried state, and the rewetting of dried cells impose physiological constraints which relatively few organisms can tolerate. The molecular basis for desiccation tolerance remains poorly understood. Mechanisms which maintain the structural integrity of membranes appear to be of importance. Certain sugars, particularly trehalose, prevent damage from dehydration not only by inhibiting fusion between adjacent membrane vesicles during drying, but also by maintaining membrane lipids in a fluid phase in the absence of water (1, 2). The experimental evidence suggests that trehalose can stabilize cell membranes for short periods in air, but it cannot do so for extended periods. In the latter respect, other mechanisms must be important in the protection of cells from long term dehydration stress. The roles of water stress proteins remain cryptic.

Of those organisms which express desiccation tolerance, certain cyanobacteria have a particularly marked capacity to do so. One form, *Nostoc commune*, has become a very useful model for the analysis of dehydration-induced stresses in photosynthetic cells (3–10). Cells of *N. commune* are subjected to acute water stress *in situ* through multiple, and often rapid, cycles of wetting and drying. These cycles interrupt often extended periods of desiccation when the cells must tolerate further oxidative- and radiation-induced stresses.

In a previous study we described the purification and partial characterization of a group of abundant water stress proteins (Wsp)[1] in *N. commune* (11). Here we present data on the structure, processing, aggregation, and localization of Wsp.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Materials—Desiccated colonies of *N. commune* var. Vauch. were collected from a variety of polar, tropical, and temperate localities (Table I). The clonal axenic isolate *N. commune* strain UTEX 584, *N. commune* strain DRH1, and *Anabaena* strains PCC7118, PCC7119, and PCC7120 were grown in BG 11_0 or BG 11 medium (12), with aeration, at 32 °C.

Isolation of Wsp Polypeptides from Extracellular Glycan—Equivalent weights of desiccated colonies were suspended in sterile water with or without the addition of one or more protease inhibitors at the specified final concentration: 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM diisopropyl fluorophosphate (DFP), 10 μM *trans*-epoxysuccinyl-L-leucylamido-[4-guanidino]-butane (E-64) or 10 mM 1,10-phenanthroline (1,10-P) and prepared according to the specifications of the manufacturer (Sigma). Following different periods of rehydration, the rehydration fluids, as well as the intact colonies, were recovered. Rehydration fluids were lyophilized, and the dried residue was reconstituted with SDS-PAGE buffer (80 mM Tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 0.001% (w/v) bromphenol blue) and boiled for 2 min prior to analysis by SDS-PAGE.

Isolation of Native Wsp—Between 5 and 20 g of desiccated colonies were rehydrated in 200–600 ml of sterile distilled water for 30 min. Aliquots of the solution were centrifuged at 20,000 × *g*, for 20 min, then the supernatant fraction was passed through a 0.2-μm filter (Millipore), and the filtrate was recovered. All further manipulations were performed at 4 °C. The filtrate was concentrated by ultrafiltration using Centriprep-10 cartridges (10-kDa cut-off, Amicon Inc., Beverly, MA).

Analytical liquid chromatography was performed using a Pharmacia fast protein liquid chromatography system (Pharmacia LKB Biotechnology Inc.) as described in the figure legends.

Cell Disruption and Isolation of Cellular Protein—Desiccated (or lyophilized) colonies were suspended in grinding buffer (50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 20 mM KCl, 1 mM sodium azide, 1 mM β-mercaptoethanol). The mixtures were boiled for 5 min, then frozen at −75 °C, thawed (three cycles), and sonicated (Fisher model 300 operated at 35% of maximum setting) for 10 min.

Total Extraction of Wsp (Preparative)—Desiccated material was ground under liquid nitrogen and 5-g (dry weight) aliquots were suspended in grinding buffer that contained 0.1 mM DFP, 1 mM PMSF, and

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[1] The abbreviations used are: Wsp, water stress proteins; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate; E-64, *trans*-epoxysuccinyl-L-leucylamido-[4-guanidino]-butane; 1,10-P, 1,10-phenanthroline; PAGE, polyacrylamide gel electrophoresis.

5 mM EDTA. The suspension was subjected to four consecutive pressings (110 megapascals) in a French pressure cell, with additions of fresh ice-cold grinding buffer. The slurry was mixed with a detergent buffer (final concentrations of 50 mM Tris-HCl, pH 7.8, 3 M urea, 2% (w/v) SDS, 1% (w/v) Nonidet P-40, and 1 mM β-mercaptoethanol). The solution was incubated at 65 °C, for 48 h, then centrifuged at 12,000 × *g* for 15 min, at 4 °C, and the supernatant fraction was retained for analysis.

Endoglycosidase Digestion—Cells were treated with detergent buffer as described above, but urea and Nonidet P-40 were omitted. The detergent MEGA 8 (Sigma) was included to prevent denaturation of the endoglycosidase by SDS (13). Aliquots of the supernatant fraction were diluted 1:20 with an endoglycosidase digestion buffer (80 mM Tris-HCl, pH 6.8, 1.7% (v/v) β-mercaptoethanol, 1% (w/v) MEGA 8) prior to the addition of 5 units of peptide-*N*-glycosidase F (25,000 units mg^{-1} protein, Boehringer Mannheim) in a final reaction volume of 50 μl. Rehydration fluids (see above) were denatured before the addition of the peptide-*N*-glycosidase F. The solutions, including replicate controls lacking the peptide-*N*-glycosidase F, were incubated at 37 °C, for 12 h.

Glycoconjugate Detection—Glycoconjugates were detected on Immobilon P (Millipore Corporation, Bedford, MA) membrane blots using a method developed by O'Shannessy *et al.* (14) with a detection kit based upon a digoxigenin hydrazide-antidigoxigenin alkaline phosphatase conjugate colorimetric assay. Reagents were obtained from Boehringer Mannheim and were used generally following the manufacturer's specifications but in accordance with the precautions and suggestions discussed by Fairchild *et al.* (15). Transferrin was used as a positive control.

Carbohydrate Analysis—Concentrations of neutral carbohydrate in column eluates were measured using a phenol-sulfuric acid colorimetric assay (16). Fluorometric carbohydrate analyses were performed as described (17).

Peptide Mapping and Sequence Analysis—Individual Wsp polypeptides were excised from SDS-PAGE gels and electroeluted. Protein samples were dialyzed exhaustively against water using 3500 cut-off dialysis membrane (Spectra/Por 3, Baxter Diagnostics Inc., McGraw Park, IL). Peptide maps were obtained by using papain, chymotrypsin, or *Staphylococcus* V8 protease (Sigma), under the conditions specified by Cleveland *et al.* (18). After blotting to Immobilon P membrane (Millipore), the digestion products were visualized through immunodetection or by staining with Coomassie Blue. Wsp polypeptides were treated with either endoproteinase Glu-C or endoproteinase Lys-C under the conditions specified by the manufacturer (Boehringer Mannheim). Treatment of Wsp polypeptides with cyanogen bromide was under a nitrogen gas phase in the dark (19). Wsp polypeptides and peptide fragments were blotted to Immobilon P membrane (20) and subjected to automated Edman degradation using an Applied Biosystems 477A Protein Sequencer. Cysteine residues were not derivatized prior to analysis.

Microheterogeneity of Wsp Polypeptides—Individual Wsp peptides were incubated at 37 °C with calf intestinal alkaline phosphatase (Sigma) in the buffer specified by Maniatis *et al.* (21) for 3 h. Samples were recovered at different time points, and the phosphatase was inactivated through the addition of EDTA.

Enzyme Assays—The capacity of native extracts of Wsp to hydrolyze 4-*O*-methyl-D-glucurono-D-xylan-remazol brilliant blue R (RBB xylan, Sigma catalog no. M-5019) was tested under the assay conditions specified by Biely *et al.* (22). Activities were quantitated using xylanase (1,4-β-D-xylanxylanohydrolase, EC 3.2.1.8) from *Trichoderma viride* (Sigma catalog no. X3876; 225 units mg $protein^{-1}$). One unit of this xylanase liberates 1 μmol of reducing sugar (xylose equivalent) from poly(β-D-xylopyranose(1→4))/min at pH 4.5, 30 °C. Xylosidase activity was tested using *o*-nitrophenyl-β-D-xylopyranoside (Sigma catalog no. N3629) as substrate and β-xylosidase (exo-1,4-β-D-xylosidase; EC 3.2.1.37) from *Aspergillus niger* (Sigma cat. no. X 5375) as the positive control.

Incubation of native gels with sugar phosphate substrates (see "Results") and the detection of the release of inorganic phosphate with malachite green followed the protocols of Zlotnick and Gottlieb (23).

Electrophoresis and Western Blotting—A colorometric dye-binding assay (Pierce Chemical Co. publication 23200) was used to estimate protein concentrations. Analytical and preparative (native and SDS-) PAGE electrophoresis, staining of proteins, electroelution of proteins, and Western blotting were as described (11, 20).

Sample Preparation for Electron Microscopy—Fixation and embedding of cells were as described (24). The resin used in the present study was EPON 812. Samples were examined using a Zeiss model EM 10. Samples were processed for scanning electron microscopy following fixation and dehydration and vacuum-infiltration with paraffin. Paraffin sections were placed on coverslips and cleared with xylene. The sections were critical-point dried, mounted on aluminum studs and coated, and examined using a Phillips model 505 scanning electron microscope.

Immunolabeling for Electron Microscopy—Thin sections were transferred to nickel grids, and the process of immunolabeling together with the use of the appropriate controls followed that described by Peat *et al.* (8).

RESULTS

Wsp Is Secreted Beyond the Outer Cell Membrane—Colonies of *N. commune* become visually conspicuous in terrestial environments from the Tropics to the polar regions (Fig. 1*A*). The analysis of desiccated colonies using light microscopy, transmitted electron microscopy and scanning electron microscopy showed that cells can retain their structural integrity despite being stored for decades in the air-dried state (see legends of Fig. 1 and Table I). Typically, a colony with a dry weight of 0.1 g will become swollen, with a wet weight of approximately 20 g, within 1 h of rehydration. A novel extracellular glycan[2] serves to maintain the cells in an immobilized state (Fig. 1, *B–F*). Wsp polypeptides were distributed throughout the glycan (Fig. 1, *D* and *E*). Very similar patterns of immunolabeling were obtained with rabbit antibodies generated against a preparation of three (33, 37, 39 kDa) purified Wsp polypeptides and with mouse antibodies generated against the single purified 39-kDa Wsp polypeptide (data not shown).

Wsp Is Stable in Desiccated Cells—Wsp was detected in every sample tested (Table I, Fig. 2). In all cases where Wsp was detected by Western blotting, it was judged to be the most abundant soluble protein following purification of cell extracts (Fig. 2, *lane I*; see also Fig. 2*a*, Ref. 11). For three samples from Aldabra Atoll (Table I), the results from immunoblotting were variable, and consistent results were achieved only when proteins were extracted in the presence of PMSF, DFP, E-64, or 1,10-P (see below).

Wsp Is Abundant in the Extracellular Glycan—Wsp was released from desiccated colonies by rehydrating them but without any form of physical disruption (Fig. 3, *A–D*). In response to the first wetting, the amounts of Wsp released from the colonies increased with time of rehydration (Fig. 3, *A* and *B*), and the use of protease inhibitors enhanced the recovery of Wsp (Fig. 3*B*). A similar, less pronounced trend was evident for the second rehydration although some higher molecular mass cross-reactive bands were observed in extracts that lacked inhibitors (Fig. 3, *C* and *D*). Signals from the 33-, 37-, and 39-kDa polypeptides were strongest following approximately 1 h of rehydration, irrespective of the presence or absence of inhibitors (Fig. 3, *A* and *B*).

Rehydration fluids in which Wsp was abundant showed no UV-induced fluorescence in the visible region, and they had a negligible spectrum in the 450–650-nm region confirming that soluble phycobiliproteins were absent (Fig. 4). The peak in absorbance at 312 nm is due to a secreted water-soluble UV-A/B-absorbing pigment (25; see below). Phycobiliproteins, as intracellular protein markers, were obtained only following cell breakage. After cell breakage the corresponding samples were all found to have very similar concentrations of Wsp in the cytosolic fractions (data not shown). Because Wsp polypeptides may still have been present in the sheath despite multiple rounds of aqueous extraction (see Fig. 1*E*) it is difficult to assess with certainty the intracellular concentration of Wsp (in relation to total Wsp and/or total soluble protein). The amounts of protein released from colonies were also difficult to quantify in view of the presence of components that interfered with the dye-binding assay. However, the proteins which are resolved in the *inset* of Fig. 4 (measured as 10 μg of total protein) represent

[2] D. R. Hill and M. Potts, unpublished data.

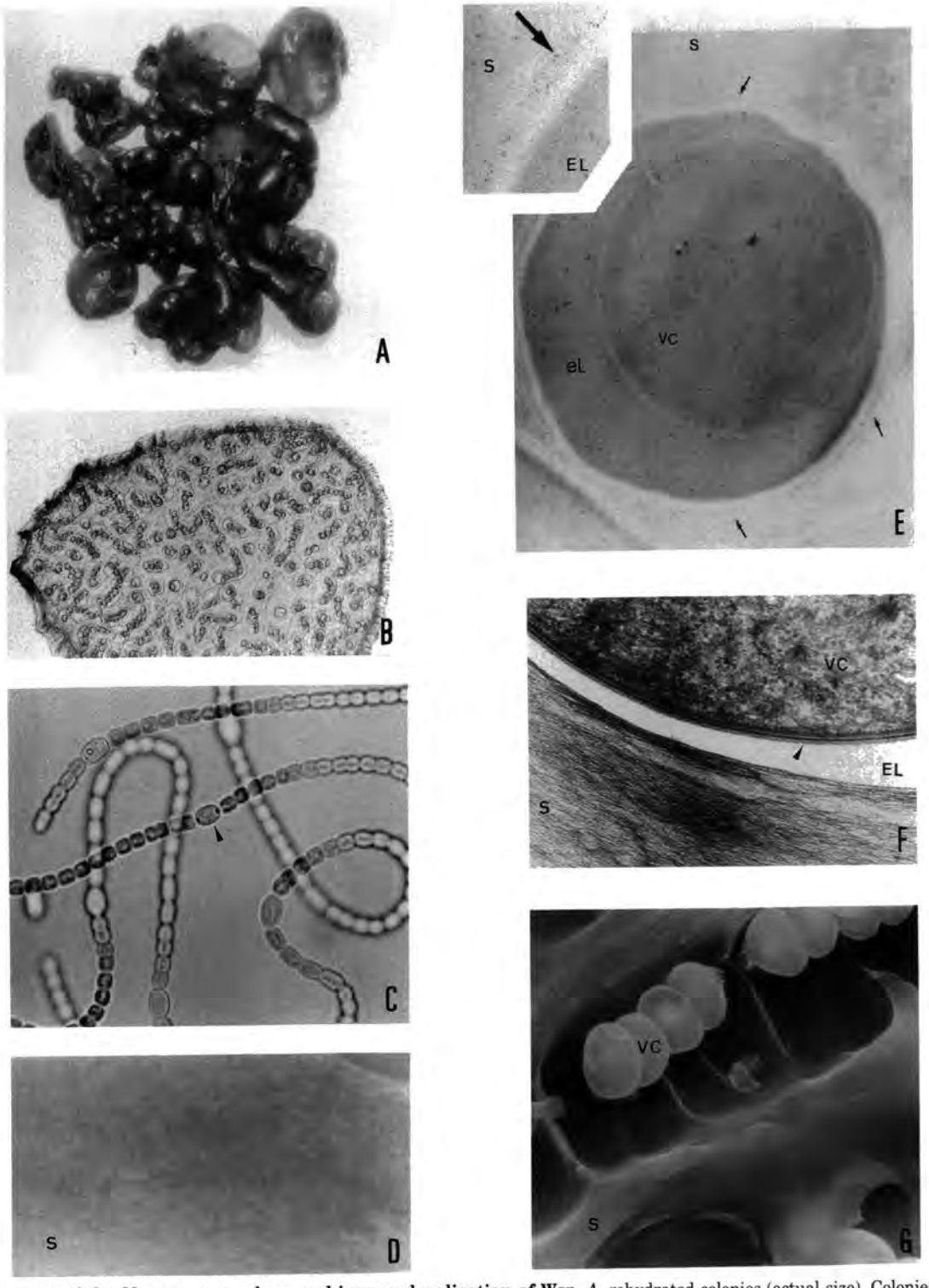

FIG. 1. **Structure of the *N. commune* colony and immunolocalization of Wsp.** *A*, rehydrated colonies (actual size). Colonies form black friable crusts when they are desiccated and an olive-green thallus, with the consistency of a rigid gel, when they are wetted. These growths become visually conspicuous over the surfaces of nutrient-poor soils and exposed limestones. *B*, cross-section through one lobe of a desiccated colony (× 160). The extracellular glycan (sheath) at the air/colony boundary appears striated and is often heavily pigmented (brown). The cells of *N. commune* develop unbranched filaments that are separated from one another and remain distributed throughout, and immobilized and embedded within, the extracellular glycan. *C*, typical appearance of *N. commune* DRH1 and UTEX 584 grown in liquid culture. Vegetative cells form filaments of 7-μm diameter with differentiated (nitrogen-fixing) heterocysts (*arrow*) dispersed regularly along their length (× 430). *D*, immunolocalization of Wsp in the extracellular sheath (*s*) from a section through a desiccated colony (no post staining). The envelope of a single cell is visible in the *top right hand corner* (× 25,000). *E*, cross-section (no post staining) through a single vegetative cell (*vc*) showing the thickened envelope (*el*) and the sheath (*s*). A discrete accumulation of Wsp (gold particles, 5-nm diameter) is apparent at the periphery of the sheath/cell envelope interface (*arrows*, × 20,000; and *inset*, × 40,000). *F*, cross-section (with post staining) through a single vegetative cell (*vc*) showing its characteristic ultrastructure, intact cell membranes (*arrow*), and the fibrous nature of the sheath (*s*); × 64,000. Compare the appearance of the envelope layer (*el*) with and without (Fig. 3*E*) post-staining. *G*, scanning electron microscopy view of a cross-section through a desiccated colony (following critical point drying) showing a filament of vegetative cells (*vc*), the envelope "tunnel" within which the filament is located, and the extensive sheath (*s*) (× 3,600).

0.003% of the total amount obtained after the rehydration of 20 g dry weight of colonies. A similar value was estimated in a second trial.

Proteolysis of Wsp—The data presented in Fig. 3 suggested that the Wsp polypeptides may result from, and be subject to, proteolysis. Incubation of total cell extracts that lacked prote-

TABLE I

Desiccated materials of N. commune and cultures of cyanobacteria

Desiccated materials were dry at the time of collection and were stored dry, in the dark until the time of analysis.

Acronymn or strain designation	Country	Locality	Year collected	Years dry at analysis	Cultures	notes/ref.
Desiccated materials						
N. commune[a] CHEN	China	Hunan Province	1981	10–12	*N. commune* UTEX 584	(4)
N. commune[a] HUN1	China	Hunan Province	1981		*N. commune* DRH 1	Isolate of *N. commune* CHEN
N. commune[a] HUN2	China	Hunan Province	1981		*Anabaena* sp. PCC 7118	Obtained from ATCC (no. 27892), Het$^-$
N. commune[a] TEN	USA	Knoxville, TN	1988	3	*Anabaena* sp. PCC 7119	Obtained from ATCC (no. 29151)
N. commune[a] TAG	Germany	Bodensee	1988	2	*Anabaena* sp. PCC 7120	Obtained from J. Elhai
N. commune[a] 8122	Seychelles	Aldabra Atoll	1974	18		
N. commune[a] ANT	Antarctica	Ross Ice Shelf	1979	11		
N. commune 776D	Seychelles	Aldabra Atoll	1974	18		
N. commune 779D	Seychelles	Aldabra Atoll	1974	18		
N. commune var. *flagelliforme* 857D	Seychelles	Aldabra Atoll	1974	18		
N. commune BBC	USA	Blacksburg, VA	1990	2		
N. commune BER	Germany	Bodensee	1988	2		
N. commune MAL	England	Malham Tarn	1989	3		

[a] See Fig. 2.

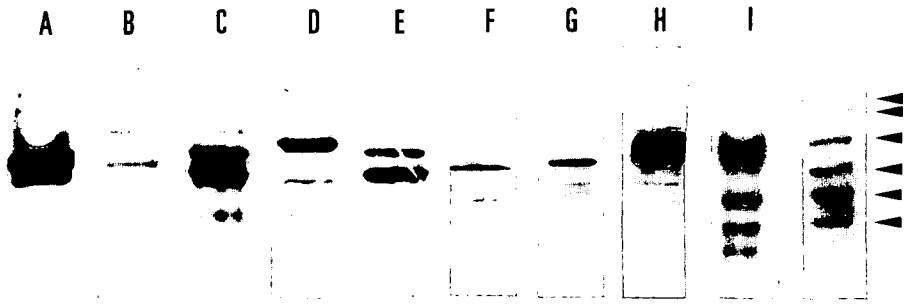

FIG. 2. **Wsp is abundant and stable in desiccated colonies.** Total cell extracts were obtained by grinding desiccated colonies (see Table I) directly with SDS-PAGE buffer with (*lane H* only) or without protease inhibitors. *Lanes A–H* are Western blots of total cell extracts containing approximately 3 μg of total protein from materials labeled CHEN, HUN1, HUN2, TEN, TAG, 8122, ANT and CHEN, respectively. *Lane I* shows a replicate silver-stained gel of the extract analyzed in *lane H* (15% (w/v) gel used for resolution of proteins). *Arrows* indicate prestained molecular mass markers in kDa (18, 27, 32, 49, 80, and 108, in ascending order).

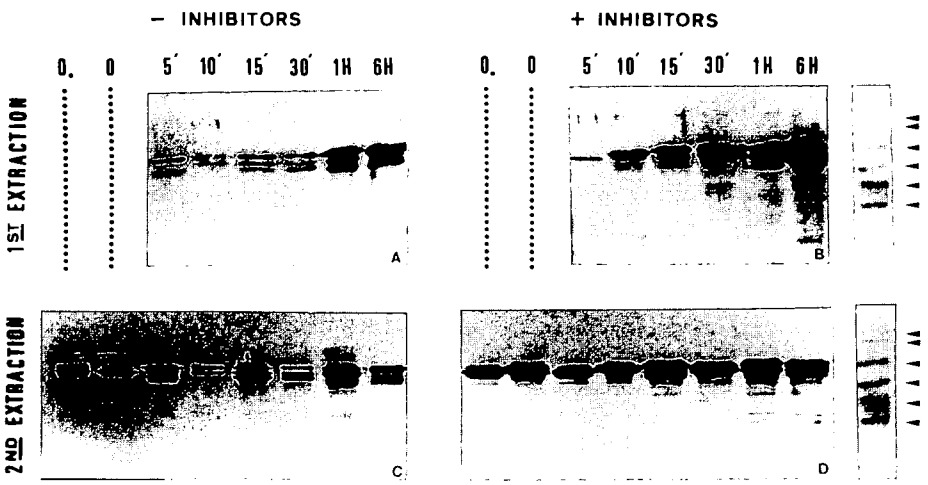

FIG. 3. **Wsp polypeptides are released from desiccated colonies upon rehydration.** Equivalent amounts (0.1 g dry weight) of desiccated *N. commune* CHEN were suspended in 5 ml of sterile water, with or without the addition of PMSF, DFP, E-64, and 1,10-P, for periods between 5 min and 6 h, at room temperature. The intact colonies were removed and lyophilized, and aliquots (3 μg of total protein) of the supernatant fractions (1st rehydration fluids) were subjected to Western analysis (*A* and *B*). All of the lyophilized colonies were then rehydrated with a further 5 ml of sterile water for 48 h, at 4 °C, and the 2nd rehydration fluids were similarly recovered and subjected to Western analysis (*C* and *D*). The first and second rehydrations released 0.50–0.55 and 0.51–0.64 μg ml^{-1} total protein, respectively. *Dotted lines* emphasize that no measurements are possible for the zero time points in the first rehydration series (desiccated colonies, 0_o; desiccated/lyophilized colonies, 0). Molecular mass markers as Fig. 2.

ase inhibitors either at room temperature, or following their freezing and thawing, lead to a selective loss of the 39-kDa Wsp polypeptide (Fig. 5, *A–D*; see also Fig. 2*A*). When total cell extracts were prepared from the same material with either one of four different protease inhibitors, a consistent banding pattern of three or four Wsp polypeptides was obtained (Fig. 5, *E–G*; see also Fig. 2, *A–G*). The results presented in Fig. 5 are representative of those obtained in repeat experiments.

Peptide Mapping of Wsp—The apparent molecular masses of the 33-, 37-, and 39-kDa polypeptides remained unchanged following treatment with cyanogen bromide for either 4 h or overnight at room temperature (data not presented). The peptide maps of the 39- and 37-kDa polypeptides after treatment with *Staphylococcus* V8 protease or chymotrypsin were virtu-

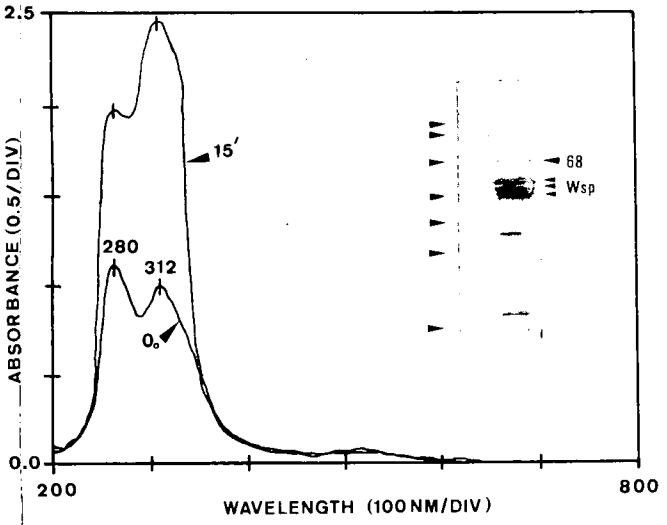

FIG. 4. **Wsp is secreted together with a UV-A/B-absorbing pigment.** 100-μl aliquots of rehydration fluids from the second extraction (times 0_o and 15 min; see Fig. 3) were diluted in rehydration buffer, and their spectra were collected using 1-cm pathlength cuvettes and a Shimadzu model UV 160U UV-visible recording spectrophotometer. *Inset*, 20 g dry weight of desiccated material was rehydrated overnight at 4°C, and Wsp was concentrated from the rehydration fluid following ammonium sulfate precipitation and ultrafiltration. Proteins were resolved by SDS-PAGE, silver staining (Wsp remain non-stained at these concentrations), and then Coomassie Blue (*right hand lane*). Positions of Wsp polypeptides (*Wsp*) and a 68-kDa protein that was sequenced are indicated. *Arrows* in ascending order indicate the solvent front and molecular mass markers as in Fig. 2.

ally identical (Fig. 6, *lanes A* and *B*, *D* and *E*). The maps of all three polypeptides were very similar following treatment with chymotrypsin (Fig. 6, *lanes D–F*).

Protein Sequence Analysis of Wsp—In addition to Wsp polypeptides, a protein with a molecular mass of 68 kDa, that did not cross-react with Wsp antiserum, was conspicuous in concentrated rehydration fluids (see Fig. 4). This 68-kDa protein was further purified. The amino-terminal sequences of the three Wsp polypeptides (Fig. 6, *lane I*) were identical: Ala-Leu-Tyr-Gly-Tyr-Thr-Ile-Gly-Glu-Gln-*X*-Ile-Gln-Asn-Pro-Ser-Asn-Pro-Ser-Asn-Gly-Lys-Gln. Residue 11 could not be identified in any of the Wsp samples in multiple trials. The amino-terminal sequence of the 68-kDa polypeptide showed no correspondence to the sequences of the Wsp polypeptides. Homology searches using the amino-terminal sequences of Wsp and the 68-kDa protein revealed similarities to carbohydrate-modifying enzymes. The sequence at the amino terminus of a 25-kDa proteolytic fragment of Wsp was Glu-Ala-Arg-Val-Thr-Gly-Pro-Thr-Thr-Pro-Ile-Asp (Fig. 6, *lane H*) and showed 66% sequence identity with a portion of the sequence of a β-xylosidase (locus XYNB_CALSA in the Swiss-Prot 23 sequence library; 33). Subsequently, quantitative Western analyses identified a strong antigenic response between the Wsp antiserum and a number of such enzymes including *N*-glycosidase F and β-D-galactoside galactohydrolase (preimmune serum showed no cross-reactions with either of these proteins).

Enzyme Assays—In view of the results of homology searches, Wsp extracts were tested for activity with a range of carbohydrate substrates. Rehydration fluids contained a weak xylosidase activity that was detected only after prolonged incubation (96 h and greater) with *o*-nitrophenyl-β-D-xylopyranoside as the substrate (the substrate showed no background hydrolysis during this time of incubation in control experiments).

Rehydration fluids hydrolyzed RBB-xylan, and the results from a typical experiment are presented as Fig. 7. The addition of Wsp antiserum to reaction mixtures caused a 10-fold reduction in xylanase activity while the Wsp antiserum had no inhibitory effect on the activity of a purified xylanase (Fig. 7, *inset*). In comparison, cell-free supernatants of liquid cultures of *N. commune* DRH1 (approximately 0.1 g dry wt liter^{-1}) had low xylanase activities, approximately 0.01 μmol of xylose released min^{-1} ml^{-1}. However, excessive amounts of viscous polysaccharides synthesized by the cultures bound the products of the colorimetric assay and made it difficult to make reliable measurements. The amounts of Wsp secreted by this culture after multiple transfers in liquid culture were detectable only through Western blotting.

Rehydration fluids did not hydrolyze *p*-nitrophenyl-β-D-galactoside, 5-bromo-4-chloro-3-indolyl galactoside or 5-bromo-4-chloro-3-indolyl glucoside, nor did they show chitinase activity (using Remazol Brilliant Violet-poly-*N*-acetylglucosamine as the substrate, Sigma C 3020, and the method of Wirth and Wolf (26); data not shown).

Malachite green staining revealed no release of inorganic phosphate in zymograms following incubations of Wsp preparations with either fructose-1,6-bisphosphate, uridine 5′-diphospho-*N*-acetylglucosamine, uridine 5′-diphospho-*N*-acetylgalactosamine, or α-D-glucose 1,6-diphosphate (data not presented).

Association of Wsp with UV-A/B-absorbing Pigments—Rehydration fluids were subjected to analytical fast protein liquid chromatography to further resolve their principal components. Two fractions of Wsp were identified through ionic exchange chromatography. One fraction failed to bind to Mono Q resin while the second fraction remained bound to the resin and was eluted only with salt concentrations of 1 M KCl or higher. Fraction one was colorless, fraction two was pale yellow at pH 7.4, and both fractions contained UV-absorbing pigments (Fig. 8). In fraction one the pigments had a narrow absorption spectrum with a maximum at 312 nm; in fraction two the spectrum was broad and extended to 450 nm. We will document elsewhere that the pigments in fraction two have the characteristics of scytonemin (27). Pigments with the spectral characteristics of those in fraction one were eluted from the column at approximately 150 mM KCl together with trace amounts of Wsp (Fig. 8). These data indicated that highly charged components were present in the rehydration fluids. There was also some suggestion that the components of the rehydration fluids form high molecular mass complexes because native Wsp failed to traverse a 100-kDa cut-off dialysis membrane. In addition, some material cross-reacted with the antiserum and was resolved as a smear, of high apparent molecular mass, that migrated slower than Wsp polypeptides during SDS-PAGE (data not shown).

To study the sizes of these complexes rehydration fluids were analyzed using gel filtration with simultaneous monitoring of the absorbance at 280 and 312 nm in the presence and absence of kosmotropic and chaotropic agents. Under native conditions (20 mM Tris-HCl, pH 7.4, 50 mM KCl), Wsp eluted in two fractions from a Superose 12 column with an apparent molecular mass between 35 and 45 kDa. Western blotting resolved two Wsp polypeptides in each fraction (Fig. 9*A*, *inset*). Silver staining detected no other proteins in those fractions that contained Wsp (data not shown). These Wsp fractions contained a peak in absorbance at 312 nm with an A_{280}/A_{312} ratio of approximately 3:2. The principal peak in absorbance at 312 nm (apparent molecular mass = approximately 11 kDa) was present in a fraction that contained no Wsp, nor any proteins that could be detected through silver staining, and was attributed to free UV-absorbing pigments. The removal of salt from the column buffer caused a marked change in the elution profile of Wsp and the UV-absorbing pigments, and both components underwent an apparent increase in molecular mass (Fig. 9*B*). Wsp was present in fractions that contained one of the four principal

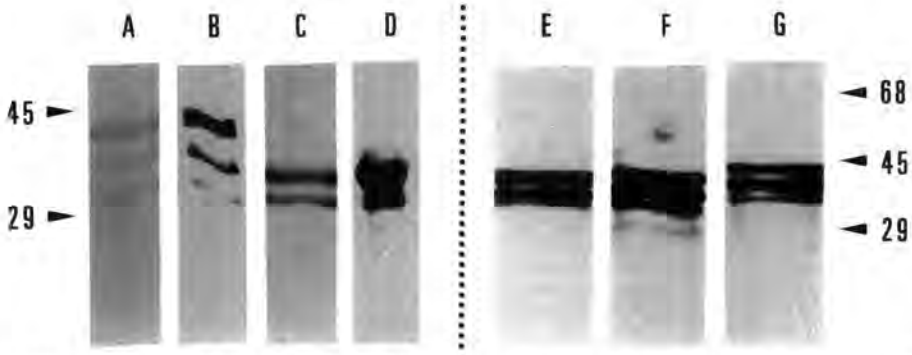

FIG. 5. **Wsp may be subject to selective proteolysis.** Total proteins were extracted from desiccated *N. commune* CHEN with SDS-PAGE buffer that lacked protease inhibitors, and extracts were either analyzed immediately (*lane A*, Coomassie-stained protein; *lane B*, equivalent Western blot) or frozen and thawed at room temperature (*lane C*, Coomassie-stained protein; *lane D*, equivalent Western blot). *Lanes E–G* are Western blots of extracts of the identical material that were obtained in the presence of either DFP, 1,10-P, or E-64, respectively. Approximately 3 μg of total protein loaded in each case on 15% (w/v) polyacrylamide gels. The running time for the gel that contained *lanes A–D* was extended to permit better resolution of the Wsp polypeptides. Molecular mass markers are in kDa.

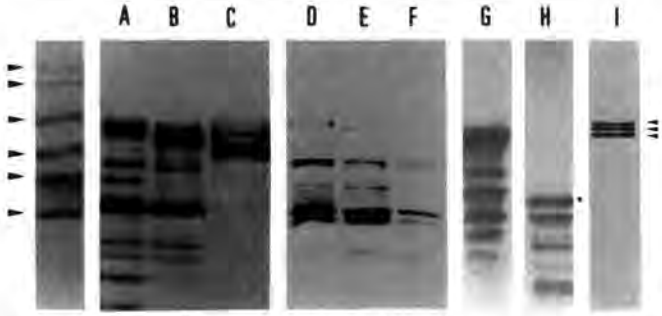

FIG. 6. **The peptide maps of Wsp polypeptides are very similar.** Western blots after treatment of purified 39-, 37-, and 39-kDa polypeptides with either V8 protease (*lanes A–C*, respectively), chymotrypsin (*lanes D–F*, respectively), endo Lys-C (*lane G*), or endo Glu-C (*lane H*, ● peptide sequenced). *Lane I* is a blot of the undigested Wsp polypeptides from which the individual polypeptides were purified through electroelution. Approximately 3 μg of polypeptide were loaded in each lane. Molecular mass markers are as in Fig. 2.

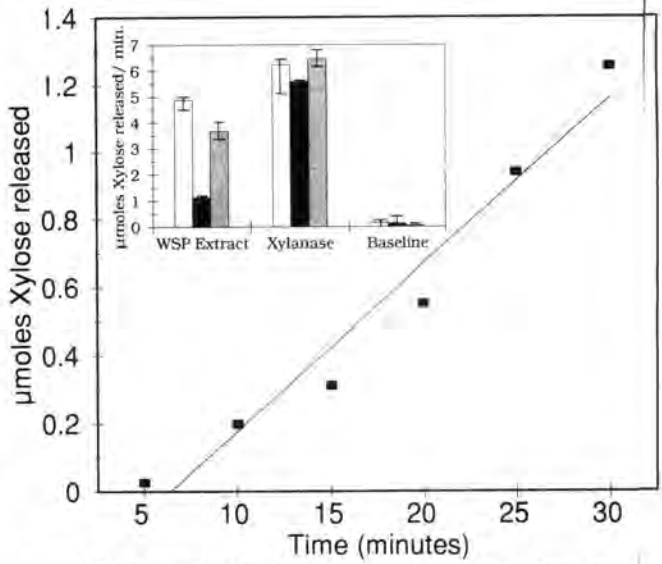

FIG. 7. **Wsp preparations have xylanase activity.** Reaction mixtures contained 200 μl (~12 μg of total protein ml^{-1}) of rehydration fluid and 200 μl of RBB substrate (see "Experimental Procedures"). Each data point in the linear regression is the mean of three replicates. The data points at 60 and 120 min (not shown) were 3.067 (±0.307) and 6.82 (±0.547) μmol of xylose released min^{-1}, respectively. *Inset*, specific activities (assayed after 25 min of incubation) of Wsp rehydration fluids and 0.25 units of purified *Trichoderma* xylanase incubated in the absence of Wsp antiserum (□), presence of Wsp antiserum (■), or presence of preimmune serum (▨). Sera were used at a final dilution of 1:200 in reaction mixtures. Reaction mixtures were incubated with the antiserum for 10 min prior to the addition of substrate.

peaks in absorbance at 312 nm and eluted with molecular masses between 60 and 80 kDa (Fig. 9*B*). In these fractions the A_{280}/A_{312} ratio was approximately 1:1. Fractions that contained the other three peaks in absorbance at 312 nm contained no proteins as judged by silver-staining. The principal peak in absorbance at 312 nm (A_{280}/A_{312} = approximately 1:5) contained material that now eluted with a molecular mass of approximately 25 kDa (Fig. 9*B*).

The elution profile of rehydration fluid in 50 mM KCNS, 20 mM Tris-HCl, pH 7.4, was very similar to that obtained with 50 mM KCl, 20 mM Tris-HCl, pH 7.4, but under these two conditions the major peak in absorbance at 312 nm appeared in fractions that eluted at 15.2 and 16.2 ml, respectively (data not shown). The elution profile, and distribution of Wsp, in the presence of 20 mM Tris-HCl, pH 7.4, 6.6 M urea, was very similar to that obtained under native conditions in the absence of salt (data not shown).

Copurification of Wsp and Xylanase—The xylanase activity present in rehydration fluids (Fig. 7) eluted from a Superose 12 column in the presence of salt and was highest in one of the two fractions that contained Wsp (Fig. 9*A*). In the second of these, the xylanase activity was approximately 50% lower, and xylanase activity was also found in fractions that contained no Wsp. Xylanase activities were lower when fractions were eluted in the absence of salt, and the highest of these activities were present in fractions that contained no Wsp, nor any other proteins as judged from silver-staining (Fig. 9*B*).

The elution profiles presented in Figs. 8 and 9 are representative of those obtained in multiple trials with different batches of rehydration fluids.

Glycosylation Status of Wsp—Wsp preparations were resolved through SDS-PAGE, and individual polypeptides were purified through electroelution. The polypeptides were freed of Coomassie Blue dye through phenyl-Sepharose chromatography and were found to have no absorbance at 312 nm (data not shown). Analysis of electroeluted Wsp polypeptides, of Wsp fractions obtained without cell disruption (rehydration fluids), and of Wsp preparations obtained through solubilization of cell wall fractions with detergents provided no evidence for glycosylation of Wsp using the glycoconjugate detection methods described under "Experimental Procedures." The apparent molecular masses of the proteins in these extracts remained unchanged following treatment with endoglycosidase F (data not presented).

Microheterogeneity of Wsp—Amino acid analysis of the three Wsp polypeptides indicated a combined serine/threonine content of approximately 20% in each one (11), and the amino-terminal sequence data presented here confirm the presence of

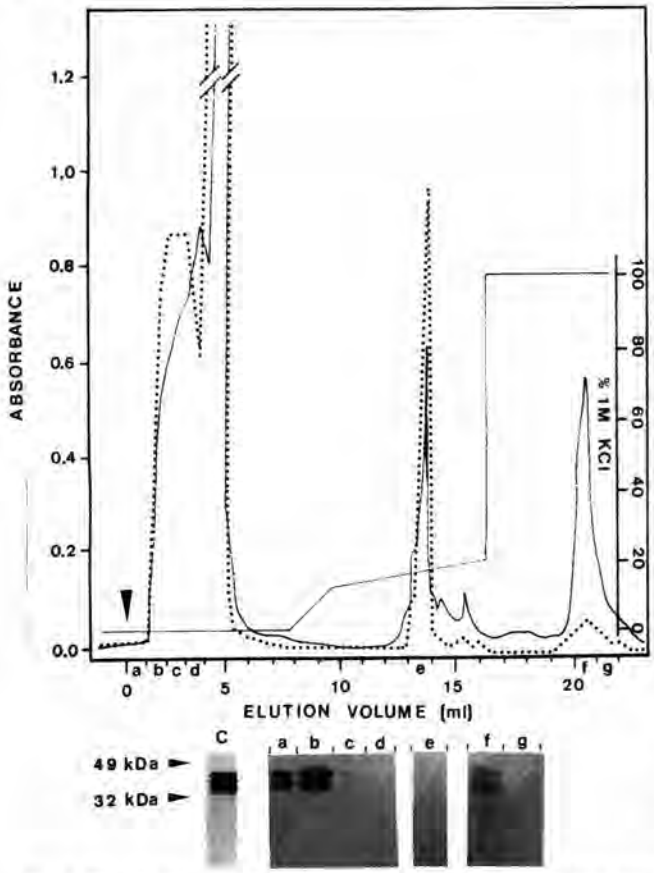

FIG. 8. **Resolution of WSP/UV-absorbing complexes.** Elution profile of 2 ml of concentrated rehydration fluid (approximately 100 μg of total protein ml^{-1}) from a Mono Q HR 5/5 column equilibrated in 20 mM Tris-HCl, pH 7.4 (buffer A) with a flow rate of 1 ml min^{-1}. Profile was developed with a gradient of buffer B (buffer A, 1 M KCl). Absorbance was monitored simultaneously at 280 nm (——) and 312 nm (.....). Aliquots from 1-ml fractions were subjected to Western blotting (*lower inset*, *C* = control; *a–g* = fractions analyzed), and assays for xylanase activity (see text).

tyrosine. As phosphorylation could account for the heterogeneity of Wsp in two-dimensional gels, and in view of our recent identification of a secreted protein tyrosine/serine phosphatase in *N. commune* UTEX 584 (28), we compared the mobilities of the Wsp polypeptides before, and at different times after, treatment with calf intestinal alkaline phosphatase using both native and SDS-PAGE electrophoresis. These experiments provided no evidence for phosphorylation of Wsp, nor was free phosphate detected after termination of the assays (data not presented).

Presence of Wsp in Filamentous Cyanobacteria—Proteins that showed a cross-reaction with the Wsp antiserum, including some in the size range 30 to 40 kDa, were detected in total cell extracts of cultures of *N. commune* UTEX 584 and *Anabaena* spp. strains PCC7118, PCC7119, and PCC7120 (data not presented). The cell-free culture fluids of these strains gave no positive signals in Western analysis. The cells of *N. commune* DRH1, and the cell-free gel-like supernatant from cultures of this strain, gave a strong reaction in Western analysis (data not presented).

DISCUSSION

The secretion of proteins by cyanobacteria has been documented in a number of studies, but the functions and characteristics of these extracellular polypeptides remain poorly understood (29). The criteria used to assess whether proteins are extracellular include whether the proteins are substantially and selectively enriched in cell-free media and, specifically for the cyanobacteria, whether the protein extract is completely devoid of phycobiliproteins (29). The latter, in view of their general abundance, visible spectra, and high extinction coefficients provide an extremely sensitive measure of cell lysis. In addition to the complete lack of phycobiliproteins in aqueous extracts of desiccated colonies, we have documented elsewhere that filaments of *N. commune* retain both their integrity during desiccation and subsequent rehydration and constitute the only cells present in the glycan sheath (9). Scanning and transmitted electron microscopy utilized in the present study confirmed the structural integrity of desiccated cells. As such, the data confirm that Wsp, the most abundant protein in desiccated *N. commune*, is secreted and distributed throughout the extracellular glycan sheath. The patterns observed after immunogold labeling of thin sections must, however, underestimate the true extent of Wsp distribution because of the finite time required to fix the dehydrated material with aqueous glutaraldehyde. The latter procedure leads to leaching of Wsp from the colonies. However, as the cells were judged to be efficiently fixed under these conditions the discrete and reproducible labeling pattern observed around the periphery of cells may be evidence for a fraction of Wsp that is not removed from the sheath during rehydration.

The 33-, 37-, and 39-kDa Wsp polypeptides are immunologically related, the amino acid sequences at their amino termini are identical, their peptide maps are very similar, and they all appear to lack methionine residues. Wsp polypeptides have very similar amino acid compositions (11). We obtained no evidence for glycosylation or phosphorylation. Fluorometric analysis indicated the carbohydrate content of electroeluted Wsp polypeptides to be no greater than 1 mol of glucose equivalent/mol of Wsp. The purified and denatured polypeptides showed no absorbance at 312 nm. Sequence analysis suggests that the amino-terminal residue of each polypeptide was unblocked, mild acid hydrolysis did not result in any change in mobility of the polypeptides, and all lacked a spectrum in the visible region. The possibility that the three polypeptides arise through the covalent modification of a single protein is, therefore, unlikely. The possibility that the 33- and 37-kDa polypeptides arise through the amino-terminal processing of the 39-kDa polypeptide is discounted on the basis of the protein sequence data. Our conclusion is that the Wsp polypeptides are either the products of separate genes (isoenzymes), or they arise through processing (proteolysis) at their carboxyl termini as has been described for the extracellular proteases of several Gram-negative bacteria (30). An appraisal of the former possibility must await completion of DNA sequence analysis. Evidence for the latter possibility is the observation of a selective loss of the 39-kDa polypeptide in total cell extracts lacking protease inhibitors (see Fig. 5). The three polypeptides may arise from a single precursor protein (a possibility given the fact that Wsp is secreted) although no Wsp polypeptides greater than 39 kDa, other than aggregates of the monomers, have ever been observed in our studies.

Accumulations of Wsp, the principal soluble protein, and the secreted UV-A/B-absorbing pigment (up to 10% of colony dry weight; 25) represent major biochemical investments on the part of desiccation-tolerant *N. commune*. Both of these products are water soluble, they accumulate in the extracellular glycan in substantial amounts, and they are released upon rehydration of desiccated colonies. Our data indicate that strong ionic interactions influence the apparent molecular masses of Wsp- and UV-absorbing pigment complexes in rehydration fluids. In the presence of salt, Wsp polypeptides appear to exist as monomers. In the absence of salt, both the Wsp polypeptides and UV-absorbing pigments undergo an apparent

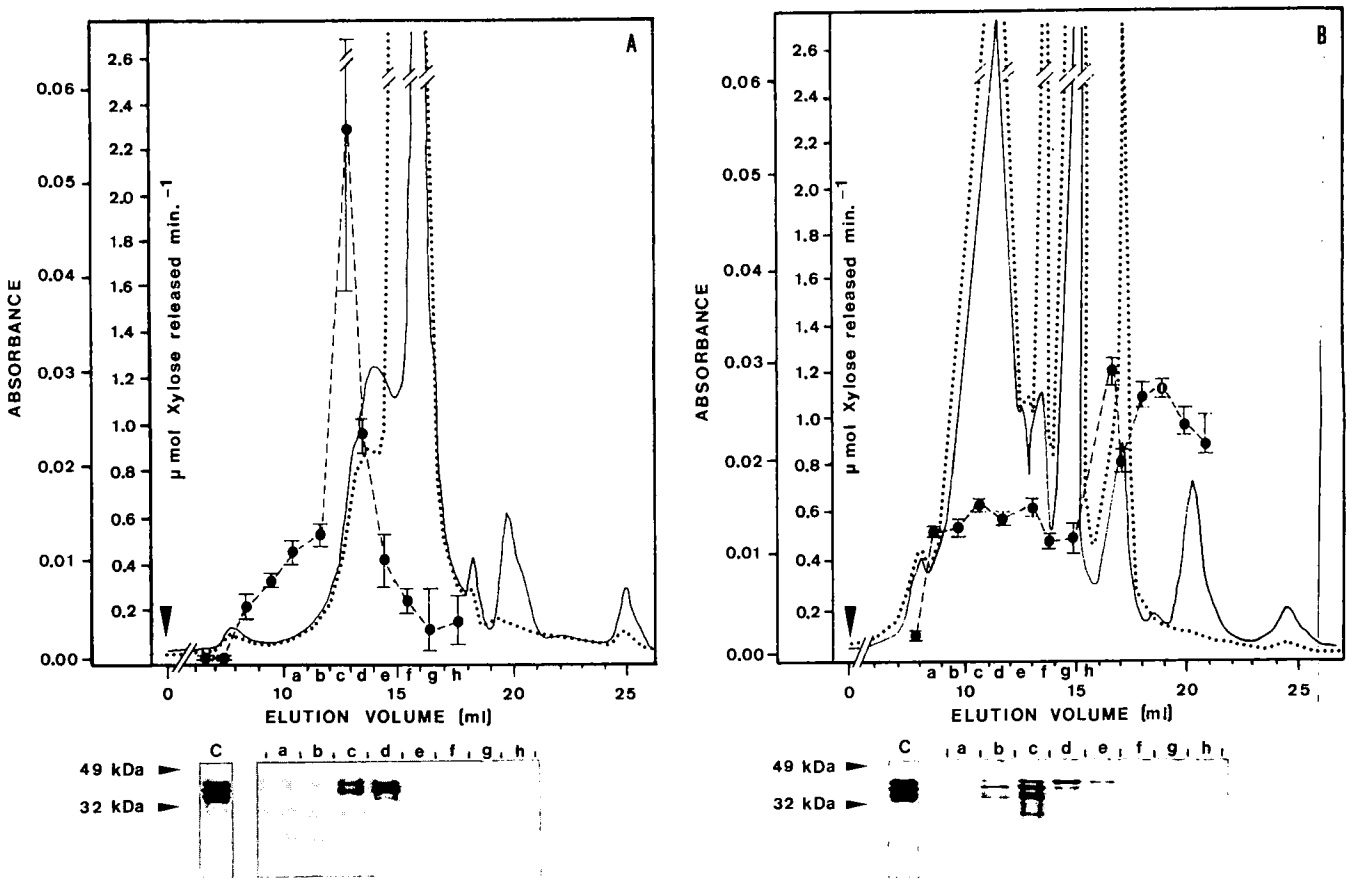

FIG. 9. **Ionic interactions of Wsp UV-absorbing pigments.** *A*, elution profile of 200 µl (approximately 100 µg of total protein ml $^{-1}$) of concentrated rehydration fluid from Superose HR 10/30 column equilibrated in 20 mM Tris-HCl, 50 mM KCl with a flow rate of 0.75 ml min^{-1}. Absorbance was monitored simultaneously at 280 nm (——) and 312 nm (.....). Aliquots from 1-ml fractions were subjected to Western blotting (*lower inset*; see Fig. 8 legend), silver staining, and assays for xylanase activity (——; see text and Fig. 7 for details). Column was calibrated using protein molecular mass markers (Boehringer Mannheim). *B*, as in *A*; column buffer 20 mM Tris-HCl, pH 7.4.

increase in molecular mass suggestive of some aggregation. There is an obvious stoichiometry in the aggregation (dimerization), and we obtained no evidence for precipitation of the complexes. These interactions may involve negatively charged components, and the presence of such components may explain why the greater fraction of Wsp polypeptides (with pI values of 4.3–4.8 (11)) were unable to bind to Mono Q resin at pH 7.4 (Fig. 8). Prokaryotic glycoproteins appear to be of limited occurrence, and only for one example, the extracellular wall component of *Halobacterium* spp. (31), has the glycoprotein identity been confirmed with certainty. Recent reports of intracellular glycoproteins (phycobilisome linker polypeptides) in the cyanobacterium *Synechococcus* sp. strain PCC 7942 (32) have been discounted after critical scrutiny (15). No evidence for glycosylation or any other detectable modification of Wsp was obtained in this study.

Partial sequence analysis of Wsp polypeptides identified a region with conspicuous homology to a bacterial β-xylosidase (33). Rehydration fluids, enriched in Wsp, had a weak xylosidase activity but a pronounced xylanase activity. Following purification of the extracts by liquid column chromatography, the xylanase activity was found in fractions that contained Wsp as well as fractions that lacked any immunogenic material. Aggregation of Wsp- and UV-pigment complexes (in the absence of salt) lead to reduction in the xylanase activity, and the resultant activity was then detected in fractions that contained or lacked Wsp. These data suggest that the xylanase activity cannot be not attributable to Wsp; rather they suggest that Wsp may form part of a protein complex that has the activity. Other proteins are present in rehydration fluids albeit at low concentrations. At least one of these, the 68-kDa protein partially characterized in this study, also shows homology with carbohydrate-modifying enzymes. The fact that Wsp antiserum did not inhibit the activity of a purified xylanase, but caused a 10-fold decrease in the xylanase activity of rehydration fluids, is consistent with the conclusion that Wsp is associated with, but not directly responsible for, the xylanase activity. Purified Wsp polypeptides showed negligible hydrolysis of *o*-paranitrophenyl-β-D-xylopyranoside although we cannot discount the possibility that a secondary xylosidase activity of Wsp may be present that was undetected under our assay conditions with the substrate in question. Xylosidases and xylanases both contribute to the hydrolysis of polysaccharides that contain 1,4-β-D-xylosyl residues, and they often exist in multiple enzyme forms (34). The basic repeating unit of polysaccharides from heterocysts and spore envelopes of the filamentous cyanobacterium *Anabaena* sp. ATCC 29414 contain xylose (35). Although we report here a discrete localization of Wsp at the periphery of the envelope of both vegetative cells and heterocysts of *N. commune* (Fig. 1), the purified glycan of desiccated colonies, and that of cultures of *N. commune* DRH 1, contain no xylose.[3] The UV-A/B-absorbing pigments of *N. commune* have a polysaccharide core, and the structure of one of the chromophores has recently been solved. It does contain xylose.[4] While these data may suggest some relationship between Wsp and the UV-absorbing pigment, it is not possible to state at this juncture whether Wsp polypeptides and UV-absorbing pigments do ag-

[3] D. R. Hill and M. Potts, manuscript in preparation.
[4] G. Böhm, S. Scherer, and P. Böger, in preparation.

gregate specifically (bind one another), or whether these molecules simply have very similar size and charge properties under the different conditions used in our experiments. In addition, these interactions may also involve other components of the glycan; the glycan and UV-absorbing pigment both contain amino sugars and Wsp clearly has similarities (both immunogenic and at the protein sequence level) to enzymes that bind such residues.

The finding of abundant Wsp in materials collected at different times from numerous locations over a period of some two decades implies that first, Wsp synthesis is constitutive *in situ* and second, the protein is very stable in desiccated colonies. One consequence of the secretion of Wsp by *N. commune* is that it must result in the loss of a substantial amount of cellular nitrogen and carbon during rehydration of colonies. This is surprising considering the extreme habitats colonized by this cyanobacterium (36). It remains to be determined whether any components secreted by the cells can be subsequently salvaged. In the laboratory, liquid cultures of *N. commune* DRH1 secrete substantially less amounts of Wsp, and the protein was not detected in cell-free culture supernatants from a number of other cyanobacterial species including *N. commune* UTEX 584. Extracts from such cultures also fail to absorb in the region of the spectrum between 310 to 341 nm (25).

One consequence of long term desiccation in nature is the need to afford protection from an increased susceptibility to UV-induced radiation damage. The ionic interactions that lead to aggregations of Wsp- and UV-absorbing pigments must be subject to attenuation *in situ*. Aggregation will be enhanced as salts, precipitated through drying of colonies, are removed upon flooding of the colonies with rainwater. Such aggregations may be required for the function of these components, may influence the absorption properties of the pigments, and/or may restrict the release of these components from rehydrating colonies.

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17. Perrini, F., and Peters, B. P. (1982) *Anal. Biochem.* **123,** 357–363
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CRYOBIOLOGY 30, 232–233 (1993)

Stabilities of Macromolecules during Water Stress in Cyanobacteria

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Desiccation tolerance of cells reflects a complex array of interactions at the structural, physiological, and molecular levels which vary with time and in response to water availability. It is unlikely that desiccation tolerance can be attributed to (or manipulated via) the properties of a single gene or its product; rather, an understanding of this trait must rely upon an appreciation of the structural biology, physiology, biochemistry, and molecular ecology of the organism.

Of those organisms which express desiccation tolerance, the cyanobacteria warrant particular attention. These photosynthetic prokaryotes share structural and biochemical affinities with the higher plant chloroplast, many synthesize nitrogenase and fix dinitrogen, they develop complex intracellular membrane systems, and some strains elaborate conspicuous extracellular layers of carbohydrates and protein. One strain, *Nostoc commune*, is often the principal component of soil crusts in terrestial karst environments from the tropics to the polar regions, especially in those subject to intermittent rains (Whitton *et al.*, *Phycologia* 18, 278–287, 1979). *N. commune*, which does not produce spores, expresses a marked capacity for desiccation tolerance.

A possible specific adaptation to desiccation tolerance. A striking feature of actively growing colonies of *N. commune* is their very regular spherical shape (0.3- to 0.5-mm diameter). Filaments of the cyanobacterium grow embedded in a homogenous gel matrix which is devoid of any other organisms. These spherical colonies presumably provide structures conducive to the retardation of water loss (evaporation). As a consequence, it is likely that they also impede the diffusion of gases. One potential molecular adaptation to this restricted gas uptake could reflect our recent finding of cyanoglobin (myoglobin) in *N. commune* (Potts *et al.*, *Science* 256, 1690–1692, 1992). Cyanoglobin synthesis is induced only under nitrogen-fixing microaerobic conditions. As such, cyanoglobin may serve to scavenge oxygen for electron transport processes if oxygen transport became restricted through, for example, flooding of colonies with oxygen-depleted water.

Carbohydrate and protein–carbohydrate stabilities. Immunocytochemical analyses using antibodies directed against sheath epitopes and extracellular protein components and TEM, SEM, and EDAX analyses confirm that the ordered structure and biochemical composition of the extracellular sheath matrix of *N. commune* undergo subtle changes in response to water deficit (Hill and Potts, unpublished data).

DNA stability. DNA of *N. commune* is subject to light-dependent strand nicking during desiccation (Stulp and Potts, *FEMS Microbiol. Lett.* 41, 241–245, 1987). DNA is recovered from desiccated cells as two distinct fractions, one hypomethylated and the other hypermethylated (Jager and Potts, *Gene* 74, 197–201, 1988). The former, but not the latter, copurifies with carbohydrate enriched in glucosamine and galactosamine. DNA from liquid cultures of *N. commune* correspond entirely with the hypermethylated fraction. In view of the damage incurred by DNA during extended des-

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iccation, the state of transcriptionally active DNA upon rehydration requires further study, particularly as cyanobacterial cells may have tens of copies of their chromosome. It seems possible that only some of these chromosomes may enter into gene expression upon rehydration.

Protein stabilities. The pivotal component of the transcriptional apparatus, the DNA-dependent RNA polymerase (Xie and Potts, *Arch. Biochem. Biophys.* 284, 22–25, 1991) remains functionally intact during prolonged desiccation while the (*rpoC1C2*) transcripts which encode two subunits of the enzyme are rapidly degraded after short-term storage of cells in the air-dry state. Upon the rewetting of desiccated cells, *rpoC1C2* expression occurs at the expense of the stable *extant* RNA polymerase holoenzyme. In contrast, a water stress protein Wsp (Scherer and Potts, *J. Biol. Chem.* 264, 12546–12553, 1989), which can account for around 50% of the total soluble protein of desiccated cells, is undetectable within minutes of cell rehydration (Hladun *et al.*, unpublished data). Nitrogenase activity ceases abruptly within 20 min of the drying of cells, yet the Fe protein nitrogenase (NifH) remains undegraded cells after prolonged (years) of desicc tion. Despite the differential susceptibiliti of proteins to water stress there is an a parently highly ordered stringent recove of metabolic capabilities during cell reh dration.

Lipid stabilities. The purified cytopla mic membrane of *N. commune* (Olie a Potts, *Appl. Environ. Microbiol.* 52, 70 712, 1986) is unusual in that it contai 20:3ω3 fatty acid as its major compone (58% of the total fatty acid). The intra- a extracellular membrane systems reta structural integrity following years of de iccation, yet the synthesis of phospholipi glycolipid, and sulfolipids resumes virtual instantaneously upon cell rehydration a steady-state levels are reached within mi utes (Taranto *et al.*, submitted for public tion).

In summary, *N. commune* expresses fe tures of physiology, biochemistry, and m lecular biology which, collectively, coul contribute to the extreme desiccation tole ance of this microorganism.

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Degrees:	B.Sc. Botany with Honors, 1973 Ph.D. Microbiology, 1977
Awards:	Royal Society - Israel Academy of Sciences Post-Doctoral Fellowship, 1977-78 C.A.N.E.E. Award for North-East Excellence, Metro Radio, UK, 1988 John Claibourne Strickland Lecturer, University of Virginia, Richmond, VA, 1993

Academic and Research Positions:

1973-77	Research Assistant; Dept. of Botany, Durham University, England (Supervisor - Dr. Brian A. Whitton)
1974-75	Resident Junior Scientist; Royal Society Research Station, Aldabra Atoll, Indian Ocean
1977-78	Post-Doctoral Fellow: Dept. of Geomicrobiology, Oldenburg University, West Germany (Supervisor Prof. Wolfgang E. Krumbein)
1978-79	Royal Society - Israel Academy of Sciences Post-Doctoral Fellow; Dept. of Microbiological Chemistry, Hadassah Medical School, Jerusalem

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1979-80	Research Associate; Dept. of Biological Sciences, Florida State University, Tallahassee, FL 32306 (Supervisor Prof. E. Imre Friedmann)
1979	Member of FSU Expedition to Antarctica, U.S. Naval Station, McMurdo Sound, Ross Island
1981-85	Assistant Professor, Dept. of Biological Sciences, Florida State University, Tallahassee, FL 32306
1985-91	Associate Professor, Dept. of Biochemistry, and Anaerobic Microbiology, Virginia Tech, Blacksburg, VA 24061
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Sponsored research:

Extramural:

The Royal Society, London: "Nitrogenase activity in non-heterocystous blue-green algae." 1978-79, $13,000 (P.I.)

NSF: "Regulation of nitrogen metabolism in cyanobacteria undergoing water stress." 1982-85, $73,898 (P.I.)

NSF: "Experimental taxonomy of blue-green algae (Cyanobacteria): A comparison and synthesis of botanical and bacteriological methods." 1982-85, $202,322 (Co-P.I.)

NSF: "Gene expression in a nitrogen-fixing cyanobacterium undergoing water stress." 1984-87, $171,500 (P.I.)

DOE-SERI: "Plasmid stability and enzyme overproduction in recombinant *E. coli* ." 1987-1992, $95,224 (P.I.)

NSF: "Regulation of water stress protein synthesis." 1988-1991, $180,000 (P.I.)

NSF: "US-Germany Collaboration on Biochemistry of water stress in cyanobacteria." 1991-92,

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NATO-Scientific Affairs Division: "Water stress proteins of desiccation-tolerant cyanobacteria." 1991-1993, $5,900 (P.I.)

NSF: "Water stress proteins in cyanobacteria." 1991-1994, $210,000 (P.I.)

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Life Technologies Inc: "Technologies for Dried Cells." 1994-1995, $12,000 (P.I.)

Foundation for Microbiology: Publication support, 1994, $750.

University:

Hatch Award: "Isolation and expression of *rpo* (RNA polymerase) genes from a cyanobacterium." 1986-1991 (P.I.)

Biomedical Research Fund: "Heat shock protein synthesis in avian erythrocytes." 1986-87 (Co-P.I.)

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Editorial Boards:

Applied and Environmental Microbiology 1990-
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Undergraduate Students:

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B.S. in Biochemistry
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Graduate Students:

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Ph.D. program in Experimental Pathology and Immunology, University of Florida, Gainsville, Florida 1991-

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Research Technician, Laboratory for Virology and Molecular Carcinogenesis, NIH Cancer Research Center, Frederick, MD.
Research Technician, Genetic Therapy Inc., Rockville, Maryland 1991-

Xie, W.-Q. (Ph.D. Biochemistry) 1989 "Molecular analysis of cyanobacterial RNA polymerase genes of cyanobacterial DNA-dependent RNA polymerase." VPI & SU
Post Doctoral Fellow, Siegfried and Janet Weis Center for Medical Research, Geisinger Clinic, Danville, Pennysylvania
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Research Scientist, Burroughs-Wellcome, North Carolina, 1995-

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Post Doctoral Fellow, Basic Research Program, NIH-NCI, Frederick, Maryland. 1992-94
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Virginia Tech Graduate Research Development Award 1989.
Technician, Enzyme Division, Life Technologies Inc., Rockville, Maryland.1992-

Hill, D. 1994 (Ph.D. Biochemistry) "Morphological, biochemical and molecular characterization of desiccation-tolerance in cyanobacterium *Nostoc commune* var. Vauch." VPI & SU
Virginia Tech Graduate Research Development Award 1989.
Graduate Travel Program Award 1989.
Post Doctoral Fellow and Research Director of EM Facility, Plant Pathology Department, VPI&SU. 1994-

Reed, D. (M.S. Biochemistry) 1989- "Structural analysis of the *iph* (indole phosphate hydrolase) operon in the cyanobacterium *Nostoc commune* UTEX 584. VPI & SU
Virginia Tech Graduate Research Development Award 1989.

Spencer, T. W. (Ph.D. Biochemistry) 1989- "Overexpression of cloned xylose isomerase (*xylA*) in *E. coli.*" VPI & SU
Senior Award in Biochemistry for Professional Development and Research
1988-1992 Resident at Solar Energy Research Institute (NREL), Golden, Colorado

Joardar V. (Ph.D. Biochemistry) 1989- "Characterization and regulation of the gene encoding water stress protein in *Nostoc commune*" VPI & SU
M.Sc. Biochemistry, St. Xavier College, Bombay, India
Virginia Tech Graduate Research Development Award, 1990.
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McCartney, B. (Ph.D. in Biochemistry) 1992- "Biochemistry of the secreted protein-tyrosine phosphatase of *Nostoc commune*"
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Thorsteinsson, M. (Ph.D. in Biochemistry) 1994 - "Structural characterization of cyanoglobin"

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Post-Doctoral Trainees:

Dr. Ben Stulp, Institute of Molecular Genetics, University of Gröningen, Netherlands.
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Dr. Jaap Olie, Dept. of Microbiology, Nieuwersluis, Netherlands.
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Sabbatical Visitors to Laboratory:

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Meetings attended (talk presented*):

1976 2nd International Symposium on Photosynthetic Prokaryotes, University of Dundee, Scotland

1976* Symposium on the Taxonomy of Blue-Green Algae, University of Durham, England (member of organizing committee)

1977 Meeting for Discussion - The Terrestial Ecology of Aldabra, The Royal Society, London

1977* 1st Oldenburg Symposium on the Taxonomy of Cyanobacteria, University of Oldenburg, Oldenburg, West Germany (member of organizing committee)

1977* Schwerpunktprogram - Nitrogen Fixation, University of Heidelberg, West Germany

1978* 10th Sedimentological Congress, Jerusalem, Israel

1978* 2nd International Congress of Ecology, Jersusalem, Israel

1978 25th Annual Meeting of the British Phycological Society, University of London, England

1979* Annual Southeastern Phycological Colloquy, Duke University Marine Laboratory, North Carolina

1979 NSF-U.S. Antarctic Research Program Meeting, Washington, DC

1981* 81st Annual Meeting of the American Society for Microbiology, Dallas, Texas

1983 13th International Congress of Microbiology, Boston, Massachusetts

1983 83rd Annual Meeting of the American Society for Microbiology, New Orleans, Louisiana

1984 100th Annual Meeting of the Society for General Microbiology, Warwick, England

1984* 3rd International Symposium on Nitrogen Fixation with Non-legumes, Helsinki, Finland

1984* S.E. Branch Meeting of the American Society for Microbiology, Clearwater, Florida

1986* Annual Meeting of the Virginia Biochemists, Mountain Lake, Virginia

1986 14th International Congress of Microbiology, Manchester, United Kingdom

1987 Molecular Biology of Photosynthetic Prokaryotes, University of Wisconsin, Madison, Wisconsin

1988* 1st New England Biolabs Meeting on DNA Modification, Gloucester, Massachusetts

1988* Biofuels Program, Solar Energy Research Institute, Golden, Colorado

1989 Biofuels Program, Solar Energy Research Institute, Golden, Colorado

1989* Gordon Conference on Plant Molecular Biology, Procter Academy, Andover, New Hampshire

1990 2nd New England Biolabs Meeting on DNA Modification, Berlin, Germany

1993 93rd Annual Meeting of the American Society for Microbiology, Atlanta, GA.

1994 94th Annual Meeting of the American Society for Microbiology, Las Vegas, NA

National and International Invitations:

1981 Institute of Pedology, University of Saskatchewan, Saskatoon, Canada. "Effects of water stress on cyanobacteria"

1981 Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia. "Marine cyanobacterial stromatolites: aspects of physiology and structure versus function"

1981 Bethesda Research Laboratories Inc., Gaithersburg, Maryland. "Cyanobacteria: physiological properties and experimental (biochemical) taxonomy"

1983 Martin Marietta Corporation, Baltimore, Maryland. "Biochemistry and genetics of nitrogen-fixing cyanobacteria"

1983 Bethesda Research Laboratories Inc., Gaithersburg, Maryland. "Cyanobacteria and algae: prospects and a perspective"

1984 Freshwater Biological Association, Windermere, England. "Evolution of cyanobacterial plasmids and cyanophage DNA's"

1984 Agricultural Research Council Group on Cyanobacteria, Department of Biological Science, University of Dundee, Scotland. "Relationships between cyanobacterial plasmids and cyanophage DNA's"

1984 Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia. "Gene expression in cyanobacteria undergoing water stress"

1988 Department of Botany, University of Tennessee, Knoxville, Tennessee. "Molecular basis for desiccation tolerance"

1988 Solar Energy Research Institute, Golden, Colorado. "Overexpression of xylose isomerase in *E. coli*"

1989 Bethesda Research Laboratories Inc., Gaithersburg, Maryland. "The water stress protein of a desiccation-tolerant cyanobacterium"

1989 Department of Biological Science, University of Durham, Durham City, England. "Molecular mechanisms for desiccation tolerance in cyanobacteria"

1989 Department of Biology, Biotechnology Centre, Sunderland Polytechnic Institute, Sunderland, England. "Desiccation tolerance in *Nostoc commune* (Cyanobacteria)"

1992 Bodega Marine Biology Laboratory, Bodega Bay, University of California, Davis, CA. "Stabilities of macromolecules in desiccated cyanobacteria."

1993 Institut für Mikrobiologie, Technische Universität München, München, Germany "Ancient proteins and enzymes"

1993 Research Division, Life Technologies Inc., Gaithersburg, MD. "Technologies for dried cells"

1993 The John Claibourne Strickland Lecture, Department of Biology, University of Virginia, Richmond, VA. "Cyanobacteria from Jurassic Park."

1995 Ecoscience Corporation, Worcester, Massachusetts "Dry-Down Technology"

1995 Southern Regional Meeting of the American Society of Plant Physiologists, Knoxville, Tennessee "The anhydrobiotic cyanobacterial cell." Symposium speaker.

1995 95th Annual Meeting of the American Society for Microbiology, Washington, D.C. Convenor and speaker - a seminar - Desiccation and the Prokaryotic Cell. "Life in the Dry Lane."

Publications:

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Undergraduate research:

1972. Holmes[1], N. T. H., E. Lloyd[1], M. Potts and B. A. Whitton. Plants of the River Tyne and future water transfer scheme. **Vasculum** 57:56-78.

Ph.D. Thesis

1977. Potts, M. Studies on Blue-Green Algae and Photosynthetic Bacteria in the Lagoon of Aldabra Atoll. Ph.D. Thesis, University of Durham, England.

Reviews:

1. 1994 - Potts, M. Desiccation tolerance of prokaryotes. **Microbiol. Rev.** 58:755-805.

2. 1995 - Potts, M. The anhydrobiotic cyanobacterial cell. **Physiol. Plant.** in press

3. 1995 - Potts, M. Ancient prokaryotes - water, water, everywhere? **ASM News** (Forum article) in press

Papers:

1. 1977 - Potts, M. and B. A. Whitton[4]. Nitrogen fixation by blue-green algal communities in the intertidal zone of the lagoon of Aldabra Atoll. **Oecologia (Berl.)** 27:275-283.

2. 1977 - Potts, M. and B. A. Whitton[4]. A study of factors influencing Eh in freshwater pools on Aldabra Atoll. **Arch. Hydrobiol.** 81:25-34.

3. 1977 - Whitton[4], B. A. and M. Potts. Observations on redox potential in freshwater pools on Aldabra Atoll. **Atoll Res. Bull.** 214:1-5.

4. 1979 - Krumbein[4], W. E. and M. Potts. Girvanella-like structures formed by *Plectonema gloeophilum* Borzi (Cyanophyta) from the Borrego Desert in Southern California. **Geomicrobiol. J.** 1:211-217.

5. 1979 - Potts, M. Nitrogen fixation (acetylene reduction) associated with communities of heterocystous and non-heterocystous blue-green algae in mangrove forests of Sinai. **Oecologia (Berl).** 39:359-373.

6. 1979 - Potts, M. Ethylene production in a hot brine environment. **Arch. Hydrobiol.** 87:198-204

7. 1979 - Potts, M. and B. A. Whitton[4]. pH and Eh on Aldabra. 1. Comparison of marine and freshwater environments. **Hydrobiologia** 67:11-17.

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