Prokaryotic Metallothionein gene isolation, Nucleotide sequence and expression

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"Prokaryotic Metallothionein Gene Isolation, Nucleotide Sequence and Expression"

by

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thesis submitted for the degree of Doctor of Philosophy
in the University of Durham

Department of Biological Sciences

April 1993

This thesis is entirely the result of my own work. It has not been accepted for any other degree and is not being submitted for any other degree.

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April 1993

ABSTRACT.

Metallothioneins (MTs) are low molecular weight, cysteine-rich, metal-binding proteins, which are proposed to have roles in essential trace metal homoeostasis and in the detoxification of metal ions. The genes encoding MTs have been isolated from a wide range of eukaryotes, although MT genes have not previously been isolated from prokaryotes.

The polymerase chain reaction (PCR) was initially used to isolate a prokaryotic MT gene fragment from *Synechococcus* PCC 6301. PCR fragments were amplified using inosine-containing primers designed from the amino acid sequence of a prokaryotic MT. Subsequent cloning and nucleotide sequence analysis revealed that the deduced amino acid sequence of the PCR product corresponded to the amino acid sequence of the prokaryotic MT. The amplified product was thus part of the gene encoding the MT, and was designated *smtA*. The same primers used in the initial amplification were subsequently utilised for anchored PCR, to amplify the remainder of the coding region and the 3' and 5' flanking regions of the *smtA* gene.

A genomic library was produced from *Synechococcus* PCC 7942 DNA and screened using the PCR products described above as probes. A genomic clone was isolated, nucleotide sequence analysis revealed the structure of the *smt* locus, two open reading frames, *smtA* and *smtB*, arranged in a divergent orientation about the *smt* operator/promoter region. The operator/promoter region contains the transcriptional and translational signals for the two genes and three regions that are candidate sites for interaction of regulatory proteins. The transcript start sites of the two genes were mapped within the operator/promoter region by primer extension analysis.

An increase in the relative abundance of transcripts of both *smt* genes was studied in response to various metal ions in a series of northern blots. Inhibitor studies confirmed that the *smtA* gene is regulated at the transcriptional level. The 5' flanking region of the *smtA* gene conferred metal specific induction of the reporter gene *lacZ*. SmtB has sequence similarity to several prokaryotic regulatory proteins and contains a putative helix-turn-helix structural domain. Deletion analysis suggests that SmtB is a repressor of *smtA* expression. Subsequent work has confirmed that SmtB is a *trans*-acting repressor of expression from the *smt* operator/promoter.

Robinson, N.J., Gupta, A., Fordham-Skelton, A.P., Croy, R.R.D., Whitton, B.A. and Huckle, J.W. (1990) Proceedings of the Royal Society of London **B 242**, 2410247.

Huckle, J.W., Morby, A.P., Turner, J.S. and Robinson, N.J. (1993) Molecular Microbiology **7**, 177-188.

ABBREVIATIONS.

°	degrees
°C	degrees Celsius
%	percent
pH	hydrogen ion potential
g	gram
mg	milligram
µg	microgram
k.b.	kilo base
b.p.	base pair
l	litre
ml	millilitre
µl	microlitre
M	molar
mM	millimolar
µM	micromolar
fM	fluoromolar
v/v	volume for volume
w/v	weight for volume
h	hour
min	minute
m	metre
nm	nanometre
OD	optical density
MT	metallothionein
MTs	metallothioneins
PCR	polymerase chain reaction
APCR	anchored polymerase chain reaction
IPCR	inverse polymerase chain reaction
rpm	revolutions per minute
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
SDS	sodium dodecyl sulphate
Tris	tris(hydroxymethyl)methylamine
UV	ultra violet
BSO	buthionine sulphoximine
DMSO	dimethyl sulfoxide
LKB	linker kinase buffer
MOPS	3-[-morpholino] propane-sulphonic acid
Cm	chloramphenicol
Cys	cysteine
Xaa	an amino acid other than cysteine

ACKNOWLEDGEMENTS.

I would like to thank Professor D. Boulter, Professor K, Bowler and Professor P.R. Evans for allowing the use of the facilities in the Department of Biological Sciences. I am especially appreciative of the effort of my supervisors Drs N.J. Robinson and B.A. Whitton for their guidance and their donation of knowledge through out the length of this study.

My gratitude also extends to Drs A.P. Morby, A.M. Tommey and A.P. Fordham-Skelton for their proficient advice and direction. I would also like to acknowledge the contribution of other laboratory colleagues, Dr W.P. Lindsay, Dr A. Gupta, Dr P. Urwin, Ms C.S.M. Furniss, Ms J.S. Turner and Mr J.R. Wilson for their motivation and friendship. I also extend my appreciation for the expert technical support provided by Mr J. Gilroy, Mrs J. Bryden, Mrs M. Snow and the photographic work of Mr D. Hutchinson and Mr P. Sidney.

Thanks also to Liz and family for their moral support and motivation during this study. This work would have been impossible without the support and persistence of my family throughout my education, many thanks to Helen, Malcolm and Sarah. Their contributions are beyond appreciation.

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Chapter 1

INTRODUCTION.

In eukaryotes, metallothioneins (MTs) are involved in the cellular responses to elevated concentrations of certain metal ions (discussed in sections 1.1.4 and 1.1.6). A number of different eukaryotic MT genes have been isolated and their nucleotide sequences determined (reviewed by Hamer 1986), but MT genes have not previously been isolated from prokaryotes. Reports have indicated the presence of low-molecular weight, cysteine-rich metal-ligands, analogous to MTs, in several prokaryotes (cited in Silver and Misra, 1988). However, there has only been one published amino acid sequence of a prokaryotic MT (Olafson *et al.*, 1988). This thesis describes the isolation, structural characterisation and analysis of expression of the first prokaryotic MT gene from *Synechococcus* PCC 7942 and *Synechococcus* PCC 6301.

1.1 Metallothioneins (MTs).

The term metallothionein (MT) was initially used to describe a Cd^{2+} and Zn^{2+}-containing sulphur-rich protein, originally isolated from equine kidney cortex by Margoshes and Vallee (1957). Since its initial isolation, similar proteins have been characterised from a wide range of eukaryotes (including vertebrates, invertebrates, higher plants, fungi) and a prokaryote. The isolation and characterisation of MTs are the subjects of several reviews (Hamer, 1986; Kägi and Kojima, 1987; Kägi and Schäffer 1988; Andrews, 1990; Riordan and Vallee, 1991).

1.1.1 Classification of MT.

The classification of MTs is based on the original characterisation of MT by Kägi and Vallee (1960). MTs are low molecular weight, have high metal and cysteine content,

contain few aromatic amino acids or histidine residues, with a characteristic distribution of cysteine residues. Class I MTs are polypeptides with the location of cysteine residues closely related to those in equine renal MT. All MTs (class I) isolated from vertebrates are subdivided into isoforms, MT-I and MT-II on the basis of ionic charge, each isoform potentially comprising of several isoproteins. Class II MTs are polypeptides in which the positions of the cysteine residues are only distantly related to those of equine MT. Class III MTs (also referred to as phytochelatin, cadystins and $(\gamma EC)_nG$) are atypical, nontranslationally synthesised metal thiolate clusters (Kondo *et al.*, 1983; Grill *et al.*, 1985; Robinson and Jackson, 1986; Steffens *et al.*, 1986; Fowler *et al.*, 1987; Hayashi *et al.*, 1988).

1.1.2 Structure of MT and metal-binding.

All MTs that have been characterised are single chain polypeptides with an abundance of cysteine residues that can total up to one third of all residues. The most conspicuous feature of all MTs is the frequent occurrence of Cys-Xaa-Cys, Cys-Xaa-Xaa-Cys (where Xaa is an amino acid other than cysteine) and Cys-Cys motifs.

Mammalian MT is a 61 or 62 amino acid polypeptide containing 20 cysteine residues. Metals bind to mammalian MT in two distinct polynuclear clusters (Boulanger *et al.*, 1982). The carboxy-terminal α domain extends from amino acid 31-61, it contains 11 cysteine residues and can bind 4 Cd^{2+} or 4 Zn^{2+}, or 6 Cu^{+}. The amino-terminal β domain extends from amino acid 1-30, it contains 9 cysteine residues and can bind 3 Cd^{2+} or 3 Zn^{2+}, or 6 Cu^{+} (Nielson and Winge, 1984). The two domains are globular and linked by a hinge region, consisting of conserved Lys-Lys-Ser, to form a prolate ellipsoid structure. The protein folding patterns are similar but of opposite chirality, the polypeptide chain forming three turns around the bound metal ions in each domain. Cu^{+} ions preferentially bind to the β domain, then to the α domain in a cooperative fashion. However, Cd^{2+} and Zn^{2+} ions preferentially bind to the α domain, then cooperatively to the β domain (Nielson and Winge, 1983). Nielson

et al. (1985) used *in vitro* substitution of metal ions bound to mammalian MTs and demonstrated the relative affinities for metal-MT complexes, to be in the order $Hg^{2+} > Cu^{+} > Cd^{2+} > Zn^{2+} > Ni^{2+} = Co^{2+}$.

The yeast *Saccharomyces cerevisiae* synthesises a copper inducible class II MT encoded by the *CUP1* locus. Yeast copper-MT is 61 amino acids long and binds 8 Cu^{+} through its 12 cysteine residues (Winge *et al.*, 1985). The proposed metal cluster structure is a cubic Cu_8S_{12} polynuclear complex in which each Cu^{+} ion is trigonally coordinated and each cysteinyl thiolate bridges two Cu^{+} ions (George *et al.*, 1988). Yeast MT is induced by copper and silver ions, and although yeast MT has been shown to bind Cd^{2+} and Zn^{2+} ions *in vitro*, it was not shown to be transcriptionally induced by these ions; therefore it was suggested that it provided no protection against them (Butt *et al.*, 1984). Recently a Cd^{2+}-resistant strain of *Saccharomyces cerevisiae* was shown to produce a Cd^{2+}-MT with similarity to CUP1 (Inouhe *et al.*, 1991). This was followed by a study of a similar Cd^{2+}-resistant strain in which the gene encoding yeast Cd^{2+}-MT was isolated (Tohoyama *et al.*, 1992), although wildtype transformants carrying this gene were resistant to copper and not Cd^{2+} ions. It was concluded that a mechanism for Cd^{2+}-specific expression of the MT gene may have been restricted to the Cd^{2+}-resistant strain (Tohoyama *et al.*, 1992).

Class III MTs, first isolated from *Schizosaccharomyces pombe*, were found to be inducible by Cd^{2+} and were designated cadystins (Murasugi *et al.*, 1981). Subsequently, class III MTs have been isolated from higher plants and from eukaryotic algae. These class III MTs have been given the name phytochelatins (Gekeler *et al.*, 1988; reviewed by Rauser, 1990). Phytochelatins are homologous, atypical polypeptides of the general structure $(\gamma Glu\text{-}Cys)_nGly$ ($(\gamma$glutamate-cysteine$)_n$glycine), where n can range from 2-11. These peptides resemble glutathione (γGlu-Cys-Gly) which can be a precursor in their synthesis.

Many metal ions (silver, gold, bismuth, Cd^{2+}, copper, mercury, nickel, antimony, tin, tungsten, Zn^{2+}, As^{5+} and Se^{4+}) induce the synthesis of phytochelatins, although only Cd^{2+} and Cu^{+} have been shown to bind *in vivo*. However, under *in vitro*

conditions Zn^{2+}, lead, mercury and silver ions have been shown to bind to phytochelatins (Wagner, 1984; Steffens, 1990). Using synthetic phytochelatins it was shown that longer peptides have a higher affinity and capacity for Cd^{2+} (Hayashi *et al.*, 1988). Reese and Winge (1988) reported that phytochelatin-Cd^{2+} complexes were isolated as a cluster composed of several phytochelatin molecules. Two distinct forms were present, one of which contained acid-labile sulphide. Acid-labile sulphide was reported to increase the affinity, and capacity, of these complexes for Cd^{2+}. It is postulated that the CdS-polypeptide complexes consist of a CdS crystallite core, surrounded by polypeptide molecules (Reese and Winge, 1988).

1.1.3 Synthesis of phytochelatins.

Phytochelatins are secondary metabolites and not post-translationally modified proteins (Robinson *et al.*, 1988). Phytochelatin synthesis in response to elevated levels of metal ions is accompanied by a depletion in the cellular pools of glutathione (Scheller *et al.*, 1987). Mutants of *Schizosaccharomyces pombe* deficient in glutathione synthesis were also unable to synthesise phytochelatin (Mutoh and Hayashi, 1988), indicating that glutathione is a precursor in phytochelatin synthesis. Furthermore, the migration of ^{35}S-cysteine from glutathione to elongated species of phytochelatin has been demonstrated by pulse-chase experiments (Robinson *et al.*, 1988). Progress has been made towards isolating and characterising the putative phytochelatin synthetase (Grill *et al.*, 1989)

1.1.4 Function of class I and class II MTs.

The capacity to bind to, and be induced by, different metal ions supports a role for MTs in metal metabolism. The ability of MTs to act as a Cd^{2+} detoxifying agent and to provide Cd^{2+}-tolerance in cultured mammalian cells has been demonstrated. Cell lines that fail to produce MT due to gene hypermethylation are sensitive to Cd^{2+}-

poisoning (Compere and Palmiter, 1981). Furthermore, cell lines selected for Cd^{2+}-resistance over-produce MT due, at least in part, to gene amplification (Crawford *et al.*, 1985), and cell lines containing high copy number MT recombinants are highly resistant to Cd^{2+} (Durnam and Palmiter, 1984). Similarly, Mehra *et al.* (1990) selected *Candida glabrata* for increased resistance to copper, and demonstrated a stable chromosomal amplification of the MT-II gene of around 30 copies. Studies using cell lines with amplified copies of MT genes have shown that MT can protect against poisoning by mercury, copper, and bismuth ions but less efficiently than for Cd^{2+} (Crawford *et al.*, 1985).

Deletion of the yeast copper-MT (*CUP1*) from *Saccharomyces cerevisiae* results in copper-hypersensitive cells (Hamer *et al.*, 1985). Transfection of *CUP1*-deleted cells with plasmids containing either the *CUP1* gene or monkey MT-I gene, gives rise to protection of the cells from copper toxicity (Theile *et al.*, 1986). Similarly, amplification of the *CUP1* gene leads to increased copper-resistance in *Saccharomyces cerevisiae* (Karin *et al.*, 1984).

Functions other than detoxification of excess metal ions have been proposed for MTs (Karin, 1985), and include the homoeostasis of copper and Zn^{2+} ions. In animals this encompasses roles in the regulation of such processes as hepatic storage, intestinal absorption and renal excretion of Zn^{2+} and copper ions. Organs such as the liver, kidney, intestine and pancreas, which play important roles in general nutrient homoeostasis, actively synthesise and accumulate Zn^{2+}- and copper-thioneins (cited in Richards, 1989). The levels of MT and the amount of bound copper and Zn^{2+} ions change dramatically in foetal and neonatal liver during the course of development. It has been proposed that MTs may function as a temporary storage site for copper and Zn^{2+} ions, required for later growth and development (Webb, 1987). The E_c protein (class I MT) is abundant in wheat-germ, the amount of this protein drops rapidly following germination. It is thought that the E_c protein may play a role in the deposition of Zn^{2+}, since Zn^{2+} requirements alter during growth and development (Lane *et al.*, 1987; Kawashima *et al.*, 1992).

A variety of stresses induce the synthesis of hepatic MT suggesting a homoeostatic role. Increase in the plasma levels of the glucocorticoid hormones and inducers of the acute phase response are known to induce the synthesis of MTs (cited in Karin, 1985). Additionally, a shift in the sub-cellular localisation of MT production in cultured hepatocytes from cytoplasm to nucleus during early S-phase has been demonstrated (Tsujikawa *et al.*, 1991). Furthermore, it has been speculated that MTs may control the activity of copper and Zn^{2+} requiring enzymes and therefore regulate many cellular processes. Zeng *et al.* (1991a) speculate that modulation of intracellular thionein (apo-MT) concentration is used for the coordinated regulation of a large subset of genes whose transcription depends on Zn^{2+} requiring transcription factors. Exchange of Zn^{2+} between apo-MT and the Zn^{2+} finger transcription factors SpI (Zeng *et al.*, 1991a) and TFIIIA (Zeng *et al.*, 1991b) has been demonstrated. MTs have also been suggested to play a role in the UV response, perhaps serving as free radical scavengers or as sources of Zn^{2+} for DNA repair enzymes which are activated after irradiation (cited in Karin, 1985). Furthermore it has recently been suggested that MT can protect DNA from oxidative damage (Chubatsu and Meneghini, 1993).

1.1.5 Function of phytochelatins.

Phytochelatins are synthesised in response to various metals (discussed in section 1.1.2) and their associations with some of the inducing metal ions suggest that these polypeptides play a role in metal ion detoxification. Cell lines of *Datura innoxia* selected for Cd^{2+}-resistance showed a direct correlation between the level of Cd^{2+}-resistance and the accumulation of Cd^{2+}-phytochelatin (Jackson *et al.*, 1987). Buthionine sulphoximine (BSO) exposure was shown to cause an inhibition of phytochelatin synthesis (Reese and Wagner, 1987). A number of species of plant showed a positive correlation between Cd^{2+} exposure and exposure to BSO in the inhibition of growth. This has been attributed directly to a decrease in the level of phytochelatin (Steffens *et al.*, 1986). It has also been speculated that phytochelatins

may play a role in metal ion homoeostasis in higher plants (Robinson and Jackson, 1986).

Phytochelatins may have a role in sulphur metabolism. Steffens *et al.* (1986) proposed that these molecules act as sulphate acceptors for adenosine 5' phosphosulphate sulphotransferase during sulphate reduction. This sulphur carrier molecule is known to be structurally similar to glutathione (Shiff, 1980). Reports that acid labile sulphur is often associated with phytochelatins support this hypothesis. The structural similarity between phytochelatin and glutathione suggests that these two molecules may be functional analogues and a putative role for glutathione itself in Cd^{2+}-tolerance should not be ignored. Furthermore, it has been proposed that phytochelatin may be a co-factor in reductive and oxidative reactions including the detoxification of free radicals (Robinson, 1989).

1.1.6 Mammalian MT gene organisation and expression.

Mammalian MT genes are encoded by three exons which are interrupted by two large introns at conserved positions (Palmiter, 1987). The coding regions are strongly homologous, whilst the non-coding regions are more divergent. However, short homologous regions, the consensus regulatory and enhancer elements, have been identified in the 5' and 3' flanking regions. Exon 1 includes the 5' untranslated region and encodes amino acids 1-8, exon 2 encodes amino acids 9-31 and exon 3 encodes amino acids 32-62 and the 3' untranslated region. The intron lengths range from 250-590 b.p. for intron 1 and 140-350 b.p. for intron 2 (cited in Hamer, 1986).

Transcription of mammalian MT genes is controlled by the activator and enhancer sequences in response to various environmental stimuli; exposure to elevated concentrations of silver, gold, bismuth, Cd^{2+}, cobalt, copper, mercury, nickel or Zn^{2+} ions, phorbol esters, iodoacetate, glucocorticoid hormones, inflammatory agents, cytokines, interferon, many toxic agents and inducers of the acute phase response (Palmiter, 1987; Kägi, 1991). Various studies have demonstrated that mammalian MT

genes are regulated at the transcriptional level (cited in Hamer, 1986), although small changes in mRNA stability have also been detected (Mayo and Palmiter, 1981). Furthermore, in chick embryo hepatocytes and fibroblasts, avian MT genes have been shown to be regulated, in part, by changes in mRNA stability (De *et al.*, 1991; McCormick *et al.*, 1991).

MT gene regulatory sequences have been identified. The mouse MT-I and human MT-II_A genes are regulated by similar control sequences, two *cis*-acting metal control sequences have been identified. A proximal element lies between bases -15 to -84, and a distal element between -84 to -151 (Carter *et al.*, 1984). These regions contain a core metal regulatory element (5'TGCXCXCG3'), present in multiple copies in both orientations in mammalian MT genes (Foster and Gedamu, 1991). Furthermore, six metal responsive elements (MREa-MREf), and elements involved in the determination of the basal level of expression (BLRs), are present in the promoter of mouse MT-I.

The MREs have been found to confer metal inducibility on heterologous promoters, with the degree of induction increasing with the number of elements present (Searle *et al.*, 1985). There are at least eight different sequence elements in addition to the TATA box that are involved in regulation of the human MT-II_A (Karin *et al.*, 1987). It has been postulated that there is an interaction between the binding proteins associated with the MREs and the binding proteins associated with other promoter elements. These act together to determine the efficiency of transcription initiation (Karin *et al.*, 1987).

The search for *trans*-acting factors has proven less straightforward than the identification of the DNA sequences to which they bind. Initial studies on the isolation of *trans*-acting factors have demonstrated Zn^{2+}-dependent binding of a liver nuclear protein to DNA sequences including the core element (Searle, 1990; Seguin, 1991). A transcription factor MBF-I (metal responsive element binding factor I) was isolated from mice by Imbert *et al.* (1989). MBF-I reacts with the MREs of eukaryotic MT genes. Point mutations in the MRE core element were created, which indicated a correlation between *in vitro* DNA binding and *in vivo* MT gene regulation. MBF-I

was shown to induce MT gene transcription *in vitro* in a mouse extract. This stimulation was shown to require Zn^{2+}, suggesting a direct role for MBF-I in MT gene transcription.

In vitro footprinting analysis using MREa-MREe of the mouse MT-1, MRE4 of human MT-II_A gene and the MREa of rainbow trout MT-B gene, showed that all of these MREs compete for the mouse nuclear protein, designated MEP-1 (metal element binding protein). Protein blotting (South Western) experiments revealed that MEP-1, which specifically binds with high affinity to mouse MREd, binds with different affinities to the other mouse MREs, mimicking their transcriptional strength in vivo: MREd>MREa=MREc>MREb>MREe>MREf (Labbe *et al.*, 1991).

Searle (1990) demonstrated the binding of a rat liver nuclear protein to the mouse MT-I MREa using fragment mobility shift assays. The binding was Zn^{2+} specific and the protein was termed ZAP (Zn^{2+}-activated protein). It was postulated that this protein is responsible for the Zn^{2+}-responsiveness of the MT genes in the liver. Additionally, a similar protein was isolated from HeLa cell nuclear extracts, which binds to the MREd of mouse MT-I in a Zn^{2+} dependent manner, and was termed MTF-1 (Westin and Schaffner, 1988). It was hypothesised that ZAP may be the rat equivalent of MTF-1 (Searle, 1990). The isolation and characterisation of genes encoding the *trans*-acting metallo-responsive factors of mammalian MT genes have not yet been reported.

1.1.7 Yeast MT gene organisation and expression.

Initial investigations of *Saccharomyces cerevisiae* copper-MT (*CUP1*) transcription demonstrated that in contrast to the wide array of metals which induce mammalian MT gene transcription, copper was the only metal capable of inducing *CUP1* transcription (Karin *et al.*, 1984; Butt *et al.*, 1984). Subsequent studies demonstrated that silver is also an effective, although less potent, inducer of *CUP1* transcription (cited in Thiele, 1992). Regulation of *CUP1* has been extensively characterised (Thiele and Hamer,

1986) and there are no sequences homologous to mammalian MREs in the region upstream of the *CUP1* gene. However, a set of six repeated sequences similar to the mammalian MREs with respect to position, number, orientation and the degree of internal homology have been identified. The consensus sequence derived for the yeast copper-MT upstream activator sequences (UAS) is (5'GAXTTTTTTGCTG3'). These sequences are found in both orientations upstream of *CUP1*, located between -105 and -230 with respect to the *CUP1* transcript start site. Reporter gene fusions have shown these elements to activate transcription in response to copper exposure (Thiele and Hamer, 1986). A detailed examination of the *CUP1* promoter was performed via deletion analysis using *CUP1/galK* fusions. It was suggested that UAS 2, 3, 4 and 5 were critical to copper induction. It was also reported that certain mutations downstream of UAS 5 resulted in increased basal expression (Etcheverry *et al.*, 1986).

Yeast nuclear proteins have been identified which bind to a promoter fragment containing UAS 2-5. Electrophoretic analysis showed that there were two discrete protein-DNA complexes. These proteins were used in DNA protection assays and found to protect UAS 2, 3 and part of 4 in a copper-dependent fashion (Furst *et al.*, 1988; Huibregtse *et al.*, 1989). Two research groups have isolated a regulatory gene responsible for the induction of *CUP1*, *CUP2* (Welch *et al.*, 1989) and *ACE1* (activation of *CUPI* expression) (Szczypka and Thiele, 1989). The nucleotide sequences of the two genes were found to be identical (cited in Buchman *et al.*, 1989).

The N-terminal 122 amino acids of the *trans*-acting activating factor (CUP2/ACE1) are responsible for specific DNA-binding (Furst *et al.*, 1988; Szczypka and Thiele, 1989). This region bears sequence similarity to CUP1, including the presence of 12 cysteinyl residues of which 10 are arranged in Cys-Xaa-Cys or Cys-Xaa-Xaa-Cys pairs and with limited numbers of hydrophobic residues. These cysteinyl residues facilitate binding of 6-7 Cu^{+} ions per ACE1/CUP2 in two polynuclear structures (Dameron *et al.*, 1991). The binding of copper to the protein allows interaction between the protein and the *cis*-elements of the DNA, and the initiation of *CUP1* transcription.

The yeast *Candida glabrata* has a more complex copper-resistance system than *Saccharomyces cerevisiae*, it comprises of a family of MT genes: a unique MT-I gene, a tandemly amplified MT-II_a gene and a single copy MT-II_b gene (Mehra *et al.*, 1990). The transcription of these genes is in agreement with *CUP1*, in that they are activated by copper and silver ions (cited in Thiele, 1992). The regulatory gene product AMT1 (Activator of MT Transcription 1) responsible for the induction of these genes in *Candida glabrata* is a 265 amino acid peptide with structural similarity to the CUP2/ACE1 protein. The sequence similarity, response to metal ions and *in vitro* DNA binding suggests CUP2/ACE1 and AMT1 bind copper and DNA by very similar mechanisms (Thiele, 1992). *Candida glabrata* also was found to synthesise phytochelatins in response to elevated concentrations of Cd^{2+} (Mehra *et al.*, 1988).

1.2 Metal resistance in prokaryotes.

Bacteria have evolved to survive in a wide variety of ecological niches. Some of these environments contain highly elevated levels of metals due to natural geochemical processes or anthropogenic factors. As a result bacteria have developed sophisticated mechanisms for surviving in the presence of elevated concentrations of metals. Silver *et al.* (1989a) categorised the basic mechanisms involved in conferring resistance to metals:

i) Reduction of the metal ions from a more toxic to less toxic form by enzymes including reductases.

ii) Dealkylation where alkylating enzymes and dealkylating lyases add or remove covalently attached components of organo-metal compounds.

iii) Sequestration and binding of the toxic metal ions either in the cell wall or intracellularly.

iv) Blocking of the cellular uptake pathway.

v) The employment of a highly specific efflux system such that the toxic metal entering the cell is rapidly pumped out (all bacterial cation efflux systems characterised to date are plasmid-encoded and metal inducible).

Some of the resistance systems that have been extensively characterised are described.

1.2.1 Arsenic.

1.2.1.1 Arsenic toxicity.

The toxicity of arsenic-containing compounds is related to the oxidation state of the arsenic atom. AsO_2^- is most toxic, whilst AsO_4^{3-} is the least toxic of the inorganic forms of arsenic. Two predominant mechanisms of arsenic toxicity have been proposed, firstly competition of AsO_4^{3-} with phosphate in phosphorylation reactions within the cell, giving rise to unstable arsenylated derivatives, and secondly reaction of AsO_2^- with the sulphydryl groups in proteins (Knowles and Benson, 1983).

AsO_4^{3-} enters the bacterial cell via chromosomally determined phosphate transport systems. There are two phosphate carrier systems, the *pst* system that is specific for phosphate, and the *pit* system which is a less specific phosphate carrier and is the main site of entry for AsO_4^{3-} (Silver *et al.*, 1981).

1.2.1.2 The arsenic resistance determinant.

The plasmid-borne resistance systems of *Escherichia coli* and *Staphylococcus aureus* are the most extensively studied of all the arsenic resistance systems. The *Escherichia coli* plasmid R773 contains the arsenical resistance (*ars*) operon that confers resistance to AsO_4^{3-}, AsO_2^- and SbO^+. In this system, arsenic uptake via the phosphate carriers is unaltered by the presence of resistance plasmids (Silver *et al.*, 1981). The resistance is proposed to be due to the rapid efflux of AsO_4^{3-} via an

energy-dependent pump, which has characteristics indicative of an ATPase (Silver and Keach, 1982).

The three genes (*arsA*, *arsB* and *arsC*) encoding the arsenic resistance efflux ATPase have been cloned and sequenced (Chen *et al.*, 1986). These genes are transcribed as a single polycistronic mRNA of 4.4 k.b. in the presence of inducer. An additional regulatory gene *arsR* located upstream of the structural genes has been identified by San Francisco *et al.*, (1989). The ArsR protein does not contain a helix-turn-helix structure as do many DNA-binding regulatory proteins (San Francisco *et al.*, 1989, San Francisco., *et al.*, 1990). *arsR-blaM* gene fusions indicate that the ArsR protein is a *trans*-acting regulatory protein (Wu and Rosen, 1991). It has been proposed that expression of *arsR* is autoregulated, expression being repressed by ArsR in the absence of arsenical inducers and induced in their presence. The ArsR protein is therefore a negatively acting regulatory protein (Wu and Rosen, 1991). Furthermore, ArsR has been shown to bind as a dimer to a DNA region upstream of and adjacent to the -35 element of the ars promoter, which includes a dyad sequence (Wu and Rosen, 1993). Wu and Rosen (1993) have also shown that AsO_4^{3-} does not give rise to derepression of the *ars* operon, it must first be reduced to AsO_2^- *in vivo* in order to do so.

Differential mRNA stability controls the relative gene expression within the *ars* operon. The polycistronic *ars* transcript is processed to generate two relatively stable mRNA species: one of 2.7 k.b., encoding the ArsR and ArsA proteins, and a second of 0.5 k.b., encoding the ArsC protein. The *arsB* region of the polycistronic transcript decays at a faster rate than the *arsR*, *arsA* and *arsC* regions. This is thought to occur because the two stable mRNA species have the potential to form secondary hairpin loop structures at the end of the transcripts, which act as decay inhibitors (Owolabi and Rosen, 1990).

The *arsA* gene encodes a AsO_2^- and SbO^+ stimulated ATPase (Rosen *et al.*, 1988). The *arsB* gene product is a membrane protein, anchoring the otherwise soluble ArsA protein to the cell membrane (San Francisco *et al.*, 1990). These two proteins are sufficient to confer resistance to AsO_2^- and SbO^+, but the *arsC* gene is needed to

confer resistance to AsO_4^{3-} (Chen *et al.*, 1986). The role of *arsC* has recently been characterised as encoding enzymatic activity converting AsO_4^{3-} to AsO_2^- (Ji and Silver, 1992b).

The AsO_2^-, AsO_4^{3-} and SbO^+ resistance systems of *Staphylococcus aureus* and *Staphylococcus xylosus* are similar in function to those of *Escherichia coli.* The encoded ArsR, ArsB and ArsC proteins show some homology to the equivalent genes from *Escherichia coli* at the amino acid level (30%, 58% and 18% respectively), although the two *ars* operons in *Staphylococcus* do not contain a gene homologous to *arsA*. It is suggested that the staphylococcal *ars* operons are dependent on a separate ATPase subunit encoded by a chromosomal gene (cited in Silver and Walderhaug, 1992; Ji and Silver, 1992b; Rosenstein *et al.*, 1992).

1.2.2 Cadmium.

1.2.2.1 Cadmium toxicity.

Cd^{2+} enters bacterial cells on micro-nutrient transporters (Tynecka *et al.*, 1981); for example Cd^{2+} accumulation in *Staphylococcus aureus* occurs via the manganese transport system (Weiss *et al.*, 1978). The mechanism by which Cd^{2+} is toxic to bacterial cells is unclear. However, it has been shown that Cd^{2+} interacts with the thiol groups, and competes with Zn^{2+} for sulphydryl or imidazole groups of proteins (Vallee and Ulmer, 1972). Therefore it is likely that the production of "Cd^{2+}-damaged" proteins is a central feature of Cd^{2+}-toxicity. Moreover, in a eukaryote (*Saccharomyces cerevisiae*) it has recently been demonstrated that the systems for "disposing" of such Cd^{2+}-damaged proteins are critical for survival following exposure to this metal (Jungmann *et al.*, 1993). Additionally, Cd^{2+} has been shown to uncouple oxidative phosphorylation and to interact with phospholipids in membranes. Furthermore, Cd^{2+} has been shown to give rise to single strand breakage of DNA in *Escherichia coli* causing a drop in cell viability of 95% (Mitra and Bernstein, 1978).

1.2.2.2 Cadmium resistance determinants.

Several mechanisms for bacterial Cd^{2+}-resistance have been reported, although only three of these have been cloned and sequenced: the *cadA* and *cadB* operons of staphylococcal plasmids (Smith and Novick, 1972) and *czc* (Cd^{2+}, Zn^{2+} and Co^{2+}) resistance system of *Alcaligenes eutrophus* (Nies and Silver, 1989).

1.2.2.3 The *cadA* and *cadB* resistance systems.

The *cadA* operon confers a high-level resistance (100-fold increase) whereas the *cadB* operon confers low-level resistance (10-fold increase) to Cd^{2+} and Zn^{2+} (Perry and Silver, 1982). Some plasmids contain only the *cadA* determinant (pI258), others contain the second resistance determinant *cadB* (pII147) (Shalita *et al.*, 1980).

1.2.2.4 The cadmium resistance *cadA* operon.

The *cadA* operon contains two open reading frames (Nucifora *et al.*, 1989a). The first open reading frame *cadC*, encodes a soluble protein of 122 amino acids in length. The function of *cadC* is unclear, the predicted amino acid sequence shows similarity to the ArsR proteins (the regulators of the As^{5+}, As^{3+} and Sb^{3+} resistance systems of *Escherichia coli, Staphylococcus aureus* and *Staphylococcus xylosus,* described in section 1.2.1.2). CadC is not thought to be involved in regulation of *cadA* (Yoon *et al.*, 1991), but is needed for high-level Cd^{2+} and Zn^{2+} resistance (Yoon and Silver, 1991; Silver and Walderhaug, 1992). CadC contains three possible cation-binding motifs leading to the suggestion that it functions to sequester intracellular Cd^{2+} and delivers it to CadA, the efflux pump (Nies, 1992a). A *cadC* homologue has recently been isolated from *Bacillus firmus*. This protein has been found to confer Na^{+}

resistance when over expressed in *Escherichia coli,* possibly by binding intracellular Na^+ and delivering it to the residual Na^+/H^+ antiporter (Mack Ivey *et al.*, 1992).

cadC overlaps the second gene *cadA* for eight nucleotides, the two genes are transcribed together as a polycistronic message (Nucifora *et al.*, 1989a). *cadA* encodes an energy dependent Cd^{2+}-efflux ATPase (Silver *et al.*, 1989b). CadA is 727 amino acids in length, the sequence of CadA includes several domains with similarity to known functional motifs and key conserved residues including: a Cd^{2+} recognition region, three transmembrane hairpin structures each consisting of two closely spaced α-helical hydrophobic regions, a region proposed to be a ATPase subunit and a putative ATP-binding site (Silver and Walderhaug, 1992). Initial reports indicated that *cadA* was expressed constitutively (Perry and Silver, 1982), although more recent studies using β-lactamase fusions revealed that the *cadA* system is inducible by a range of divalent cations at micromolar concentrations (Yoon *et al.*, 1991). The transcript start of *cadA* lies in the middle of an inverted repeat sequence that is a candidate binding site for the hypothetical CadR protein (Yoon *et al.*, 1991).

1.2.2.5 The Cadmium resistance *cadB* operon.

The *cadB* system is less well characterised than the *cadA* system, it is thought to encode an inducible Cd^{2+}-binding protein (Perry and Silver, 1982). The *cadB* operon contains two open reading frames *cadB* and *cadX* (Silver and Walderhaug, 1992). CadX shows homology to CadC, although a function for these proteins has yet to be assigned.

1.2.2.6 The cadmium, zinc and cobalt *czc* resistance determinants.

The *Alcaligenes eutrophus* strain CH34 was isolated from a Zn^{2+} decantation tank (Mergeay *et al.*, 1978) and contains at least seven metal resistance determinants. These are either located on the bacterial chromosome or on the two indigenous

plasmids pMOL28 and pMOL30 (Mergeay *et al.*, 1985; Nies *et al.*, 1987; Neis *et al* ., 1989). Co^{2+}, Zn^{2+} and Cd^{2+} are transported into *Alcaligenes eutrophus* by relatively non-specific divalent cation transporters (Nies and Silver, 1989). These ions are pumped out of the cell by the CzcABC protein complex, an energy dependent efflux system.

The *czc* operon is made up of five genes: *czcA*, *czcB*, *czcC*, *czcD* and *czcR*. Deletion analysis of the three structural genes was used to produce a working model in which CzcA is the central membrane protein and Zn^{2+} is the central cation. CzcB and CzcC are ancillary proteins, adding to the range of cation specificities of the efflux system. Cd^{2+} resistance was most readily lost followed by Co^{2+} then Zn^{2+}; (reviewed by Silver and Walderhaurg, 1992). *czcR* is a regulatory gene which has been suggested to encode an activator. This gene is transcribed in a divergent orientation to the *czcCBAD* genes. A region of dyad symmetry is located upstream of the *czcC* gene and is thought to be the binding site of a *trans*-acting regulatory protein. The regulation of *czc* by CzcR is metal dependent (shown by reporter gene studies). The CzcR protein contains three potential metal binding sites and is thought to be a DNA-associated protein (Nies, 1992b). The *czcD* and *czcR* genes are thought to form a two component regulatory system, where *czcD* encodes a membrane protein sensor and *czcR* encodes the *trans*-acting regulatory protein (Nies, 1992a; Nies, 1992b; Silver *et al.*, in press).

1.2.3 Copper.

1.2.3.1 Copper toxicity.

Copper is an essential trace metal that is required by a limited number of metallo-proteins. Copper becomes toxic when present at elevated concentrations. A mechanism of resistance cannot therefore merely exclude or detoxify the metal ion, but

must be integrated with the mechanisms of copper homoeostasis in the cell (Brown *et al.*, 1991).

The chemical properties of copper underlie both its role in metallo-proteins and its toxicity. Copper can undergo redox reactions between Cu^{+} and Cu^{2+} under physiological conditions, and thus can act as an electron donor and acceptor in the electron transport chain (cytochrome oxidases) and in redox-active enzymes which use molecular oxygen as a substrate. Copper can also catalyse adverse redox reactions in the cell, giving rise to free radicals which damage membranes and cause oxidation of proteins (Brown *et al.*, 1992). Copper also binds to proteins, nucleic acids, polysaccharides and lipids causing a change in structure and interfering with the function of these macro molecules (Brown *et al.*, 1992).

1.2.3.2 Copper resistance determinants.

Copper resistance in bacteria is widespread and many bacterial strains resistant to high levels of copper have been isolated (cited in Trevors, 1987). Two different plasmid copper resistance determinants have been extensively characterised. The *cop* operon of *Pseudomonas syringae* and the *pco* determinant of *Escherichia coli*.

1.2.3.3 The copper resistance *cop* operon.

The copper resistance determinant of the plant pathogen *Pseudomonas syringae* consists of four open reading frames *copA*, *copB*, *copD* and *copC* (Mellano and Cooksey, 1988a). The four genes are transcribed from one promoter upstream of the first gene *copA*, as a single polycistronic message (Mellano and Cooksey, 1988a; 1988b). The first two genes, *copA* and *copB* are needed for partial copper resistance, whilst *copC* and *copD* are also required for full resistance. The *copB* gene product is predicted to contain five direct repeats of an octapeptide Asp-His-Ser-Gln/Lys-Met-Gln-Gly-Met. Furthermore, a related but less conserved octapeptide sequence appears

five times in the *copA* gene product. These motifs have been suggested to be involved in copper binding. The *copA* gene product binds 11 copper ions per polypeptide and shows similarity to the copper containing proteins azurin and plastocyanin (Mellano and Cooksey, 1988a; Cooksey, 1993; Silver *et al.*, in press).

The *cop* system is induced solely by copper and confers resistance only to copper (Mellano and Cooksey, 1988b). The regulation of the *cop* system is proposed to be controlled by two genes transcribed separately, that are located upstream of *copD, copR* and *copS.* These genes are thought to encode a two component regulatory system (Silver *et al.*, in press). The regulation is proposed to be of a negative type, involving a chromosomally encoded repressor that binds to the promoter in the absence of copper but is released in the presence of copper (cited in Cooksey, 1993). The copper-inducible release of the repressor requires *copR* and *copS* (cited in Cooksey, 1993). The CopA and CopC proteins are found in the periplasmic space, and CopB is located in the outer membrane (cited in Brown *et al.*, 1992). The CopD polypeptide is a inner membrane protein which has been speculated to have a role in copper transport (Silver *et al.*, in press). Copper accumulation by induced resistant cells was 100 times that of uninduced unexposed cells. Copper accumulation in the periplasm plus cytoplasm increased 2.4 times in low-copper exposed resistant cells, compared to that in similarly exposed sensitive cells (Cha and Cooksey, 1991). It is proposed that copper resistance is due to periplasmic binding and extracellular sequestration of the copper cations (Trevors and Cotter, 1990; Brown *et al.*, 1992; Silver and Walderhaug, 1992). A second plasmid encoded copper resistance determinant has been isolated from a plant pathogen, *Xanthomonas campestris.* This has genes similar in number and characteristics to those in *Pseudomonas syringae* (Bender *et al* ., 1990; cited in Silver and Walderhaugh, 1992; Brown *et al.*, 1992).

1.2.3.4 The copper resistance *pco* operon.

A different plasmid (pRJ1004) encoded copper resistance system appeared in *Escherichia coli* isolated from piggeries, in which animals were fed diets containing $CuSO_4$ as a probiotic growth stimulant (Tetaz and Luke, 1983). This system has recently been shown to be related to the *cop* system via DNA sequencing analysis (Silver *et al.*, in press). Insertional mutagenesis of pRJ1004 produced two classes of mutants. The first class of mutant showed only a slight reduction in copper resistance. This was found to be mutated in the resistance determinant *cdr*, the functions of which are associated with repair of copper induced damage to DNA. The second class of mutant showed a marked decrease in resistance to copper and was associated with a mutation in the resistance determinant *pco* (Rouch *et al.*, 1989).

The *pco* determinant was thought to be composed of at least four genes *pcoA*, *pcoB*, *pcoC* and *pcoR*. All four genes are required for expression of copper resistance, although recent DNA sequence analysis of the *pco* system suggests that there are seven genes *pcoA-E, pcoR* and *pcoS* (mapping in the order *pcoABCDRSE*) (Silver *et al.*, in press; Cooksey, 1993). The *pcoR* gene encodes a *trans*-acting repressor (Rouch *et al.*, 1989). The *pcoS* gene product is thought to be a membrane protein with homology to the sensors of two component regulatory systems (Silver *et al.*, in press). The mechanism of resistance conferred by the *pco* system has not been fully characterised. The resistance mechanism involves an increase in the rate of copper efflux from the cell in an energy dependent manner. The copper exported from the cell is modified but the nature of modification is unknown. However, it has been suggested that the modification renders this copper unavailable to the copper uptake systems. A model for *pco* has been described in which PcoC acts as a cytoplasmic copper storage/transport protein and PcoA and PcoB proteins are responsible for excess copper modification and transport (Rouch *et al.*, 1989).

1.2.3.5 The copper uptake *cut* system.

The copper uptake system of *Escherichia coli* has also been characterised and has been shown to interact with the resistance system. A number of mutants were studied which respond differently to the wild type *Escherichia coli* in response to external copper concentrations. These mutants mapped to seven complementation groups *cutA-cutF*. The *cutA* and *cutB* genes encode copper influx proteins, CutB is specific for copper whereas CutA also participates in Zn^{2+} uptake. CutE and CutF are two copper storage/transport proteins preventing intracellular damage by copper ions, whereas CutC and CutD are copper export proteins. The genes of the *cut* system are present on the chromosome unlike the *pco* genes which are plasmid encoded (reviewed by Brown *et al.*, 1992).

1.2.3.6 Interaction between the *pco* copper resistance system and the *cut* copper uptake system.

The expression of the *cut* genes is regulated in order to maintain copper homoeostasis. This involves interaction between the chromosomal and plasmid-determined copper resistance genes. At high copper concentrations the *pco* gene products are proposed to modify the action of the proteins for normal copper metabolism. This gives rise to an increase in the export of copper in an energy dependent manner, and modifies the copper such that it is no longer biologically available. The *pco* system is still regulated even if the *pcoR* gene is disrupted (Rouch *et al.*, 1989). Due to this observation it has been speculated that there is a chromosomal regulatory locus *cutR* which has a *trans*-acting role in the maintenance of copper homoeostasis under high external copper. This regulation is proposed to involve a two component regulatory system (Silver *et al.*, in press). It is suggested that copper metabolism and resistance genes in *Escherichia coli* are co-ordinately regulated via chromosomal and plasmid genes. The *pcoR* gene also shows sequence

similarity to a number of prokaryotic regulatory proteins which are part of two-component regulatory systems (cited in Brown *et al.*, 1992). These regulatory proteins respond to signals generated by the sensor proteins. The copper sensor has yet to be identified (Brown *et al.*, 1991; Brown *et al.*, 1992; Silver and Walderhaug, 1992; Silver *et al.*, in press).

1.2.4 Mercury.

1.2.4.1 Mercury toxicity.

Mercury has no known biological function, its toxicity has been suggested to be due to a number of mechanisms. Hg^{2+} ions have high affinities for thiol groups of proteins and interact with lipids, nucleic acids and polysaccharides. They often inactivate the biological molecules to which they are bound, alter membrane permeability and interact with DNA causing a conformational change (cited in Brown *et al.*, 1991).

1.2.4.2 Mercury resistance determinants.

Mercury participates in a biological cycle in which microbes play a significant role in the methylation of mercury to methlymercury and dimethylmercury, and in the reduction of Hg^{2+} to elemental mercury (Trevors, 1986). This reduction of Hg^{2+} forms the basis of the most well known of the bacterial mercury resistance systems (Brown, 1985). Bacteria carrying out this reduction can be divided into two groups on the basis of substrate specificity; narrow and broad spectrum. Narrow spectrum resistance is conferred to Hg^{2+} and a limited number of organomercurials, and is widespread. Broad spectrum resistance is conferred to Hg^{2+} and a larger number of organomercurials. Broad spectrum resistance is plasmid encoded and is less prevalent than narrow spectrum resistance (Brown, 1985).

Eight bacterial mercury resistance operons have been cloned and sequenced. These operons consist of a number of genes, whose products carry out a carefully regulated process of Hg^{2+} uptake by the cell, delivery of the toxic Hg^{2+} ions to the intracellular enzyme mercuric reductase and the subsequent volatilisation of the product, gaseous Hg^0. The mercury reduction resistance systems of *Pseudomonas* transposon Tn*501* and the plasmid R100 (originally isolated from *Shigella flexneri*) have been extensively characterised. These operons are very similar at the protein and DNA level.

The mercury resistance operon of Tn*501* consists of 5 genes, *merRTPAD* (R100 possesses an additional *merC* gene between *merP* and *merA*). *merT* and *merP* encode transport proteins, *merA* encodes the mercuric reductase, whilst *merD* and *merR* encode regulatory proteins. The deletion of *merT* and *merP* genes leads to an almost complete loss of the resistance phenotype, even in the presence of mercuric reductase. However, expression of the *merT* and *merP* genes in the absence of mercuric reductase produces a Hg^{2+} super-sensitive phenotype. Initial induction from the *mer* promoter is independent of the presence of the *merT* and *merP* transport genes, as seen by reporter gene fusion assays (Lund and Brown, 1987).

The predicted protein structure of MerT is a hydrophobic polypeptide containing three α-helices capable of spanning the cytoplasmic membrane (Brown, 1985; Silver and Misra, 1988). The *merT* product lies in the inner membrane (Dr. A.P. Morby personal communication). Futhermore, two pairs of cysteine residues present on each side of the membrane were speculated to participate in the transport of Hg^{2+} (Brown, 1985). It has since been established that Cys24 and Cys25 are necessary for Hg^{2+} resistance (cited in Silver and Walderhaug, 1992). MerC is membrane associated, shows strong homology to MerT and has been postulated to play a role in Hg^{2+} transport (Summers, 1986; Brown *et al.*, 1991).

The product of *merP* is not essential for transport and resistance. However, deletion of *merP* results in a partial loss of resistance and it is suggested that MerP is a periplasmic protein that increases resistance by acting as an external scavenger of Hg^{2+} (Brown *et al.*, 1991). MerP has two cysteine residues, Cys36 and Cys33. The

alteration of Cys36 to serine causes no change in transport or resistance; however a Cys33 substitution to serine decreases both transport and resistance (cited in Brown *et al.*, 1991). Other mutations in the structure of the MerT and MerP proteins suggest that specific binding between MerT and MerP is involved in Hg^{2+} uptake (cited in Brown *et al.*, 1991).

A model has been proposed for *mer* function by Silver and Misra (1988), in which Hg^{2+} ions are bound by MerP outside the cell, transported across the cell membrane by MerT and directly transferred to the mercuric reductase enzyme encoded by *merA*. Furthermore, it is speculated that the transfer of a Hg^{2+} ion is mediated by cysteine pairs in the proteins via a redox-exchange where a pair of protons are transported in the opposite direction to the Hg^{2+} ion (Brown, 1985). MerA is a cystolic, NADPH-dependent, flavin adenine dinucleotide-containing disulphide oxidoreductase. Electrons are transferred from NADPH to enzyme-bound FAD and subsequently to the Hg^{2+} ion, which is reduced to volatile Hg^{0} and diffuses out of the cell (Brown, 1985; Summers, 1992).

The *mer* operon is transcribed from two overlapping divergent promoters. The *merR* regulatory gene is transcribed in a divergent orientation to the *merTPAD* structural genes. The transcription of *merTPAD* is repressed in the absence of Hg^{2+} and activated in the presence of Hg^{2+}, by the regulatory protein, MerR. Furthermore, MerR represses its own expression regardless of the presence of Hg^{2+}. MerR has a dual role as the metal receptor and transcriptional effector (Foster and Brown, 1985; Lund *et al.*, 1986). Mutation of *merR* results in operons that exhibit a depressed level of synthesis of the detoxification gene products and operons that cannot become fully induced (Ni'Bhriain *et al.*, 1983). The behaviour of MerR mutants revealed distinct domains for its binding to DNA and for its interaction with the Hg^{2+} inducer. A predicted helix-turn-helix domain (residues 9-30) is associated with DNA binding (cited in Summers, 1992).

MerR binds as a dimer to a DNA region located between the two divergently orientated transcription units in the operator/promoter region of the *mer* operon

(O'Halloran and Walsh, 1987). The MerR DNA binding region is a 7-4-7 b.p. perfect dyad symmetrical repeat (O'Halloran *et al.*, 1989). The MerR dimer binds only one Hg^{2+} and binds to the dyad in the presence and absence of Hg^{2+} (Shewchuk *et al.*, 1989).

DNA footprinting experiments suggest that when MerR is bound to the operator/promoter, σ-70 RNA polymerase is also bound to the -35 region irrespective of the presence of Hg^{2+}, although MerR prevents the formation of an open complex by RNA polymerase in the absence of Hg^{2+} (cited in Park *et al.*, 1992; Summers, 1992). The operator/promoter region contains an unusually long 19 b.p. spacing between the -35 and -10 transcript initiation sites (Lund and Brown, 1989; Parkhill and Brown, 1990). This long spacing prevents the formation of the open complex by RNA polymerase since the -35 and -10 transcript initiation sites are offset by approximately 70°. The binding of Hg^{2+} to MerR on the operator/promoter causes a conformational change in MerR, which distorts the DNA, changing the spacing between the -35 and -10 transcript initiation sites, twisting them to lie on the same face of the DNA. This process allows the formation of the open complex by RNA polymerase (Ansari *et al.*, 1992; reviewed by Summers, 1992). Furthermore, specific MerR mutants have been created that allow the protein to stimulate transcription in the absence of Hg^{2+}, and there is a direct correlation between the degree of activation caused by the mutants and DNA distortion (Parkhill *et al.*, 1993).

The *merD* gene product has a similar structure to that of MerR, particularly in its amino-terminal helix-turn-helix region (Brown *et al.*, 1986). This leads to the speculation that MerD might be a coregulator, although deletion of *merD* has little or no effect on the expression of mercury resistance. However, *mer-lac* fusions in *trans* to a multicopy operon resulted in lower β-galactosidase activity in comparison to cells containing an equivalent construct with a deletion in *merD* (Nucifora *et al.*, 1989b). Consistent with this hypothesis is the fact that MerD binds to the *mer* promoter-operator, although it has a considerably lower affinity than MerR (cited in Summers,

1992). Hg^{2+} binding by MerD is as yet unstudied, although there are three cysteine residues in the protein which may be involved.

1.3 Metalloregulatory proteins.

Metalloregulatory proteins have been characterised in prokaryotic and eukaryotic systems. Other than the regulatory proteins of the metal resistance determinants described above (section 1.2), only a limited number of other metalloregulatory proteins have been characterised in prokaryotic systems (reviewed by Hennecke, 1990).

The Fur protein controls the expression of many operons of *Escherichia coli* in response to the iron levels in the cell. Fur is an iron responsive repressor protein which was the first metalloregulatory protein to have been isolated (Bagg and Neilands, 1985; Bagg and Neilands, 1987). Fur controls genes involved in specific iron chelation and uptake into the cell. Fur also controls genes not directly involved in iron uptake, but for which the iron concentration in the cell is important for the functioning of their products. In all Fur-regulated promoter regions, Fur-Fe^{2+} binds to a 19 b.p. consensus sequence DNA recognition region 5'GATAATGATAATCATTATC 3' (9-1-9 b.p inverted repeat) designated the "iron box" (Hennecke, 1990).

The *iucA* promoter of the aerobactin operon is the most extensively studied promoter under the regulation of Fur. This promoter contains two contiguous repressor-binding sites which are required for full repression. The primary binding site overlaps the -35 region, were as the secondary site overlaps the -10 region and transcript start site (de Lorenzo *et al.*, 1988). At low iron concentrations Fur has low affinity for the operator DNA and transcription of the aerobactin promoter occurs. At high iron concentrations, Fur binds tightly to the operator DNA and transcription is blocked. The Fur complex is thought to wrap around the DNA in a screw-like fashion (de Lorenzo *et al.*, 1988). Fur is 148 amino acids in length and lacks a consensus

helix-turn-helix domain characteristic of many DNA-binding regulatory proteins. The amino acid sequence reveals the presence of two potential metal-binding motifs rich in histidine and cysteine sequences (Hennecke, 1990).

Metal regulatory proteins also exist in which the metal ion is not the signal, but acts as the sensor for the signal. The positive regulatory proteins of NifA from rhizobia (nitrogen fixation) and Fnr in *Escherichia coli* (anaerobically induced genes) act in this manner. These proteins respond to the oxygen concentration in the environment. They are active in low oxygen tension and inactive in high oxygen tension. NifA binds to the upstream activator sequence (5' TGT-N_{10}-ACA 3') located 100-200 b.p. from the transcript start sites of nitrogen fixation genes via a helix-turn-helix domain (cited in Hennecke, 1990). Fnr also contains a helix-turn-helix domain and functions as an activator and a repressor of genes concerned with anaerobic energy metabolism. The promoter regions of the positively induced genes contain a 22 b.p. consensus sequence which is the Fnr binding site. This region of dyad symmetry, designated the Fnr box (5' AAANTTGAT-N_4-ATCAANTTT 3') is located 39-49 b.p. upstream of the transcript start sites (Bell *et al.*, 1989). These promoters contain -10 regions but no recognisable -35 regions consistent with positively controlled genes (Raibaud and Schwartz, 1984). In the negatively controlled genes the Fnr-binding site overlaps the transcript start sites, which is consistent with them being repressed (Eiglmerier *et al.*, 1989). Both of these proteins contain potential metal-binding domains and are thought to function via the redox state of the bound metal ion sensing the presence of oxygen (Fischer *et al.*, 1988; Spiro *et al.*, 1989).

1.4 Prokaryotic MT.

A number of reports in recent years have alluded to the existence of low-molecular weight metal-binding MT-like ligands in prokaryotes (Higham *et al.* 1985; Khazaeli and Mitra, 1981; MacLean *et al.*, 1972; Mitra *et al.*, 1975; Olafson *et al.*, 1980). A series of 3 related cation-binding polypeptides have been isolated from a Cd^{2+}-

resistant isolate of *Pseudomonas putida* (Higham *et al.* 1985). Metal ion analysis revealed 4-7 Cd^{2+}, Zn^{2+} or Cu^{+} ions per polypeptide. NMR studies and amino acid composition suggested a possible tetrahedral arrangement of bound Cd^{2+} ions and a 10-20% cysteine content. The ability of the strain to produce these polypeptides was lost before they were fully characterised. An earlier report of MT-like Cd^{2+}-binding protein from *Escherichia coli,* (Mitra *et al.*, 1975) was followed by preliminary characterisation of the peptide (Khazaeli and Mitra, 1981). The protein was associated with 60% of the cytoplasmic Cd^{2+} in cells adapted to inhibitory levels of Cd^{2+}. However, a similar protein was not detected in non adapted cells.

1.4.1 Prokaryotic MT from *Synechococcus* species.

MacLean *et al.* (1972) correlated the production of a MT-like protein from *Anacystis nidulans* with Cd^{2+}-resistance. Since this early observation, Olafson has isolated class II MTs from a marine strain of *Synechococcus* (strain RRIMP-NT) (Olafson *et al.*, 1979; Olafson *et al.*, 1980) and from fresh water strains *Synechococcus* UTEX-625 and TX20 (these strains, and very closely related strains, are also referred to as *Anacystis nidulans*, *Synechococcus* PCC 6301, *Synechococcus* PCC 7942 and *Synechococcus* R2; refer to section 2.1.2) (Olafson, 1984; Olafson *et al.*, 1988).

The amino acid compositions of the MTs from the two fresh water strains of *Synechococcus* were similar, although different to the marine strain RRIMP-NT. However, the cysteine content (18%) of the MTs from all three strains was similar, although the histidine content was high for MTs. By far the most marked departure from the amino acid compositions of eukaryotic MTs was the abundance of hydrophobic residues making these the most hydrophobic MTs to be described. The MT from *Synechococcus* TX20 was sequenced (Olafson *et al.*, 1988) and compared with a selection of eukaryotic MTs. Despite the high frequency of cysteine residues

increasing the probability of chance alignments, data base comparisons indicate that there is no significant homology.

Reverse phase HPLC analysis of the protein prepared from Cd^{2+}-exposed *Synechococcus* cells resolved 7 peaks, all of which contained isoforms of MT with ratios of Cd^{2+} : protein ranging from 2.2:1 to 3.1:1. Furthermore, amino acid analysis of the peaks revealed no significant differences in the various MT isoforms. Additionally, spectral data obtained for Zn^{2+}-MT and MT substituted with copper ions, suggested that the prokaryotic protein may have a metal thiolate cluster similar to that of eukaryotic MT, but in a single domain (Olafson *et al.*, 1988). Furthermore, addition of copper ions to the metal-stripped apo-MT resulted in alteration of the CD spectrum to one with characteristics resembling yeast MT.

Synechococcus MT was found to be induced by Cd^{2+} and Zn^{2+} but not copper ions, although this MT was shown to complex with all three metal ions (Olafson *et al.*, 1980). It was suggested that copper resistance in this organism was due to membrane exclusion rather than binding to the cyanobacterial MT (Olafson *et al.*, 1988). This is of particular relevance since an energy dependent copper efflux mechanism has recently been demonstrated in *Nostoc calcicola* (Verma and Singh, 1991), although a similar system has not yet been reported for *Synechococcus* species. Inhibitor studies using chloramphenicol and actinomycin D indicated that, in common with eukaryotic MTs, metal induction is regulated at the transcriptional level (Olafson, 1984; Olafson *et al.*, 1986).

1.5 The aim of this research.

MTs have been isolated from such a wide range of eukaryotes that they are often considered ubiquitous, yet genes encoding equivalent proteins have not previously been isolated from prokaryotes. The aim of this research was to isolate and characterise a prokaryotic MT gene.

Chapter 2

MATERIALS AND METHODS.

2.1 Materials.

2.1.1 *Escherichia coli* strains.

The *Escherichia coli* (K12) strains used in this research were: JM101 [*sup*E, (*lac-pro*AB), {F'*tra*D36, *pro*AB, *lac*IqZ M15}, (rk+, mk+), *mcr*A(+)]; DH5α {F'/*end*AI, *hsd*17, (rk- mk+), *sup*E44, *thi*1, *rec*A1, *gyr*A, (NaIr), *lac*ZYA, *-arg*F, U169, 80d*lac* (*lac*Z M15)}. Both strains were obtained from Northumbria Biologicals Ltd., Cramlington, Nothumbria.

2.1.2 *Synechococcus* strains.

The *Synechococcus* strains used in this study were: *Synechococcus* D33, *Synechococcus* D839 (Durham University Culture Collection) and *Synechococcus* PCC 7942 (PIM8) (R2-PIM8) (Pasteur Culture Collection). Strains D33 and D839 had been obtained from sources other than the Pasteur Culture Collection, although these strains were originally subcultured from similar stocks as the cultures held in the Pasteur Culture Collection. For simplicity, in this thesis, *Synechococcus* D33 (=PCC 6301), *Synechococcus* D839 (=PCC 7942) are referred to by their Pasteur Culture Collection designation.

The taxonomy and origin of these strains is confused. In the literature, different taxonomic names have been applied to the same strain: *Synechococcus* PCC 6301 (*Anacystis nidulans* TX-20, *Anacystis nidulans* UTEX 625, *Anacystis nidulans* UTEX 1550 *Synechococcus leopoliensis* CCAP 1405/1) all originate from the same

isolate of Kratz and Myers (1955) and are therefore identical (Herdman, 1982); *Synechococcus* PCC 7942 (*Anacystis nidulans* R2, *Anacystis nidulans* CALU 895, *Synechococcus leopoliensis* UTCC 100). *Synechococcus* strains PCC 6301 and PCC 7942 have been said to belong to one and the same species (Wilmotte and Stam, 1984). However, PCC 7942 has superior transformation properties, when compared to those of PCC 6301 (Golden *et al.*, 1989), and is thus the organism of choice for genetic manipulations. *Anacystis nidulans* strains adapt quickly to environmental change, so it is likely that there are genetic differences among strains held by different collections (Whitton, 1992).

Synechococcus PCC 7942 (PIM8) (van der Plas *et al.*, 1990) is a variant strain of PCC 7942 that has been cured of the 7.5 k.b. plasmid present in some *Synechococcus* strains. This enables transformation of this strain with vectors containing sequences derived from the 7.5 k.b. plasmid, reducing the risk of homologous recombination.

2.1.3 Plasmids.

The following commercially supplied plasmids were used: pUC19, mp18, mp19 and pGEM4z. pUC19, mp18 and mp19 were obtained from Boehringer Mannheim, Lewes, UK. pGEM4z was obtained from Promega Ltd., Enterprise Rd., Southampton, UK. Full descriptions of these plasmids are given in Sambrook *et al.* (1989), in which original references are cited. pLACPB2 (a *Synechococcus* shuttle vector containing ampicillin and chloramphenicol resistance) was obtained from the University of Warwick (Scanlan *et al.*, 1990).

2.1.4 Chemicals, reagents and laboratory consumables.

Unless otherwise stated, general laboratory chemicals were obtained from Sigma Chemical Co. Poole, Dorset. The suppliers of other chemicals and reagents are as stated below:

Taq polymerase; Perkin-Elmer/Cetus, ILS Ltd., Newbury St., London and Promega Ltd., Enterprise Rd., Southampton, UK.

Calf intestinal alkaline phosphatase, deoxynucleotide triphosphates; Boehringer Mannheim UK, Lewes, Sussex.

Radiochemicals, hybridisation membranes ("Hybond N"); Amersham International Ltd., Bucks., UK.

Sephadex G-50; Pharmacia LKB, Milton Keynes, UK.

Nitrocellulose filter discs BA85 (0.45 μm); Schleicher and Schluell, Dassel, FRG.

3MM chromatography paper, 2.5 cm GF/C filter discs; Whatman Ltd., Maidstone, Kent, UK.

Electrophoresis grade agarose; GIBCO-BRL Ltd., Paisley, Scotland.

Yeast extract, Bacto-Agar; Difco, Detroit, Michigan.

Trypticase peptone; Beckton Dickinson, F-38240, Maylan, France.

Fuji RX X-ray film; Fuji Photo Film Co. Ltd., Japan.

Phenol (redistilled); International Biotechnologies Inc., Newhaven, Connecticut.

Restriction enzymes, DNA modification enzymes, IPTG, Xgal; Northumbria Biologicals Ltd., Cramlington, Co. Northumbria.

Silica fines were a gift from Dr R.G Alexander.

Other commercially supplied consumables and equipment are acknowledged at the first reference to use.

The water used in growth media and for DNA manipulations was double-deionised (MilliQ). Water used for RNA manipulations was further treated for the inhibition of RNAses by addition of diethylpyrocarbonate (1/1000), incubation at 25 °C for 16 h, followed by autoclaving.

2.1.5 Metal salts.

Metals were used as the following salts: $Ag(N0_3)$, $Na(AuCl_4).2H_20$ (obtained from the Johnson Massey Technology Centre, Reading), $CdCl_2.H_20$, $CoCl_2.6H_20$, $CrCl_2$, $CuCl.2H_20$, $HgCl_2$, $NiCl_2.6H_20$, $Pb(N0_3)_2$, $ZnCl_2$ (Analar).

2.2. Media and buffers.

2.2.1 Buffers used in DNA and RNA manipulations.

Restriction enzyme and DNA modification enzyme reaction buffers were supplied with the enzymes. Those buffers not described in individual protocols were as described by Sambrook *et al.*, (1989).

2.2.2 NaI solution.

90.8 g NaI and 1.5 g Na_2SO_3 were added to 100 ml distilled water (final volume is greater than 100 ml). The solution was filter sterilised and a further 0.5 g Na_2SO_3 added (the final solution should be saturated). The resulting solution was stored in the dark at 4 °C.

2.2.3 *Escherichia coli* growth media.

Luria-Beltrami (LB) medium, terrific broth and 2 XL medium were prepared as described in Sambrook *et al.*, (1989).

TSS (transformation and storage solution for preparation of competent cells); LB broth at pH 6.5 containing 10 % w/v PEG 8000 and 50 mM MgSO4, 5 % DMSO v/v was added to this prior to use.

2.2.4 Maintenance of *Synechococcus* cultures.

Synechococcus PCC 6301 and PCC 7942 were cultured in AC medium (Kratz and Myers, 1955) as modified by Shehata and Whitton (1982). *Synechococcus* PCC 7942 (PIM8), a methionine auxotroph, was cultured in Allen's medium (Allen, 1968) supplemented with DL-methionine (30 $\mu g\ ml^{-1}$) and streptomycin (5 $\mu g\ ml^{-1}$). Cultures were maintained either on 1.5 % agar plates or in liquid media under constant light conditions (100 mmol photon $m^{-2}s^{-1}$) at 32 °C. Generally, cultures were maintained in 50 ml flasks without aeration. For experiments requiring larger volumes of cells (500 ml), filter-sterilised air was bubbled through the culture. Strains were also maintained in long-term storage by freezing 1 ml aliquots of culture in liquid nitrogen, both with and without added DMSO (10 $\mu l\ ml^{-1}$ of culture).

2.2.5 Estimation of cell numbers.

Where it was necessary to determine *Synechococcus* cell numbers, two methods were employed. Direct counts were carried out using a haemocytometer, using standard procedures. Where comparative data were required (e.g. when monitoring cell growth), it was possible to estimate cell numbers by measurement of absorbance at 600 nm. This was performed using the micro-titre plate reader. Using this method of measurement of cell numbers, 1 absorbance unit at 600 nm is equivalent to 1.22 x 10^{10} *Synechococcus* cells ml^{-1}.

2.3 Methods.

2.3.1 Atomic absorption spectrophotometry (AAS).

Atomic absorption analysis for measurement of the concentration of metal ions in solution was performed using a Perkin Elmer Model HGA spectrophotometer. Analyses were performed according to manufacturer's protocols.

2.3.2 Determination of minimum inhibitory concentration (MIC) and maximum permissive concentration (MPC) of metal salts for *Synechococcus* PCC 7942 (PIM8) cells.

Triplicate cultures of *Synechococcus* PCC 7942 (PIM8) were inoculated into Allen's medium at a cell density of 1×10^6 cells ml^{-1} and cultured as described in 2.2.4, with added metal salts at different concentrations. The cultures were left to grow for 2 days, then the MIC and MPC for each metal was recorded following visual determination for growth.

2.3.3 Determination of β-galactosidase activity.

2.3.3.1 Determination of β-galactosidase activity, as described by Miller (1972).

β-galactosidase activity was assayed using o-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma, Dorset, UK) as the substrate as described by Miller (1972).

Synechococcus cultures were grown in Allen's medium (as in section 2.2.4) until the OD 600 = 0.3-0.6 (the OD 600 was recorded). 0.5 ml aliquots of culture were induced with metal ions at various concentrations and times prior to the β-galactosidase assay. 0.5 ml of induced culture was added to 0.5 ml of Z buffer (0.06

M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol, pH 7.0) and the cells were lysed by the addition 1 drop of 0.1 % SDS and 2 drops of chloroform followed by vortexing for 10 seconds.

β-galactosidase assays were carried out in triplicate using micro-titre plates, 176 µl of lysed cell solution was added to 35 µl of ONPG 4 $mgml^{-1}$ (in 0.1 M phosphate buffer, pH 7.0) and incubated at room temperature for a recorded time period over which a yellow colouration developed. Reactions were then terminated by the addition of 88 µl of 1 M Na_2CO_3 and the optical density of each reaction was measured at OD 414 and OD 540 using a micro-titre plate reader. The reactions were blanked against a control in which the cells had not been exposed to metal ions. The activity was defined relative to values obtained for the *Synechococcus* PCC 7942 (PIM8) transformants with no added metal.

The activity was calculated using an equation derived by Miller (1972):

$$\text{Units} = 1000 \times \frac{\text{OD 414} - 1.75 \times \text{OD 540}}{t \times v \times \text{OD 600}}$$

were v = the volume of culture used in the assay (in ml).

t = the time of the reaction (in minutes)

2.3.3.2 A modified method for the determination of β-galactosidase activity.

β-galactosidase activity was also measured using a modification to the protocol described above. Changes in the pigmentation of cultures were observed upon the addition of metal ions. This may affect the result of the β-galactosidase assay. This was not taken into account using the unmodified protocol of Miller (1972), but it is taken into account in the modified protocol. The activity is calculated based upon the rate of change in ONP production for each sample individually. The assays had been carried out in triplicate when using the unmodified protocol. The triplicates did not show a large variation. The modified protocol involved carrying out two assays for

each sample which made it technically more difficult to carry out experiments in triplicate, although two sets of data were obtained for each experiment.

Synechococcus cultures were grown and lysed as in section 2.3.3.1. 176 µl of lysed cell solution was added to 35 µl of ONPG 4mgml^{-1} (in 0.1M phosphate buffer, pH7.0) in a micro-titre plate. A similar reaction was also set up for each reading where the reaction was terminated by the addition of 88 µl of 1M Na_2CO_3 at t = 0. The remaining reactions were incubated at room temperature for a recorded time period (t=t) over which a yellow colouration developed. These reactions were then terminated by the addition of 88 µl of 1M Na_2CO_3. The optical density of each reaction was measured at OD 414 using a micro-titre plate reader.

The activity was calculated using an equation:

$$\text{Units} = \frac{\text{change in OD 414 (t=t - t=0) x 300}}{\text{t x v (1.83 x OD 595)}}$$

Units = n moles o-nitrophenol/min/mg protein.

were: t=t = the OD 414 reading of the sample assayed at time t.

t=0 = the OD 414 reading of the sample assayed at time 0.

v = the volume of culture used in the assay (in ml).

t = the time of the reaction (in minutes).

300 n moles of ONP = 1 OD unit at OD 414 (calculated from a standard curve).

1.83 = the OD 600 of a *Synechococcus* culture that is equivalent to 1mg of protein (calculated assuming that 10^9 cells yields approximately 150 µg of protein).

2.3.4 General molecular biology methods.

(Those methods not described in detail in this section were performed as described by Sambrook *et al.*, 1989).

2.3.4.1 Plasmid mini-preps from *Escherichia coli* by alkaline lysis.

(Modified from Birboim and Doly 1979).

Escherichia coli cultures (5 ml) were grown in LB broth for 16 h at 37 °C with appropriate antibiotic selection (dependent on the plasmid concerned). An aliquot (1.5 ml) was removed to an Eppendorf microfuge tube and the cells harvested by microcentrifugation (MSE microcentaur microcentrifuge). The supernatant was then removed. The cells were resuspended in 100µl of an ice cold solution of 50 mM glucose, 10 mM EDTA, 25 mM tris-Cl (pH 8.0). After a 5 min incubation at room temperature, 200µl of a solution of 0.2 N NaOH, 1 % SDS was added and the contents of the tube were mixed by inversion and incubated on ice for 10 min. 150µl of an ice cold solution of potassium acetate (5 M potassium acetate 60ml, glacial acetic acid 11.5ml, H_2O 28.5ml) was then added and the tube was incubated on ice for a further 10 min. After microcentrifugation at high speed for 5 min, the supernatant was removed to a fresh tube, RNAse A was added to a final concentration of 20µgml^{-1} and the tube was incubated at 37 °C for 30 min. 500µl of phenol/chloroform (1 : 1) was then added, the contents mixed, centrifuged at high speed and the aqueous phase transferred to a fresh tube. 2.5 volumes of 100 % ethanol were added and the tube was incubated at -80°C for 10 min. Plasmid DNA was rescued by microcentrifugation at high speed for 5 min. The pellet was then washed in ice cold 70 % ethanol and dried briefly under vacuum. The plasmid DNA was resuspended in 16µl of water. 4µl of 4 M NaCl was added followed by 20µl of 13 % polyethylene glycol, and the tube was incubated on ice for 30 min. The purified plasmid DNA was recovered by microcentrifugation, washed using 70 % ethanol, dried under vacuum and resuspended in 20µl of water.

2.3.4.2 Plasmid maxi-preps from *Escherichia coli.*

Large-scale preparations of plasmid DNA from *Escherichia coli* were performed essentially as a scale up of steps 1 to 10 of the mini-prep protocol as given in section 2.3.4.1. 500 ml of culture was used (the cells were collected by centrifugation at 4000 rpm; Beckman centrifuge). Further centrifugation steps were performed using 30 ml glass corex tubes at 10 000 rpm using a Beckman JA-20 rotor. When greater purity of plasmid DNA was required, the following procedure was included: The dried DNA pellet was resuspended in 8 ml of TE buffer (pH 8.0) and 8.6 g of CsCl was added followed by 0.45 ml of a 10 $mgml^{-1}$ solution of ethidium bromide. The solution was placed in two 1/2 x 2 inch quick-seal centrifuge tubes which were then heat sealed. The tubes were then centrifuged at 50000 rpm for 16 h using a Sorval OTD65B ultracentrifuge. The nucleic acid bands were visualised under UV illumination and the plasmid band removed using a 10 ml syringe. The ethidium bromide was removed by extraction with isoamyl alcohol saturated with CsCl, and salts removed by dialysis against TE buffer (pH 8.0). The plasmid DNA was precipitated using 2.5 volumes of 100 % ethanol at -20 °C.

2.3.4.3 Preparation and transformation of competent *Escherichia coli* cells.

The method used to prepare transformation competent cells was as described by Chung *et al.*, (1989). *Escherichia coli* cells were grown in LB broth to early exponential phase and diluted 1 : 1 with TSS. A 1 ml aliquot of the cells in this solution was transferred to a cold Eppendorf tube, mixed with plasmid DNA and incubated at 4 °C for 30 min. The cells were then diluted by the addition of 0.9 ml of TSS and incubated at 37 °C for 1 h. Aliquots of transformed cells were then plated onto LB agar containing the desired selective agent.

2.3.4.4 Preparation and transformation of high efficiency competent *Escherichia coli* cells.

The method to prepare high efficiency competent cells was essentially as described by Alexander *et al.*, (1984).

5 ml of *Escherichia coli* cells were grown in 2 XL overnight at 30 °C. An aliquot (1 ml) was used to inoculate 100 ml 2 XL prewarmed to 30 °C in a 500 ml Erlenmeyer. This was cultured (with shaking) at 30 °C until the OD 600 = approximately 0.2, then sterile 2 M $MgCl_2$ was added to a final concentration of 20 mM. Growth was allowed to continue until OD 600 = 0.5 (0.45-0.55). The cells were then incubated in ice-water for 2 h. 40-50 ml aliquots were spun down in sterile blue-capped Falcon tubes at 3000 rpm for 5 min in the bench-top centrifuge, and the supernatant was aspirated off. The resulting pellets were resuspended gently in one half of the original volume of ice-cold Ca/Mg medium, 100 mM $CaCl_2$, 70 mM $MnCl_2$, 40 mM NaAc, pH 5.5. This solution was prepared fresh and filter sterilised (start with a stock NaAc solution of about pH 7, the Ca^{2+} and Mn^{2+} salts will drop the pH considerably and the pH must be adjusted down). The cells were then incubated on ice for 1 h and collected by centrifugation at 3000 rpm in the bench top centrifuge for 5 min. The resulting pellet was resuspended (very gently) in 1/20 the original culture volume of Ca^{2+}/Mn^{2+} solution containing 15% (v/v) glycerol. Aliquots (0.2 ml) of cells were collected in 1.5 ml Eppendorfs, frozen in liquid nitrogen and stored at -80 °C until required.

The cells were thawed on ice and transformed immediately by the addition of DNA in 100 µl TE and incubation on ice for 30 min. The transformation sample was subjected to heat shock (37 °C for 5 min), diluted to 4 ml with 2 XL broth (pre-warmed to 37 °C) and grown with shaking for 1.5 h at 37 °C. The transformed cells were then plated out onto LB agar containing the desired selective agent.

2.3.4.5 Agarose gel electrophoresis of DNA.

Agarose gel electrophoresis was performed as described by Sambrook *et al.*, (1989). Generally 0.8 % agarose gels were used, although higher concentrations up to a maximum of 2 % were used, as specified for each experiment, where small fragments (to a minimum of 100 b.p.) were to be separated. Maxi-gels were cast using tris-acetate buffer (40 mM tris-acetate [pH 7.7], 2 mM EDTA), and mini-gels using tris-borate buffer (89 mM tris-borate, 2 mM EDTA). DNA was loaded into the wells of the gel after addition of loading dyes (0.25 % each of bromophenol blue and xylene cyanol, 15 % w/v Ficoll 400).

2.3.4.6 Isolation of restriction fragments from agarose gels.

Gel slices containing fragments to be isolated were cut from agarose gels using a clean scalpel blade. The DNA was purified from the gel block by binding to silica fines as described below.

The required band was excised and placed in an Eppendorf tube. 1 ml of NaI solution was added and the tube was incubated 60 °C for 10 min. 5 µl of silica fines was then added, followed by incubation at room temperature for 10 min. The fines were collected by spinning for 15 seconds in a microcentrifuge, followed by the aspiration the supernatant. The pellet of fines was washed in 1 ml 70 % ethanol (70 % ethanol: 30 % TE buffer) and centrifuged for 15 seconds in a microcentrifuge. The ethanol was then aspirated off and the fines were resuspended in 50 µl TE buffer. The DNA was then eluted off by incubation at 37 °C for 10 min. The fines were spun down by centrifugation for 15 seconds in a microcentrifuge and the supernatant containing the DNA was used in ligations or used to make radioactive probes.

2.3.4.7 Cloning of DNA fragments.

General methods employed for cloning of DNA fragments (restriction and ligation) were as described by Sambrook *et al.*, 1989).

2.3.4.8 Radioactive labelling of DNA.

DNA was radioactively labelled with ^{32}P using one of the two following methods, dependent on the source of the DNA. Chemically synthesised oligonucleotides were end-labelled using (γ-^{32}P)dATP with the enzyme T4 polynucleotide kinase, as described by Sambrook *et al.*, (1989). Double-stranded DNA fragments were labelled by random priming using (α-^{32}P)dCTP with Klenow polymerase, as described by Feinberg and Vogelstein (1985). Following the labelling reaction, unincorporated radioactivity was separated from the DNA fragments by Sephadex G-50 gel permeation chromatography using 10 ml (total volume) columns.

2.3.4.9 Formaldehyde-agarose gel electrophoresis of RNA.

Formaldehyde-agarose gels were prepared and run as described by Sambrook *et al.*, (1989). Ribosomal RNA bands were used as size markers for these gels.

2.3.4.10 Southern and northern blotting.

DNA and RNA was transferred to nylon hybridisation membranes essentially as described by Sambrook *et al.*, (1989). DNA was denatured prior to transfer by soaking agarose gels in an excess of denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 1 h, followed by neutralisation of the gel by soaking in an excess of neutralisation solution (1.5 M NaCl, 0.5 M tris; pH 7.5) for 1 h. Gels were blotted for 16 h using 10X SSC, after which time complete transfer of nucleic acids had occurred. Filters

were then placed under vacuum at 80 °C for 1 h prior to hybridisation of radioactive probes.

2.3.4.11 Hybridisation of radioactive DNA probes to filter-immobilised nucleic acids.

All hybridisation reactions were carried out in heat-sealed polythene bags which were contained in plastic boxes. Hybridisation of probe to northern blots was carried out at 42 °C, and to Southern blots at 65 °C. In both cases, filters were pre-hybridised for 1 h prior to the addition of probe. Solutions used for both prehybridisation and hybridisation were as described by Sambrook *et al.*, (1989). Hybridisations were carried out for 16 h. Filters were washed using 3 changes of 1X SSC, 0.1 % SDS over a period of 1 h, after which the radioactive filter was placed on 3MM paper, orientated by the addition of several spots of radioactive ink, and exposed to X-ray film. Film cartridges were maintained at -80 °C for the required exposure time. After film development, the filter could be washed to a greater stringency, or completely stripped of radioactivity by incubation at 90 °C in 0.1 % SDS which allowed the filter to be re-probed as desired.

2.3.4.12 *In situ* hybridisation of bacterial colonies.

The method used was based on that described by Sambrook *et al.* (1989). *Escherichia coli* were grown on nitrocellulose filter discs, whereas *Synechococcus* cultures were dot blotted onto nitrocellulose filters. The filters were placed colony side up on a piece of 3MM paper saturated with 10 % (w/v) SDS and left for 3 min. The filter then was transferred to 3MM paper soaked in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 5 min, followed by 5 min on 3MM paper saturated with neutralising

solution (1.5 M NaCl, 0.5 M Tris.HCl pH 8.0). Then the filter was placed on a sheet of dry 3MM paper and allowed to dry at room temperature for 30-60 min before being baked for 2 h at 80 °C in a vacuum oven. Prior to hybridisation, filters were prewashed at 65 °C for 1-2 h (with shaking) in 100 ml of washing solution (1 M NaCl, 1 mM EDTA, 0.1 % (w/v) SDS, 50 mM Tris.HCl pH 8.0). Prehybridisation and hybridisation were carried out as described in section 2.3.4.11.

2.3.4.13 Use of the polymerase chain reaction (PCR) for *in vitro* amplification of DNA.

PCR reactions were carried out essentially as described by Saiki *et al.*, (1988). Reaction conditions were as follows: 200 μM each of dATP, dTTP, dGTP and dCTP, 50 mM KCl, 10 mM tris-Cl [pH 8.3], 1.5 mM $MgCl_2$, 0.001 % gelatin (final reaction volume 50 μl). The concentration of each primer and the amount of template DNA added varied according to the particular experiment concerned. Taq polymerase was added to the reaction last, and the contents mixed and overlaid with mineral oil. Reactions were carried out using a Hybaid Intelligent Heating Block. Details of temperatures and time settings used to control denaturing, annealing, extension, and the number of amplification cycles, are stated for individual reactions.

2.3.4.13.1 Design of PCR primers for the amplification of *smtA*.

PCR primers were designed based upon the amino acid sequence of a prokaryotic MT isolated from *Synechococcus* TX-20 by Olafson *et al.*, (1988). Inosine-containing PCR primers were synthesised corresponding to two peptide regions shown in figure 2.1. These peptide regions were selected to minimise primer redundancy, yet maximise the proportion of the coding region of the gene that would be amplified. Inosines were incorporated into the primers at base positions with greater than two

fold redundancy. Restriction endonuclease recognition sites were included at the 5' ends of the primers to facilitate cloning of the PCR products.

2.3.4.13.2 Conditions for the amplification of *smtA*.

PCR was carried out as described in section 2.3.4.13, using the primers described above and template DNA from *Synechococcus* PCC 6301. *Synechococcus* PCC 6301 DNA was used as the source of template DNA because this strain is identical to *Synechococcus* TX-20, the strain from which Olafson had originally isolated the prokaryotic MT (refer to section 2.1.2). The PCR contained 10 μg of each primer and 0.5 μg of template DNA. Reactions were subject to 28 cycles of the following series of temperatures and times: denaturation at 92 °C for 1.5 min, annealing at 55 °C for 1.5 min and elongation at 72 °C for 1.5 min.

2.3.4.13.3 APCR (Anchored PCR) strategy for the amplification of regions flanking *smtA*.

The APCR strategy entailed ligating a genomic fragment onto a vector to produce a known primer binding site at the end of the genomic fragment. This would allow subsequent amplification using a "generic" primer to the vector in conjunction with a specific *smtA* genomic primer.

4 μg of pUC19 was digested with *Pst*I. The cut vector was then recovered from an agarose gel as described in 2.3.4.6, and digested with *Hin*d III in order to obtain plasmid DNA with only one *Hin*d III compatible end. This linear plasmid was then ligated to 10 μg of *Hin*d III digested, total genomic DNA from *Synechococcus* PCC 6301. After ligation, 0.35 μg aliquots of the ligation mix were used to set up 4 APCRs. The APCRs used combinations of one of the two *smtA* specific primers, in conjunction with one of the two generic M13 universal primers which hybridise to pUC19.

The ligation population used as the template in the APCRs contains diverse *Hin*d III fragments ligated to pUC19. The M13 primers will hybridise to all of these ligation products. However, only fragments containing *smtA* will amplify exponentially when the ligation mix is used as a template for APCR due to the use of the *smtA* primer. Furthermore, only APCRs containing the M13 reverse primer and a *smtA* primer will give rise to exponential amplification, as only this primer is adjacent to the *Hin*d III ligation site. The M13 forward primer is adjacent to the incompatible *Pst* I site and will not give rise to amplification products (figure 2.2).

APCR with the C-terminal *smtA* primer (figure 2.1) and the M13 reverse primer will amplify upstream through the coding region of the *smtA* gene and into it's 5' flanking region until the first 5' *Hin*d III site. Amplifications with the N-terminal *smtA* primer and the M13 reverse primer will amplify downstream through the coding region of the gene and into the 3' flanking sequence to the first 3' *Hin*d III site (figure 2.2).

2.3.4.13.4 APCR amplification conditions.

APCR was performed using conditions as described in section 2.3.4.13 with some modifications. The template DNA used was 0.35 μg of the ligation mix described in the previous (section 2.3.4.13.3). 10 μg of *smtA* specific primer and 0.5 μg of universal M13 primer were used in 100 μl reactions, and reactions were subject to 32 cycles of the following series of temperatures and times: 92 °C for 1.5 min, annealing at 55 °C for 2.0 min and elongation at 72 °C for 5 min.

2.3.4.13.5 Amplification strategy for the fusion of the *smtA* 5' region to the reporter gene *lacZ* to produce the vector pLACPB2(*smt*-5').

PCR was used to couple approximately 600 b.p. of 5' *smtA* DNA sequence to the reporter gene *lacZ*. Figure 2.3 shows the amplification strategy. The PCR primer used in the reaction is shown in figure 2.3 C. This primer incorporates a *Bam*H I

restriction site adjacent and upstream to the *smtA* ribosome binding site which allows the cloning of the PCR product into pLACPB2 to produce a transcriptional fusion as shown in figure 2.3 D, designated pLACPB2(*smt*-5').

2.3.4.13.6 Amplification conditions and cloning of the products for the construction of pLACPB2(*smt*-5').

PCR was performed essentially as described previously 2.3.4.13, using 1 μg of pJHNR49 as the template and only 12 cycles of amplification to minimise the chance of PCR-mediated errors. Reactions were subject to 12 cycles of the following series of temperatures and times: 92 °C for 1.5 min, annealing at 55 °C for 2.0 min and elongation at 72 °C for 5 min.

2.3.4.13.7 PCR conditions and cloning of the 5'*smtA* truncated fusion products.

Promoter deletion analysis was used in an attempt to identify the functional elements within the *smt* operator/promoter region.

PCR was used to produce truncated fusion products. The PCR conditions were essentially as described in 2.3.4.13. 1μg of pGEM4Z containing the insert of pLACPB2(*smt*-5') was used as template in the reactions. All the truncated fragments were subcloned into pGEM4Z and sequenced and no PCR-mediated errors were detected. The fragments were subsequently cloned into pLACPB2 and used to transform *Synechococcus* PCC 7942 (PIM8) cells to Cm resistance as described in 2.3.4.23. Transformants were checked by plasmid recovery into *Escherichia coli* followed by restriction mapping and sequence analysis.

The sequence of the largest truncation which was produced via the digestion of the insert of pLACPB2(*smt*-5') with *Pst* I and *Bam*H I is shown in figure 2.4. This resulted in a fusion construct designated pLACPB2(*smt*-5'del1) which contained the *smt* operator/promoter region but did not contain a functional *smtB* gene upstream of

smtA (refer to section 4.1.2). A truncated fusion designated pLACPB2(*smt*-5'del2) was also produced, using PCR to generate a fragment that included the *smt* operator/promoter region up to and including inverted repeat1 (refer to section 4.1.2) as shown in figure 2.5. The smallest fusion pLACPB2(*smt*-5'del3) was produced using PCR to generate a construct in which inverted repeat1 was deleted (figure 2.6). A summary of these deleted fusion constructs is shown in figure 2.7.

2.3.4.13.8 Amplification strategy for the fusion of *smtB* 5' regions to the reporter gene *lacZ* to produce the vectors pLACPB2(*smtB*-5'1.2) and pLACPB2(*smtB*-5'0.2).

PCR was used to produce two constructs, one of which contained approximately 1200 b.p. of 5' *smt*B DNA sequence fused to the reporter gene *lacZ*, designated pLACPB2(*smtB*-5'1.2). The other contained approximately 240 b.p. of 5' *smt*B DNA sequence fused to the reporter gene *lacZ*, designated pLACPB2(*smtB*-5'0.2). Figures 2.8 and 2.9 show the amplification strategies. PCR was used to incorporate a *Bam*H I restriction site adjacent to and upstream of the *smtB* ribosome binding site. This allowed the cloning of the PCR products into pLACPB2 to produce transcriptional fusions. As a control, equivalent constructs to the two described above were produced in which there was a one base deletion in the *smtB* -10 sequence (*smtB* -10=TATGGT, deletion -10=TAGGT). These constructs were produced using a similar PCR strategy to that described above, although the primer contained a deletion in the -10 sequence. These were designated pLACPB2(*smtB*-5'1.2c) and pLACPB2(*smtB*-5'0.2c).

PCR was performed essentially as described previously (2.3.4.13), using 1 μg of pJHNR49 as template and only 12 cycles of amplification to minimise the chance of PCR-mediated errors. Reactions were subject to 12 cycles of the following series of temperatures and times: 92 °C for 1.5 min, annealing at 55 °C for 2.0 min and

elongation at 72 °C for 5 min. The PCR fragments produced were subcloned into pGEM4Z and sequenced. No PCR-mediated errors were detected. The fragments were then cloned into pLACPB2 and used to transform *Synechococcus* PCC 7942 (PIM8) cells to Cm resistance as described in 2.3.4.23. Transformants were checked by the plasmids being recovered from *Synechococcus* and introduced into *Escherichia coli*. Plasmid preparations were then carried out and the constructs were checked by restriction mapping and sequence analysis.

2.3.4.14 DNA sequencing.

Direct sequencing of plasmid clones was performed by the dideoxy-sequencing method of Sanger *et al.*, (1977) using fluorescent dye-linked universal M13 primers. Sequences were analysed using an Applied Biosystems 370A DNA sequencer. Reactions were prepared according to protocols provided by the manufacturer (Model 370A DNA Sequencing System, User's Manual Version 1.3A, October 1988).

2.3.4.15 Synthesis of DNA oligonucleotides.

Oligonucleotides were synthesised using an Applied Biosystems 381A DNA synthesiser operated using standard protocols.

2.3.4.16 Annealing of DNA oligonucleotides.

After resuspension of the oligonucleotides in TE buffer (pH 8.0), the DNA was quantified by measurement of absorbance at 260nm. 5 μg of each DNA strand was then added to a reaction containing 1X LKB, 10 mM ATP and 5 units of T4 polynucleotide kinase. After incubation at 37 °C for 1 h, the reaction tube was placed in a waterbath and heated to 90 °C. This was then left for 5 h to return to room temperature. The annealed oligonucleotides were then ready for ligation.

2.3.4.16.1 The addition of inverted repeat1, to a strong hybrid promoter in *Synechococcus* PCC 7942 (PIM8).

A hybrid promoter was designed containing the 7-2-7 inverted repeat1 (refer to section 4.1.2). The λ P_R - 35 region (TTGACT) was chosen as, Ferino and Chauvat (1989) had shown this promoter sequence to function in cyanobacterium. The - 10 sequence chosen was the *tac* (- 10 consensus sequence, TATAAT). The spacing between the two regions was 18 b.p. which was used to keep the spacing as close to the optimum for *Escherichia coli* promoters (17 b.p.) (refer to 4.1.2), although allowing the incorporation of the inverted repeat (figure 2.10). An equivalent promoter was also designed without the inverted repeat but including the λ P_R sequence in its place (figure 2.10). The promoters were produced from oligonucleotides which were annealed together as described above. Half *Sal*I and *Bam*HI restriction sites were included on the ends of the primers to enable cloning of the promoter fragments into pLACPB2 in order to produce transcriptional fusions. The promoter sequences were subcloned into pGEM4Z and sequenced prior to ligation into pLACPB2.

2.3.4.17 Transcript mapping by primer extension.

Primer extension was carried out as described by San Francisco *et al.*, (1990), the RNA template being isolated from cultures that were pre-induced with 2.5μM $CdCl_2$ for 2 h prior to extraction.

Synthetic oligonucleotides complementary to the *smtA* and *smtB* transcripts were end labelled using T4 polynucleotide kinase as described in section 2.3.4.8. RNA (10 μg) was annealed to labelled oligonucleotide (5 fM) for 20 min at 55 °C in a total volume of 10 μl of a buffer consisting of 40mM PIPES, pH 6.5, 0.4M NaCl, and 1mM EDTA. The RNA hybrids produced (10μl) were added to 90 μl of extension mix containing 50mM Tris-HCl, pH 8.5, 6mM $MgCl_2$, 0.1M KCl, 2 mM dithiothreitol, 2.5

μg actinomycin D, 100 units RNasin (Promega), 0.5 mM of each dATP, dCTP, dGTP, dTTP, and 30 units of AMV reverse transcriptase (Promega) and incubated at 42 °C for 90 min. The reactions (0.1ml) were precipitated with ethanol and suspended in 10 μl of dye (95 % formamide, 0.04 % xylene cyanol, 0.04 % bromophenol blue). The cDNA products were heated at 85 °C for 5 min, cooled on ice and separated by electrophoresis on a 6% polyacrylamide gel containing 7M urea. mp18 and mp19 containing the *Sal* I *Hind* III genomic fragment from pJHNR49 were used to prepare single stranded DNA corresponding to both strands of the genomic fragment as described by Sambrook *et al.*, (1989). The single stranded templates were then used to produce sequencing ladders using the same primers as in the primer extension reactions (Sanger *et al.*, 1977) and this was run on gels in parallel with the products of the primer extension. The gels were transferred on to 3MM chromatography paper and dried under vacuum, prior to exposure to X-ray film. Film cartridges were maintained at -80 °C for the required exposure time.

2.3.4.18 Isolation of DNA from *Synechococcus* cells.

Cells were harvested by centrifugation at 3000 rpm using Beckman bench top centrifuge. The supernatant was discarded and the cells resuspended in an equal volume of extraction buffer. The resuspended cells were added drop-wise to liquid nitrogen and ground to a fine powder using a mortar and pestle. 200 μl aliquots of ground cells were placed in an Eppendorf tube containing 2 μl of β-mercaptoethanol, followed by the addition of 200 μl of DNA extraction buffer (1.4 M NaCl, 100 mM tris, 20 mM Na_2EDTA; pH 8.0). 400 μl of phenol/chloroform was then added and the tube contents thoroughly mixed. The mixture was allowed to return to room temperature and then microcentrifuged at top speed for 5 min. The supernatant was removed to a fresh tube and re-extracted once more using phenol/chloroform, followed by several extractions using chloroform alone, until no protein contamination was visible at the aqueous/organic interface. After the final extraction with chloroform

the supernatant was made up to 320 µl followed by the addition of 80 µl of 5 M ammonium acetate and 1 ml of 100 % ethanol was added. The nucleic acids were then allowed to precipitate overnight at -20 °C. Total nucleic acid was recovered by microcentrifugation for 10 min at high speed, briefly washed using 70 % ethanol and dried under vacuum. This material was resuspended in 40µl of sterile water and quantified by measurement of absorbance at 260 nm.

2.3.4.19 Production of a size fractionated genomic library.

A genomic clone (designated pJHNR49) was isolated from a *Sal* I, *Hin*d III partial genomic library using this procedure. These enzymes were selected for the genomic DNA digestion in the library production because a *Sal* I *Hin*d III digestion of *Synechococcus* PCC 7942 gave a fragment of suitable size (1.8 k.b.) on a Southern blot (Dr. W.P. Lindsay unpublished observation).

Genomic DNA was isolated as described above (section 2.3.4.18). DNA (100µg) was digested with *Sal*I (overnight) in conditions according to the restriction enzyme manufacturer. The digest was then extracted with phenol/chloroform, followed by a further digestion with *Hin*d III (overnight). A second phenol/chloroform extraction was carried out and then the DNA was precipitated at -20°C as described in Sambrook *et al.*, (1989). The resulting DNA pellet was resuspended in 1x TE buffer and heated for 10 min at 68°C before loading on a sucrose gradient.

A 25-50% sucrose density gradient was produced in Beckman polyallomer tubes. Sucrose solutions were made up in a buffer containing 1M NaCl, 20mM Tris-HCl, pH 8.0, and 5mM EDTA. The DNA sample was fractionated by sucrose density gradient centrifugation at 20 000rpm for 18 h at 20°C in a MSE prepspin 65 centrifuge, using a 43127-104 MSE swing out rotor. The centrifuge tube was then punctured at the bottom and 0.5ml fractions were collected. The fraction containing *smtA* was identified by dot-blot hybridisation to *smtA* probe prepared from gel purified fragments from pJHNR11 (a pUC19 clone containing a PCR fragment corresponding to part of

smtA). The DNA within the identified fraction was ligated to *Sal*I, *Hin*d III digested pGEM4Z (Promega, Madison, USA) and introduced into *Escherichia coli* JM101 competent cells (Alexander *et al.*, 1984; section 2.3.4.4). The resulting library was screened with *smtA* probe prepared from pJHNR11 and pJHNR21 to isolate clone pJHNR49.

2.3.4.20 Exposure of cells to metal ions for northern analysis of metal-induced gene expression.

Cells (1 litre) were cultured to mid log phase in Allen's medium. Aliquots (50 ml) of culture were removed to 50 ml Falcon tubes and different concentrations of metal ions (0, 1, 2.5, 5, 10 μM) were added for a designated time period. The cells were collected by centrifugation at 3000 rpm (MSE Centaur centrifuge) and frozen in liquid nitrogen prior to RNA extraction, performed as outlined below.

2.3.4.21 Isolation of total RNA from *Synechococcus* cells.

(From Dzelzkalns *et al.*, 1988).

Cells were harvested and ground to powder as for extraction of DNA (section 2.3.4.18). The ground cells (200 μl) were added to an equal volume of pre-warmed (37 °C) lysis buffer and incubated at 37 °C for 30 min. The lysed cells were extracted twice using phenol/chloroform followed by several extractions using chloroform until no contaminating proteins were visible at the aqueous/organic interface. The supernatant was then removed to a fresh Eppendorf tube and nucleic acids precipitated in the same manner as for DNA extraction. The isolated nucleic acid was quantified both by absorbance at 260 nm and by visualisation on a 1.2 % agarose mini-gel. The RNA was further treated prior to electrophoresis by mixing 10-20 μg of RNA (quantities were standardised for each gel to give equal amounts in each lane) with 50X MOPS and 100 % formamide to give final concentrations of 1X MOPS and 50 %

formamide. This was then incubated at 60 °C 10 min before electrophoresis on 1.4 % agarose-formaldehyde gels as described in 2.3.4.9.

2.3.4.22 The use of rifampicin to inhibit transcription.

Rifampicin was used to inhibit transcription in *Synechococcus* cultures as described by Wealand *et al.* (1989). Cultures were exposed to rifampicin (400 μgml^{-1}) alone, rifampicin (400 μgml^{-1}) and $CdCl_2$ (2.5 μM) and to $CdCl_2$ alone (2.5 μM). RNA was then extracted from each culture every 15 minutes over a 1 h period and used for northern blots as described above, section 2.3.4.21.

3.4.23 Transformation of *Synechococcus* cells with plasmid DNA.

Cells were grown to late log phase in Allen's medium, counted using a haemocytometer, and a total of 10^8 cells pelleted (3000 rpm) in a 10 ml sterile test tube. The pelleted cells were resuspended in 100 μl of Allen's medium and 10 μg of plasmid DNA added, followed by incubation at 30 °C for 40 min to allow DNA uptake. Aliquots of transformed cells (between 5 and 40 μl) were spread on 1.5 % agar Allen's plates and incubated under conditions described previously (section 2.2.4). After incubation for 16 h, Cm was added to the plates to give a final concentration of 10 μgml^{-1} Cm (pLACPB2, the plasmid used in these transformations, contains a chloramphenicol (Cm) resistance gene). Antibiotic was added by gently lifting the agar disc from the bottom of the petri dish using a 1 ml sterile glass pipette and pipetting 0.5 ml of a 100 X stock of the antibiotic underneath the agar. The plates used for transformation contained 50 ml of Allen's agar per plate to allow 100X dilution of the antibiotic. After a further incubation period of 5-10 days, isolated single colonies were picked from the original plates and re-streaked onto fresh plates containing the selective agent. Colonies from these plates were used to set up 50 ml cultures for further sub-culture and for long term storage of transformants.

2.3.4.24 Plasmid rescue from *Synechococcus* cells.

In order to check that the plasmids introduced into *Synechococcus* cells had not undergone rearrangement, plasmids were re-isolated from the transformants and analysed by restriction digestion and sequencing. In order to rescue the plasmids from *Synechococcus* cells, a standard DNA preparation was performed (as described in section 2.3.4.18) and the total DNA used to directly transform *Escherichia coli* cells to Cm resistance (as described in 2.3.4.4). The plasmids were isolated from *Escherichia coli* transformants using standard procedures. In no case was the plasmid DNA found to have been altered during the procedure.

Figure 2.1 The amino acid sequence of the *Synechococcus* TX-20 MT isolated and sequenced by Olafson *et al.*, (1988). The arrows marked 1 and 2 indicate the peptide regions to which the PCR primers shown below were designed. The arrows show the relative location and orientation of the two PCR primers. The restriction endonuclease recognition sites (*Sal* I and *Eco*R I), which enabled cloning of the PCR products, are underlined.

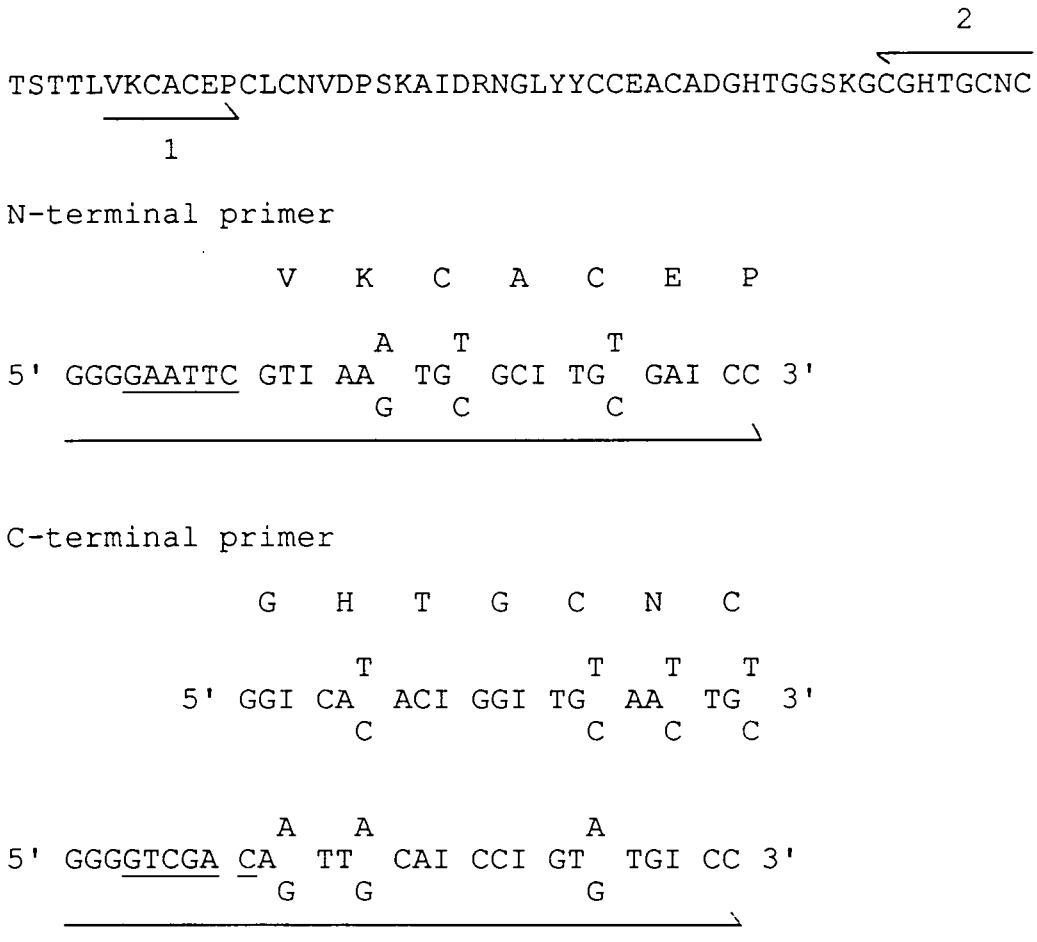

2
TSTTLVKCACEPCLCNVDPSKAIDRNGLYYCCEACADGHTGGSKGCGHTGCNC
1
N-terminal primer
V K C A C E P
A T T
5' GGGGAATTC GTI AA TG GCI TG GAI CC 3'
G C C
C-terminal primer
G H T G C N C
T T T T
5' GGI CA ACI GGI TG AA TG 3'
C C C C
A A A
5' GGGGTCGA CA TT CAI CCI GT TGI CC 3'
G G G

Figure 2.2 Diagrammatic representation of APCR. Total genomic DNA, digested with *Hin*d III, was ligated to pUC19 digested with *Hin*d III and *Pst* I (represented as a partial circle). APCR is carried out with one *smtA* specific primer in conjunction with one "generic" primer to the vector. The universal M13 reverse primer (1) will prime unidirectional single stranded synthesis of all ligated fragments but only the *Hin*d III fragments containing the *smtA* gene will be exponentially amplified (long arrows). Genomic fragments containing *smtA* will be ligated in both orientations A and B. In reactions containing the *smtA* N-terminal primer (N) there will be exponential amplification of fragments ligated in orientation B of part of the *smtA* coding region (black box) and the 3' flanking sequence (open box) to the first 3' *Hin*d III site. In reactions containing the C-terminal primer (C) there will be exponential amplification of fragments ligated in orientation A of the *smtA* coding region and the 5' flanking sequence (shaded box) to the first 5' *Hin*d III site. There will be no amplification of *smtA* fragments in reactions containing the M13 forward primer (2). Primers that will not give exponential amplification of *smtA* fragments in a particular ligation are indicated as short arrows.

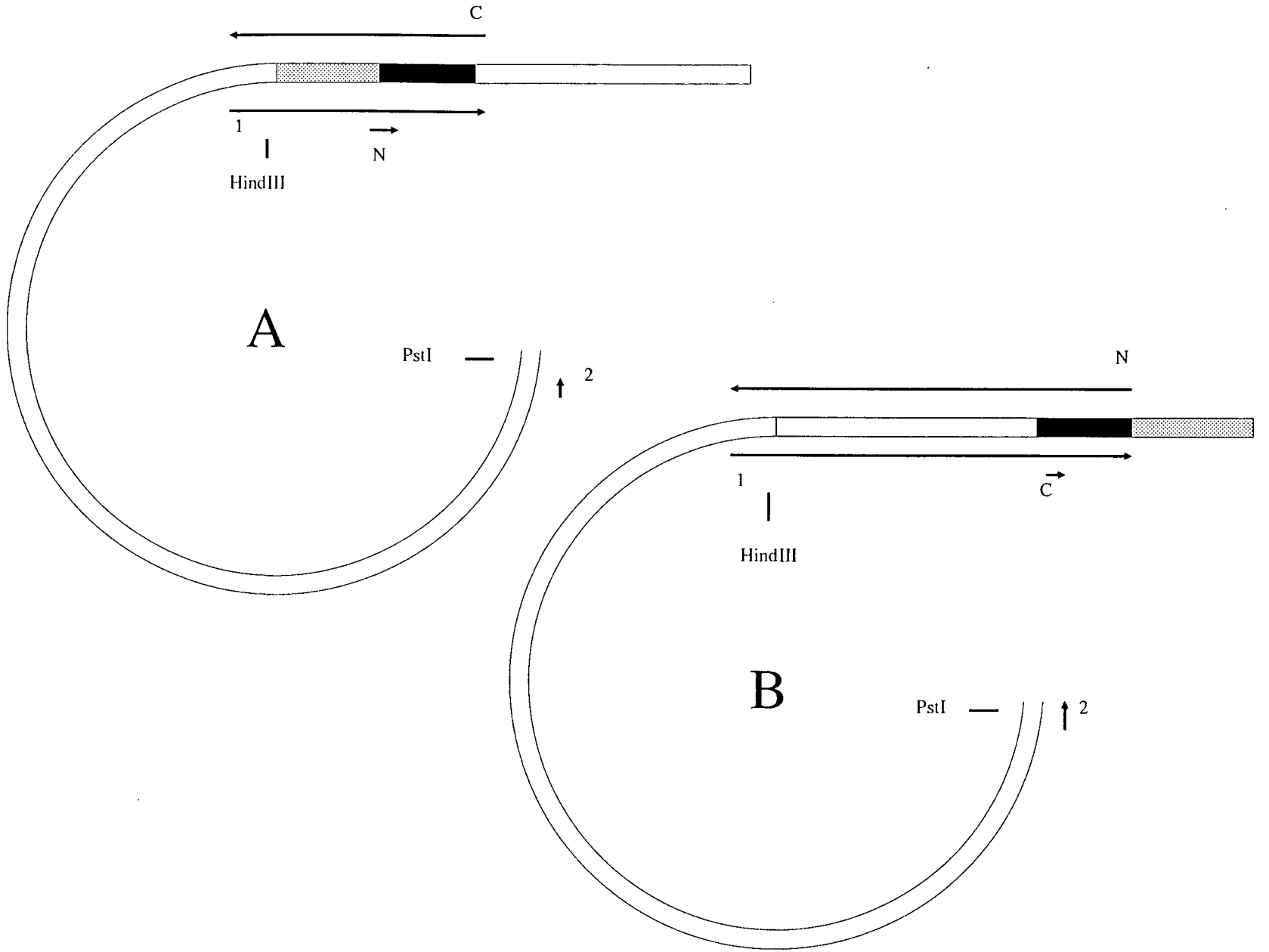
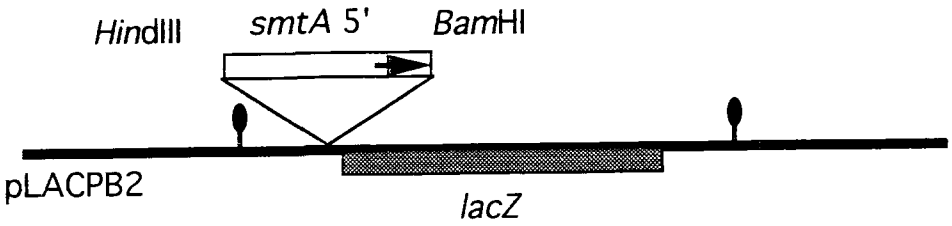

C
1
N
HindIII
A
PstI
2
N
1
C
HindIII
B
PstI
2

Figure 2.3 Fusion of the 5' region of the *smtA* gene to a promoterless *lacZ* gene to produce the vector pLACPB2(*smt*-5'). Panel A is a schematic representation of the *smtA* 5'/*lacZ* gene construct. Panel B is the nucleotide sequence of part of the pLACPB2 vector showing the restriction sites which allow the creation of a transcriptional fusion to the *lacZ* gene. Panel C is the nucleotide sequence of part of the 5' flanking region of the *smtA* gene and the PCR primer used for amplification of the *smtA* 5' region which includes a *Bam*H I site to allow cloning of the PCR product into pLACPB2. Panel D is the nucleotide sequence of the fusion between the *smt* 5' region and the region of translation initiation in *lacZ*. The *smt* sequence is shown in bold, inverted repeat1, the direct repeat and the -10 sequence are underlined.

A.



Transcription terminator

B.

5'CTGCAGGTCGACGGATCCCCGGGAATTCATCGAGCAACATATTAATGAGCCAGAGAAATGCTGGCGGCACT
*Pst*I *Sal*I *Bam*HI *Sma*I *Eco*RI

GAAAGTTTTTGTACAAGCCGATGAAAGCGGCGACGCGCAGTTAATCCCACAGCCGCCAGTTCCGCTGGCGGCA

TTTTAACTTTCTTTATCACACAGGAAACAGCT ATG ACC ATG ATT ACG GAT TCA CTG GCC GTC3'
Met Thr Met Ile Thr Asp Ser Leu Ala Val
lacZ

C.

Primer *Bam*HI
3'GTCAATAAGTCTATAAGT*CCTAGG*TTG5'

5'**CCACCCACCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGATATTCAAGGAGT**

GCTGTC ATG ACC TCA ACA3'

Met Thr Ser Thr
smtA

D.

5'*smtA* *Bam*HI

5'**CCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGATATTCA***GGATCC*CCGGGA

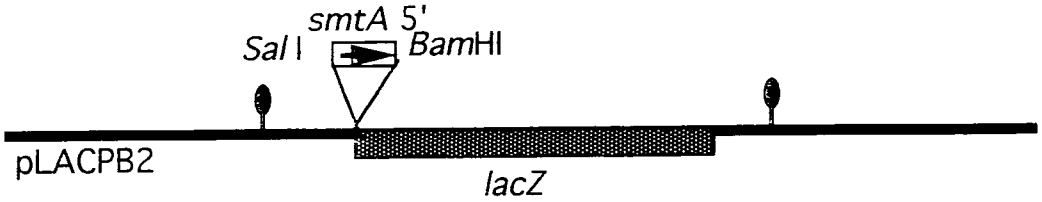
ATTCATCGAGCAACATATTAATGAGCCAGAGAAATGCTGGCGGCACTGAAAGTTTTTGTACAAGCCGATGAAAGC

GGCGACGCGCAGTTAATCCCACAGCCGCCAGTTCCGCTGGCGGCATTTTAACTTTCTTTATCACACAGGAAACAG

CT ATG ACC ATG ATT ACG GAT TCA CTG GCC GTC3'
Met Thr Met Ile Thr Asp Ser Leu Ala Val
lacZ

Figure 2.4 Production of a truncated 5' region of the *smtA* gene, fused to a promoterless *lacZ* gene. Panel A is a schematic representation of the *smtA* 5'deletion1/*lacZ* gene construct. Panel B is the nucleotide sequence of part of the insert of pLACPB2(*smt*-5') showing the restriction sites which allow the creation of a truncated fusion to the *lacZ* gene. Panel C is the nucleotide sequence of the 5' truncation of the *smtA* gene fused to pLACPB2, designated pLACPB2(*smt*-5'del1) which includes a *Bam*H I site to enable cloning of the digestion product into pLACPB2. The *smt* sequence is shown in bold, inverted repeat1, the direct repeat and the -10 sequence are underlined.

A.



Transcription terminator

B.

Pst I *smtA 5'*

5'**CTGCAGCACTGGTTTGTCATGAGCCAATCACGGTTTGTCCACCCACCATACCTGAATCAAGATTCAGATGT**

TAGGCTAAACACATGAACAGTTATTCAGATATTCA*GGATCC*3'

BamH I

C.

Pst I *smtA 5'*

5'**CTGCAGCACTGGTTTGTCATGAGCCAATCACGGTTTGTCCACCCACCATACCTGAATCAAGATTCAGATG**

TTAGGCTAAACACATGAACAGTTATTCAGATATTCA*GGATCCCCGGGAATTCATCGAGCAACATATTAATGA*

BamH I

GCCAGAGAAATGCTGGCGGCACTGAAAGTTTTTGTACAAGCCGATGAAAGCGGCGACGCGCAGTTAATCCCA

CAGCCGCCAGTTCCGCTGGCGGCATTTTAACTTTCTTTATCACACAGGAAACAGCT ATG ACC ATG ATT

Met Thr Met Ile

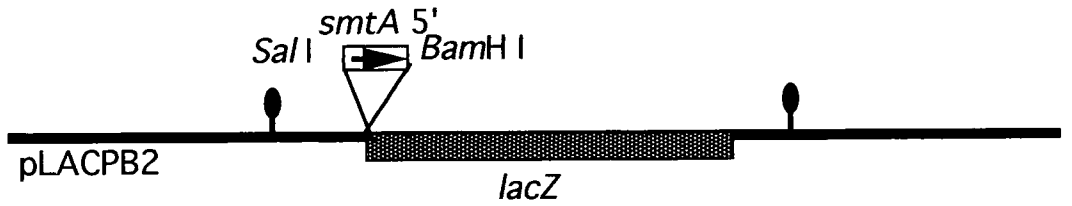
ACG GAT TCA CTG GCC GTC3'

Thr Asp Ser Leu Ala Val

lacZ

Figure 2.5 Production of a truncated 5' region of the *smtA* gene, fused to a promoterless *lacZ* gene. Panel A is a schematic representation of the *smtA* 5'deletion2/*lacZ* gene construct. Panel B is the nucleotide sequence of part of the 5' flanking region of the *smtA* gene in pGEM4Z. The PCR primer used for amplification of the *smtA* 5' truncated region includes a *Sal* I site. This primer was used with the M13 universal forward primer to amplify to a fragment that includes the *Sal* I restriction site and a *Bam*H I site which allow cloning of the truncated product into pLACPB2. Panel C is the nucleotide sequence of the 5' truncation of the *smtA* gene fused to pLACPB2, designated pLACPB2(*smt*-5'del2). The *smt* sequence is shown in bold, inverted repeat1, the direct repeat and the -10 sequence are underlined.

A.



Transcription terminator

B.

M13 forward primer

Sal I Primer

3'——5'

5'*GTCGAC*CTGAATCAAGATTCAGATGTTAGG3'

5'**CACCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGATATTCA***GGATCC* 3'

5'*smtA* *BamH I* pGEM4Z

C.

Sal I 5'*smtA* *BamH* I

5'*GTCGAC***CTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGATATTCA***GGATCC*CCGGGA

ATTCATCGAGCAACATATTAATGAGCCAGAGAAATGCTGGCGGCACTGAAAGTTTTTGTACAAGCCGATGAAAGC

GGCGACGCGCAGTTAATCCCACAGCCGCCAGTTCCGCTGGCGGCATTTTAACTTTCTTTATCACACAGGAAACAG

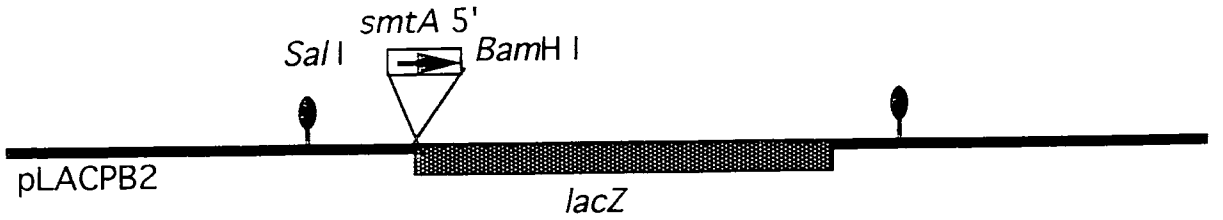
CT ATG ACC ATG ATT ACG GAT TCA CTG GCC GTC3'

Met Thr Met Ile Thr Asp Ser Leu Ala Val

lacZ

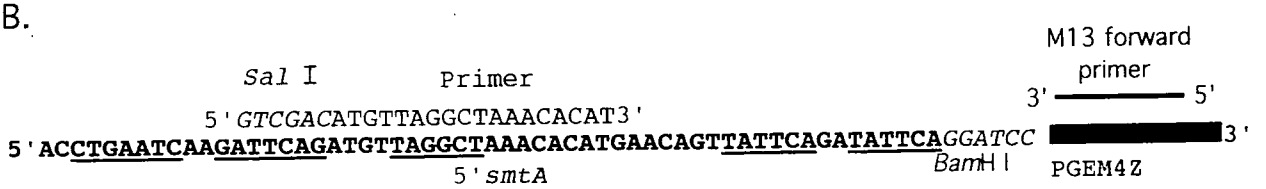
Figure 2.6 Production of a truncated 5' region of the *smtA* gene, fused to a promoterless *lacZ* gene. Panel A is a schematic representation of the *smtA* 5'deletion3/*lacZ* gene construct. Panel B is the nucleotide sequence of part of the 5' flanking region of the *smtA* gene in pGEM4Z. The PCR primer used for amplification of the *smtA* 5' truncated region includes a *Sal* I site. This primer was used with the M13 universal forward primer to amplify to a fragment that includes the *Sal* I restriction site and a *Bam*H I site which allow cloning of the truncated product into pLACPB2. Panel C is the nucleotide sequence of the 5' truncation of the *smtA* gene fused to pLACPB2, designated pLACPB2(*smt*-5'del3). The *smt* sequence is shown in bold, inverted repeat1, the direct repeat and the -10 sequence are underlined.

A.



Transcription terminator

B.



C.

```
   Sal I                  5' smtA                          BamH I
5' GTCGACATGTTAGGCTAAACACATGAACAGTTATTCAGATATTCAGGATCCCCGGGAATTCATCGAGCAACAT
ATTAATGAGCCAGAGAAATGCTGGCGGCACTGAAAGTTTTTGTACAAGCCGATGAAAGCGGCGACGCGCAGTTAA


TCCCACAGCCGCCAGTTCCGCTGGCGGCATTTTAACTTTCTTTATCACACAGGAAACAGCT ATG ACC ATG

                                                                Met Thr Met

ATT ACG GAT TCA CTG GCC GTC3'
Thr Asp Ser Leu Ala Val Ile
          lacZ
```

Figure 2.7 Summary of the *smtA* 5' reporter gene fusion constructs. Panel A is a schematic representation of the *smt* locus. The divergent genes *smtA* and *smtB* are shown as black rectangles. The 100 b.p. operator/promoter region is expanded to show the sequence including inverted repeat 1 (converging arrows) and the direct repeat (unidirectional arrows). Other features include the determined transcript start sites (bent arrows), -10 motif (hatched box) and putative terminators (circle). Panel B is a schematic representation of the fusion of the *smtA* 5' region to *lacZ* as in the vector pLACPB2(*smt*-5'). Panel C is a schematic representation of the fusion of the truncated *smtA* 5' region to *lacZ* as in the vector pLACPB2(*smt*-5'del1). Panels D, E and F show the regions of the *smt* operator/promoter region fused to *lacZ* in the constructs pLACPB2(*smt*-5'del1), pLACPB2(*smt*-5'del2) and pLACPB2(*smt*-5'del3), respectively.

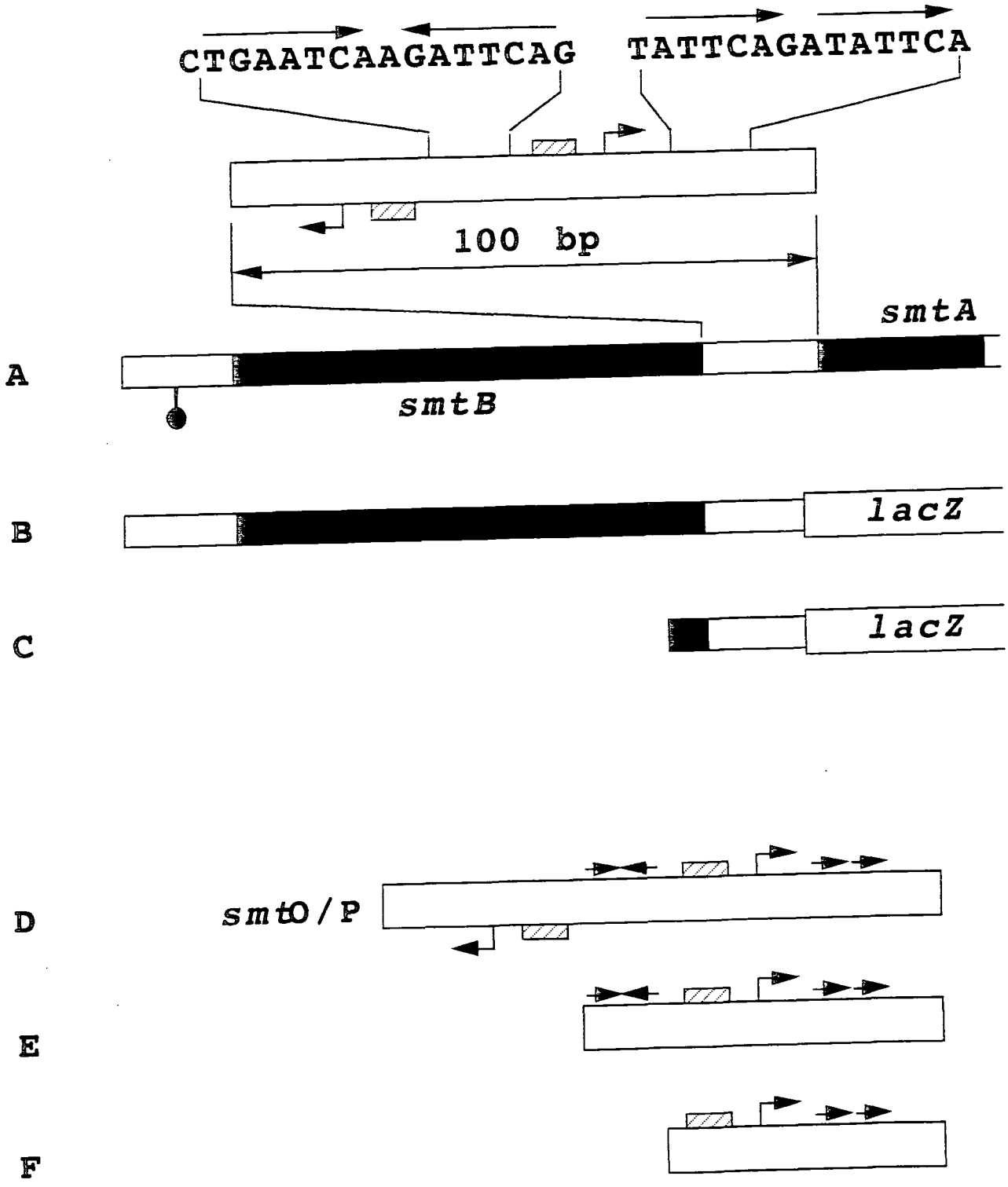
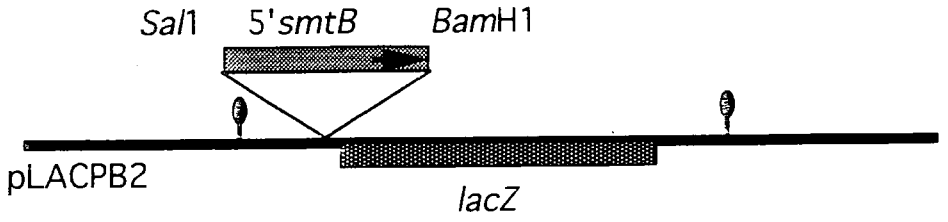

CTGAATCAAGATTCAG
TATTCAGATATTCA
100 bp
smtA
A
smtB
B
lacZ
C
lacZ
D
smtO/P
E
F

Figure 2.8 Fusion of the 5' region of the *smtB* gene to a promoterless *lacZ* gene to produce the construct pLACPB2(*smtB*-5'1.2). Panel A is a schematic representation of the *smtB* 5'/*lacZ* gene construct. Panel B is the nucleotide sequence of part of the 5' flanking region of the *smtB* gene and the PCR primer used for amplification of the *smtB* 5' region which includes a *Bam*H I site to allow cloning of the PCR product into pLACPB2. Panel C is the nucleotide sequence of the fusion between the *smtB* 5' region and the region of translation initiation in *lacZ*. The *smt* sequence is shown in bold and the -10 sequence is underlined.

A.



B.

Primer BamH1

3'ATACCACCCACCTGTTTGG*CCTAGG*5'

5'**AATAACTGTTCATGTGTTTAGCCTAACATCTGAATCTTGATTCAGGTATGGTGGGTGGACAAACC**

GTGATTGGCTC ATG ACA AAA CCA GTG CTG3'

Met Thr Lys Pro Val Leu

smtB

C.

5' *smtB* BamH1

5'**AATAACTGTTCATGTGTTTAGCCTAACATCTGAATCTTGATTCAGGTATGGTGGGTGGACAAACC***GGATCCC*

CGGGAATTCATCGAGCAACATATTAATGAGCCAGAGAAATGCTGGCGGCACTGAAAGTTTTTGTACAAGCCGA

TGAAAGCGGCGACGCGCAGTTAATCCCACAGCCGCCAGTTCCGCTGGCGGCATTTTAACTTTCTTTATCACAC

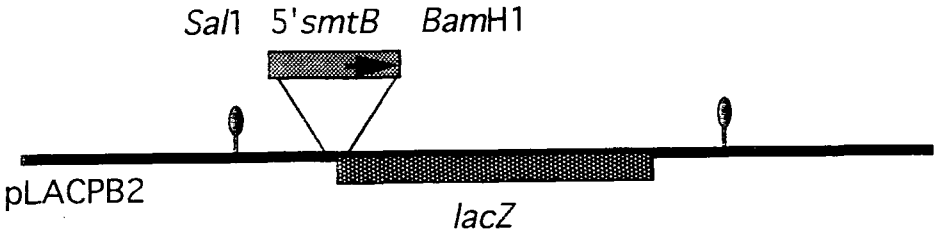
AGGAAACAGCT ATG ACC ATG ATT ACG GAT TCA CTG GCC GTC3'

Met Thr Met Ile Thr Asp Ser Leu Ala Val

lacZ

Figure 2.9 Fusion of a truncated 5' region of the *smtB* gene to a promoterless *lacZ* gene to produce the construct pLACPB2(*smtB*-5'0.2). Panel A is a schematic representation of the *smtB* 5'/*lacZ* gene construct. Panel B is the nucleotide sequence of part of the 5' flanking region of the *smtB* gene and the PCR primers used for amplification of the *smtB* 5' region which include *Bam*H I and *Sal* I restriction sites to allow cloning of the PCR product into pLACPB2. Panel C is the nucleotide sequence of the fusion between the *smtB* 5' region and the region of translation initiation in *lacZ*. The *smt* sequence is shown in bold and the -10 sequence is underlined.

A.



B.

5'**AGTTACAGCCGGTGTGGCCGCAGCCTTTGCTACCACCGGTGTGGCCATCGGCACAGGCTTCGCT**
A G A

5'*GTCGAC*AGTTACAICCIGTGTGICC3'

Sal1smtA C-terminal Primer
GCAGTAGTACAGACCGTTGCGATCGATCCGCTTTGCTGGGATCGACGTTGCAGAGACAGGGCTCAC

AAGCGCATTTGACCAACCGTTGTTGAGGTCATGACAGCAACTCCTTTGAATATCTGAATAACTGTT

Primer *BamH1*
3'ATACCACCCACCTGTTTGGC*CTAGG*5'

CATGTGTTTAGCCTAACATCTGAATCTTGATTCAGGTATGGTGGGTGGACAAACCGTGATTGGCTC

ATG ACA AAA CCA GTG CTG3'
Met Thr Lys Pro Val Leu
smtB

C.

Sal1
5'*GTCGAC***AGTTACAGCCGGTGTGGCCGCAGCCTTTGCTACCACCGGTGTGGCCATCGGCACAGGCTTCGCT**

GCAGTAGTACAGACCGTTGCGATCGATCCGCTTTGCTGGGATCGACGTTGCAGAGACAGGGCTCAC

AAGCGCATTTGACCAACCGTTGTTGAGGTCATGACAGCAACTCCTTTGAATATCTGAATAACTGTT

BamH1
CATGTGTTTAGCCTAACATCTGAATCTTGATTCAGGTATGGTGGGTGGACAAACC*GGATCC*CCCGGG

AATTCATCGAGCAACATATTAATGAGCCAGAGAAATGCTGGCGGCACTGAAAGTTTTTGTACAAGC

CGATGAAAGCGGCGACGCGCAGTTAATCCCACAGCCGCCAGTTCCGCTGGCGGCATTTTAACTTTC

TTTATCACACAGGAAACAGCT ATG ACC ATG ATT ACG GAT TCA CTG GCC GTC3'
Met Thr Met Ile Thr Asp Ser Leu Ala Val
lacZ

Figure 2.10 Construction of hybrid promoters. Panel A i) and ii) are sequences of oligonucleotides which were annealed to produce the hybrid promoter containing inverted repeat1, (represented by the arrows) panel A iii). An equivalent promoter was produced without the inverted repeat via the annealing of the oligonucleotides shown in Panel B i) and ii), this produced the promoter shown in panel B iii). *Sal* I and *Bam*H I half restriction sites were included on the ends of the promoters, which enable cloning into pLACPB2. The λ P_R - 35 sequence (TTGACT) and the *tac* - 10 sequence (TATAAT) are indicated in bold.

A.

i) 5'TCGACTTGACTGAATCAAGATTCAGCGGTTATAATGTGTGGAAG3'

ii) 5'GATCCTTCCACACATTATAACCGCTGAATCTTGATTCAGTCAAG3'

*Sal*1
half site

iii) 5'TCGAC**TTGACT**GAATCAAGATTCAGCGGT**TATAAT**GTGTGGAAG3'
3'GAACTGACTTAGTTCTAAGTCGCCAATATTACACACCTTCCTAG5'
*Bam*H1
18b.p. half site

B.

i) 5'TCGACTTGACTATTTTACCTCTGGGCGGTTATAATGTGTGGAAG3'

ii) 5'GATCCTTCCACACATTATAACCGCCCAGGGTTAAAATAGTCAAG3'

*Sal*1
half site

iii) 5'TCGAC**TTGACT**ATTTTACCTCTGGGCGGT**TATAAT**GTGTGGAAG3'
3'GAACTGATAAAATGGAGACCCGCCAATATTACACACCTTCCTAG5'
*Bam*H1
18b.p. half site

Chapter 3

RESULTS.

3.1 Structural characterisation of a prokaryotic MT gene.

Prior to the results described in this thesis, preliminary experiments had shown that PCR products of the appropriate size could by amplified using degenerate primers designed from the sequence of the *Synechococcus* MT and DNA from *Synechococcus* PCC 6301 (R.Marshall, MSc dissertation, University of Durham).

This chapter describes the structural characterisation of a prokaryotic MT gene, initially via the polymerase chain reaction (PCR), followed by the isolation of a genomic clone. The nucleotide sequence of the genomic clone contained two open reading frames. The open reading frame encoding the prokaryotic MT gene is designated *smtA*, the second open reading frame is designated *smtB* and the locus, *smt*. The expression of the *smt* genes was also studied using northern analysis and reporter gene constructs.

3.1.1 Characterisation of the prokaryotic MT gene *smtA* via PCR.

PCR was performed as described in section 2.3.4.13.1. The products of the PCR were separated on a 2.0 % agarose gel (figure 3.1). Two amplification products of 144 b.p. and 116 b.p. were obtained. The fragments were isolated from agarose gels as described in section 2.3.4.6, digested with *Sal* I and *Eco*R I restriction endonucleases (restriction sites were included near the 5' ends of the primers), ligated to *Sal* I and *Eco*R I cut pUC19 and introduced into *Escherichia coli* DH5α competent cells (as described in section 2.3.4.4). The resulting transformants were subject to *in situ*

colony screening as described in section 2.3.4.12. Positive colonies were used for plasmid preparations (plasmids were designated pJHNR11 and pJHNR12). Both strands of the plasmid inserts were sequenced.

The nucleotide, and encoded amino acid, sequence of the 144 b.p. product, is shown in figure 3.2. The nucleotide sequence of the 116 b.p. fragment revealed that it was a product of primer mismatch due to the presence of an internal gene region that has homology to the C-terminal primer-binding site, giving rise to a truncated product (refer to figure 3.2).

3.1.2 Chromosome crawling by APCR.

A genomic Southern blot of restricted DNA from *Synechococcus* PCC 6301 was probed with the 144 b.p. insert of plasmid pJHNR11 (figure 3.3). A single hybridising band was observed for each digest. A *Hind* III digestion was chosen to produce the template DNA for APCR, as the restriction fragment containing the *smtA* gene was considered to be short enough (2.8 k.b.) to be spanned by *Taq* polymerase (figure 3.3). APCR was performed as described in section 2.3.4.13.3.

The products of the APCRs were separated on an 0.8% agarose gel (figure 3.4). A variety of non-specific products were produced by APCR; however, specific *smtA* products were revealed when the gel was used for Southern analysis and probed with the insert of pJHNR11, refer to section 3.1.1 (figure 3.4). Subsequent APCRs were performed and the specific products were isolated from agarose gels as described in section 2.3.4.6. Several unsuccessful attempts were made to clone the 3' APCR product. The 5' product was cloned, after treatment with T4 polymerase and blunt end ligation to pUC19. The 3' APCR product was never cloned. PCR fragments are difficult to clone due to 3' overhangs caused by *Taq* DNA polymerase having terminal deoxynucleotidyl transferase activity, although treatment with T4 polymerase should facilitate blunt end cloning of PCR products.

The 5' product was cloned and sequenced, and the consensus sequence derived from 4 independent clones in pUC19, (pJHNR21, pJHNR22, pJHNR23 and pJHNR24) each from independent APCRs is shown in figure 3.5. The sequence contained the 15 b.p. that were missing from the partial sequence, (figure 3.1) the correct sequence of the N-terminal primer binding site, the ribosome binding site and the translational start codon. No further sequence analysis was carried out because a genomic clone was subsequently isolated.

3.1.3 Isolation of a genomic clone containing *smtA* from *Synechococcus* PCC 7942.

A genomic clone (designated pJHNR49) was isolated using the method described in section 2.3.4.19. The genomic clone was restriction mapped and fragments of suitable size were subcloned into pGEM4Z and used for sequencing (figure 3.6). A 6 phase open reading frame map was produced and putative open reading frames were analysed using the computer program DNA Strider. The *smtA* open reading frame and a divergently transcribed open reading frame designated *smtB* were identified (figure 3.7). The genomic sequence is shown in figure 3.8. The *smtA* encoded protein sequence was compared to the protein sequence of Olafson *et al.* (1988) (figure 3.9). The DNA region containing the two open reading frames was designated the *smt* locus, (refer to 4.1.2). The OWL 14.0 data base was searched for sequences similar to SmtB, the ArsR and CadC sequences were most significant, figure 3.10 (analysis carried out by Dr A.P. Morby) (refer to 4.2.3). SmtB shows 54% similarity 31% identity to 267ArsR (*Staphylococcus xylosus* plasmid pSX267) and 47% similarity 30% identity to 258CadC (*Staphylococcus aureus* plasmid pI258).

3.2 Determination of the *smtA* and *smtB* transcript start sites by primer extension.

The transcript start sites of *smtA* and *smtB* were determined via primer extension as described in 2.3.4.17. The result of the *smtA* primer extension reaction is shown in figure 3.11 and the transcriptional start site is marked on figure 3.8. This predicts a *smtA* transcript length of 285 bases. The result of the *smtB* primer extension analysis is shown in figure 3.12 and the transcriptional start site is marked on figure 3.8. This predicts a *smtB* transcript length of 491 bases. The predicted lengths of these transcripts agree with the sizes observed on northern blots (figures 3.13 and 3.16).

3.3 Characterisation of expression of *smt* genes.

3.3.1 The relative abundance of *smt* transcripts in response to metal ion exposure.

3.3.1.1 Northern blot analysis of *smtA* transcript abundance in *Synechococcus* PCC 6301.

In order to ascertain whether *smtA* transcripts increase in abundance with exposure to Cd^{2+}, cultures of *Synechococcus* PCC 6301 were exposed to 2.5 μM $CdCl_2$ for different times (0, 5, 10, 15, 20, 30 and 60 min) as described in 2.3.4.20. RNA was purified and used for northern analysis as described in 2.3.4.21, the insert of pJHNR11 was used as the *smtA* probe. The relative abundance of *smtA* transcripts increased with time of exposure reaching a maximum after 30 min (figure 3.13).

Synechococcus PCC 6301 cultures were also exposed to a range of metal salts ($CdCl_2$, $CoCl_2$, $CrCl_2$, CuCl, $HgCl_2$, $NiCl_2$,$PbCl_2$ and $ZnCl_2$) at various concentrations (0, 1, 2.5, 5, 10 μM) for 2 h prior to carrying out northern blot analysis as described above. An increase in transcript abundance was observed in response to exposure to $HgCl_2$, $CoCl_2$, $CdCl_2$, $ZnCl_2$ and CuCl, (figure 3.14). A heat shock control was also included, where *Synechococcus* PCC 6301 cultures were exposed to

conditions that are known to give rise to the production of heat shock proteins in this organism, figure 3.14.

3.3.1.2 Northern blot analysis of the relative abundance of *smtA* transcripts in *Synechococcus* PCC 7942.

Synechococcus PCC 7942 (PIM8) cultures were exposed to $CdCl_2$ at various concentrations, and RNA extraction and northern analysis were performed as described for *Synechococcus* PCC 6301 (section 2.3.4.21). A similar pattern in the relative abundance of *smtA* transcripts was observed for *Synechococcus* PCC 7942 (PIM8) as for *Synechococcus* PCC 6301, figure 3.15.

3.3.1.3 Northern blot analysis of *smtB* transcripts from *Synechococcus* PCC 7942.

Synechococcus PCC 7942 (PIM8) cultures were used for northern analysis (as described in section 2.3.4.21). The probe was to a region of the *smtB* gene (the extreme 5' *Pst* I fragment, figure 3.6). The gel used for the northern blot had to be very heavily loaded with RNA before the *smtB* transcripts could be detected (figure 3.16), suggesting that *smtB* transcripts were of lower abundance than *smtA* transcripts. *smtB* transcripts, as is the case with *smtA* transcripts, increase with increased exposure to $CdCl_2$, figure 3.16.

3.3.2 The use of rifampicin to analyse *smtA* transcript stability.

Rifampicin was used block transcription in *Synechococcus* PCC 7942 (PIM8) cultures as described in 2.3.4.22. *smtA* transcript abundance increased with increased time of exposure to $CdCl_2$, figure 3.17 panels B and C. In cultures exposed to $CdCl_2$+ rifampicin and cultures exposed to rifampicin alone, *smtA* transcripts could not be detected when the northern blot was exposed to film for 4 days, figure 3.17 panel

B. Upon prolonged exposure (figure 3.17 panel C), it was observed that the rate of *smtA* transcript degradation was the same in cells whether treated with $CdCl_2$ + rifampicin or rifampicin alone.

3.3.3 Fusion of *smt* DNA regions to the reporter gene *lacZ*.

3.3.3.1 Fusion of the 5' *smtA* region to the reporter gene *lacZ*.

The PCR product described in section 2.3.4.13.5 was subcloned into pGEM4Z and sequenced. No PCR-mediated errors were detected. The fragment was then cloned into the *Sal* I and *Bam*H I restriction sites in pLACPB2 to produce the vector pLACPB2(*smt*-5'). pLACPB2 and pLACPB2(*smt*-5') were used to transform *Synechococcus* PCC 7942 (PIM8) cells as described in 2.3.4.23. Transformants were checked by plasmid recovery into *Escherichia coli* and restriction mapping.

3.3.3.1.1 β-galactosidase activity of pLACPB2(*smt*-5')- containing *Synechococcus* PCC 7942 (PIM8).

Figure 3.18 shows the β-galactosidase activity of cultures of pLACPB2- and pLACPB2(*smt*-5')- containing *Synechococcus* PCC 7942 (PIM8) relative to the Synechococcus transformant not exposed to metal. The β-galactosidase assays were performed initially as described by Miller (1972) (section 2.3.3.1). These cultures were exposed to the same metal salts at the same concentrations as for northern analysis (refer to section 2.3.4.20) for 2 h prior to the β-galactosidase assay. An increase in β-galactosidase activity (relative to the transformant that was not exposed to added metal) was observed for cells carrying pLACPB2(*smt*-5') on exposure to $ZnCl_2$, $CdCl_2$, CuCl and $HgCl_2$ the most potent induction being form $ZnCl_2$ exposure. The control strain containing pLACPB2 without the *smt* promoter showed no increase in β-galactosidase activity on exposure to any metal salt. Exposure to $CrCl_2$, $CoCl_2$,

$PbCl_2$ and $NiCl_2$ did not increase β-galactosidase activity in cells carrying pLACPB2(*smt*-5').

β-galactosidase assays were also carried out on cultures that had been exposed to minimum inhibitory concentrations (MIC) and maximum permissive concentrations (MPC) of the respective metal salts (refer to table 3.1). $PbCl_2$ gave very high and variable MPC/MIC values, probably because of the poor solubility of lead salts, and therefore was not used in subsequent studies.

Figure 3.19 shows the relative β-galactosidase activity detected in extracts of cultures exposed to metal salts at these biologically significant levels. The most potent induction of β-galactosidase activity was observed upon exposure to $ZnCl_2$ at these levels followed by CuCl=$CdCl_2$. Slight induction was observed with $CoCl_2$ and $NiCl_2$. There was no significant induction of activity in response to $HgCl_2$ and $CrCl_2$ exposure.

3.3.3.1.2 β-galactosidase activity of pLACPB2(*smt*-5') containing *Synechococcus* PCC 7942 (PIM8) using the modified β-galactosidase assay.

Cultures of pLACPB2 and pLACPB2(*smt*-5') transformed *Synechococcus* PCC 7942 (PIM8) were used for β-galactosidase assays using the modified protocol 2.3.3.2. The metal salts used and their concentrations were the same as used previously (3.3.3.1.1) with the addition of $Ag(N0_3)$ and $Na(AuCl_4)$. An increase in β-galactosidase activity was observed upon exposure to $ZnCl_2$, $CdCl_2$ and a slight response observed for CuCl figures 3.20a and 3.20b. No other metals gave an induction of β-galactosidase in duplicate experiments.

β-galactosidase assays were also carried out on cultures that were exposed to MIC and MPC levels using the modified assay. Exposure to $ZnCl_2$ gave the most potent induction at these levels followed by $CdCl_2$, CuCl and $HgCl_2$ (figure 3.21). In situ hybridisation was used as described in 2.3.4.12, to examine whether there were any effects of metal ions on plasmid copy number. No significant change in plasmid copy

number was observed for *Synechococcus* transformed with pLACPB2(*smt*-5'), upon exposure to $ZnCl_2$ or $CdCl_2$ (figure 3.22).

3.3.3.1.3 β-galactosidase activity from the 5' *smtA* truncated constructs.

Promoter deletion analysis was used in an attempt to map the functional regions within the *smtA* promoter region. Cultures containing pLACPB2(*smt*-5') and the truncated fusions described in section 2.3.4.13.7 were used for β-galactosidase assays using the modified protocol. The cultures were exposed to $ZnCl_2$ at 0, 5, 11 and 12 μM for 2 h prior to the assay. The results of the β-galactosidase assays are shown in figure 3.23. The pattern of activity of the truncated fusion constructs varied in the two experiments. Although, there were trends in the activity, the basal level (no added metal) of all the truncated fusions in both experiments was higher than the basal level of pLACPB2(*smt*-5'). Cells containing all of the truncated fusions gave decreased metal inducibility compared to pLACPB2(*smt*-5').

3.3.3.2 Expression from a hybrid promoter with and without inverted repeat1.

Inverted repeat1 was added to a hybrid promoter (as described in section 2.3.4.16.1) in an attempt to verify if this was a *cis*-acting sequence in the *smt* promoter. *Synechococcus* transformants containing the hybrid promoter fused to *lacZ* and the hybrid promoter with inverted repeat1 fused to *lacZ* were analysed for β–galactosidase activity using the modified protocol (section 2.3.3.2). The cultures were exposed to $ZnCl_2$ at 0, 5, 11 and 12 μM for 2 h prior to the assay.

The hybrid promoter was very active in *Synechococcus* PCC 7942 with levels of activity of over four times greater than that observed for maximally induced pLACPB2(*smt*-5'). The addition of inverted repeat1 to the promoter gave very low β-galactosidase activity, figure 3.24. The constructs gave similar activities in

Escherichia coli and *Synechococcus* suggesting the effect of the inverted repeat was the same in both hosts (figure 3.25).

3.3.3.3 β-galactosidase activity from the 5' *smtB* constructs.

Cultures containing pLACPB2(*smt*-5') and the *smtB* 5' fusion constructs (described in 2.3.4.13.8) were used for β-galactosidase assays using the modified protocol. The cultures were exposed to $ZnCl_2$ at 0, 5, 11 and 12 μM for 2 h prior to the assay.

The *smtB* constructs did not show $ZnCl_2$ mediated induction of *lacZ*. The activity of the *smtB* fusion constructs was slightly higher than that of pLACPB2(*smt*-5') with no added metal, regardless of the addition of $ZnCl_2$, (figure 3.26). The control constructs in which there was a deletion in the -10 consensus sequence of the *smtB* promoter showed lower activity than the undeleted constructs suggesting that the *smtB* promoter was active in the undeleted constructs.

Table 3.1 Maximum permissive concentration MPC and minimum inhibitory concentration MIC, of the respective metal salts for *Synechococcus* PCC 7942 (PIM8) cultures.

Metal salts	MPC	MIC
$CrCl_2$	25.0μM	30.0μM
$HgCl_2$	0.025μM	0.03μM
$NiCl_2$	3.0μM	4.0μM
$CoCl_2$	3.0μM	4.0μM
$CdCl_2$	1.5μM	2.0μM
CuCl	9.0μM	10.0μM
$ZnCl_2$	11.0μM	12.0μM

Figure 3.1 Visualisation of PCR amplified fragments on a 2.0 % agarose gel stained with ethidium bromide.


bp
ORIGIN
144
116

Figure 3.2 The nucleotide sequence of the 144 b.p. PCR fragment (generated from *Synechococcus* PCC 6301 genomic DNA) shown in figure 3.1 (the insert of plasmid pJHNR11) and the encoded amino sequence. The serine is underlined because this amino acid residue differs from the published MT amino acid sequence by Olafson *et al.*, (1988). A region of internal homology to the C-terminal primer binding site is also underlined, this corresponds to the 3' end of the truncated 116 b.p. product. The bases corresponding to inosine in the PCR primers are shown in bold as they may be incorrectly assigned, (refer to 4.1.1).

Olafson *et al.*, 1988 T S T T L V K C A C E P C L C N V

SmtA **M** T S T T L V K C A C E P C L C N V

D P S K A I D R N G L Y Y C C E A C A D G H T G G S K G

D P S K A I D R N G L Y Y C **S** E A C A D G H T G G S K G

C G H T G C N C

C G H T G C N C **H G**

Figure 3.3 A genomic Southern blot of DNA isolated from *Synechococcus* PCC 6301. DNA was digested to completion with the following restriction endonucleases: lane 1, *Sal* I and *Nco* I; lane 2, *Sal* I and *Bgl* II; lane 3, *Hin*d III; lane 4, *Pst* I; lane 5, *Sal* I; lane 6, *Bam*H I; lane 7, *Kpn* I; lane 8, *Eco*R I. The DNA was then run out on a 1.0% agarose gel, subject to Southern transfer and the blot was probed with ^{32}P-labelled *smtA* probe prepared from the insert of plasmid pJHNR11.

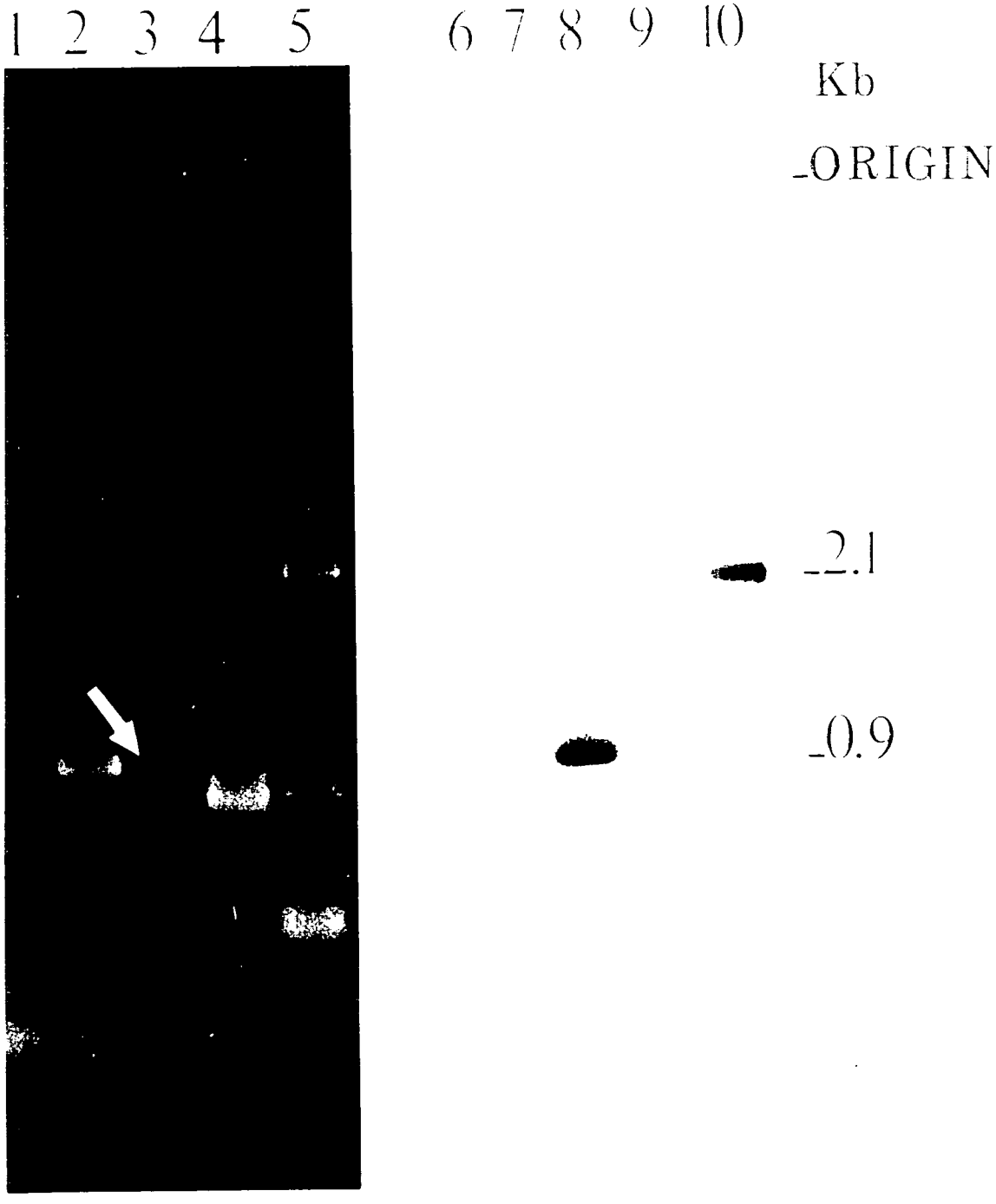

1 2 3 4 5
6 7 8 9 10
Kb
ORIGIN
2.1
0.9

Figure 3.4 Visualisation of APCR fragments on a 0.8% agarose gel. The primers used in the reactions were: lane 1, *smtA* N- and C-terminal primers, no template control ; lane 2, M13 forward and *smtA* C-terminal; lane 3, M13 reverse and *smtA* C-terminal; lane 4 M13 forward and *smtA* N-terminal; lane5, M13 reverse and *smtA* N-terminal. Lanes 6-10 are a Southern blot of lanes 1-5 probed with ^{32}P-labelled *smtA* probe prepared from the insert of plasmid pJHNR11. Two specific products are revealed in lanes 8 and 10. The amplification product which was subsequently cloned and sequenced is indicated in lane 3.

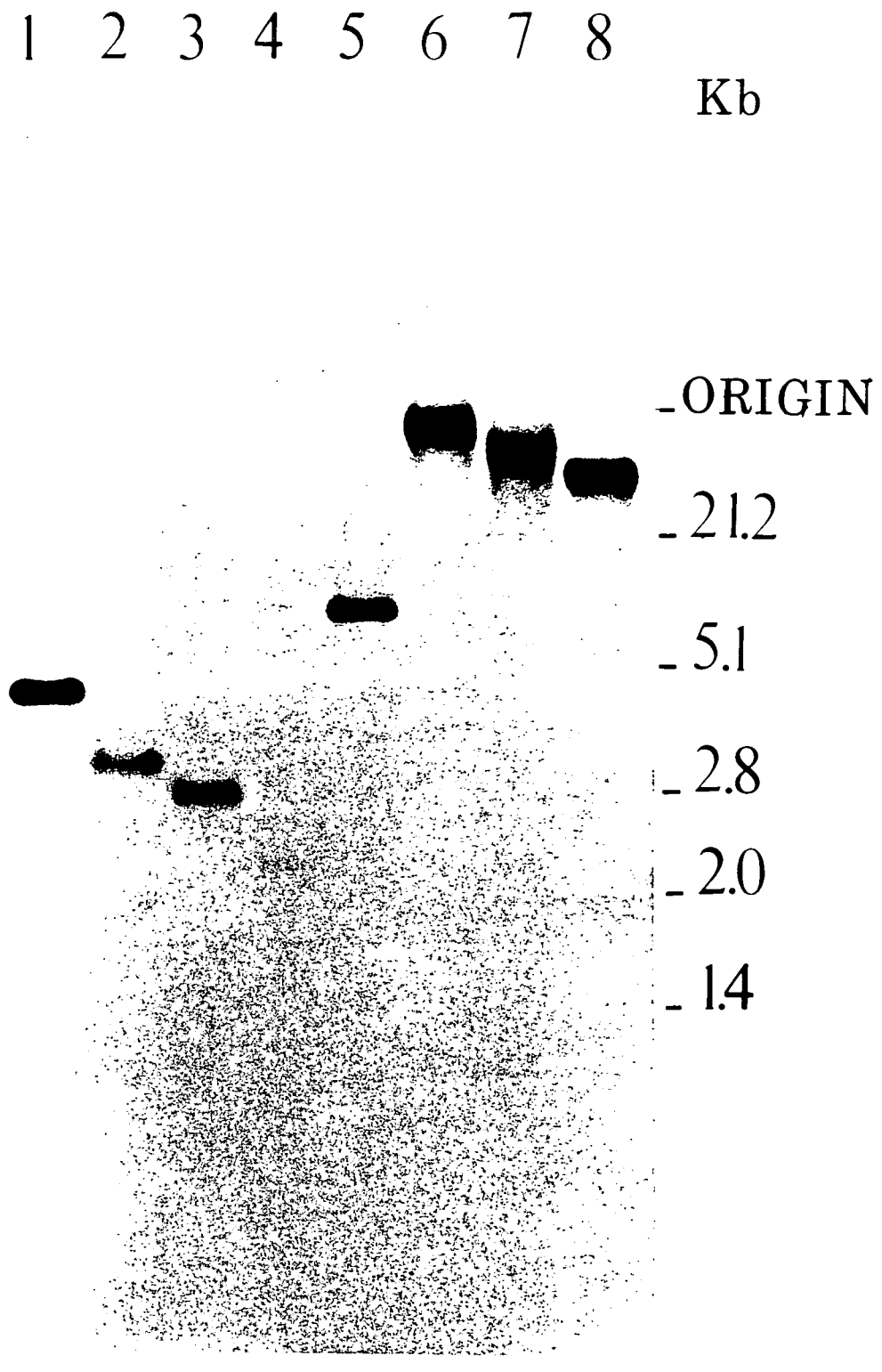

1 2 3 4 5 6 7 8
Kb
ORIGIN
21.2
5.1
2.8
2.0
1.4

Figure 3.5 The sequence of the 5' APCR product, which contains the diagnostic *smtA* coding sequence with the encoded amino sequence. Within the 5' flanking sequence the ribosome binding site and the translational start codon are underlined.

ACTACAACGGGCGCCGCTGATCTACAGCAACTCGATCAAGAACGCTGCCTGAATCCCCAA

GCATTCTTGGGCATGACATATCACGATGCTACTGCGATCGCCCCGACCACTCCCCAGCCG

ATTTCTGCCTAAGGTGCATCTCTAGCGACACTCTTGTAAGTGATCGAGGGCGTTTTGATA

AAGCGCCACAATGTGATGATCCTGTAGCTGGTAGTAGACATGCCGCCCTTGCTTGCGATA

CTCACCAGCCGCAGATTACGGAGCGATCGCAATTGGTGAGGACACCGTCGATTCGGAAAC

ACCAATTGCCTGGGCCAAATCCCCAACACAGAGCTCCGATCGCGCTAAACAGGGACAGCA

ACCGCAGTCGATTTTGGATCGGGCCAGCACTGCAAAAAATTTCGGGTACACCGATTGGGA

ACTTTCGGGTACGATCGCCTTGAAGTCCGAGGCGATCGCCGCAATGAGTCCCTTGGAAAG

ACTACCGTCTCACCCGTCCTGCAGCACTGGTTTTGTCATGAGCCAATCACGGTTTGTCCA

CCCACCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGAT

ATTCAAAGGAGTTGCTGTCATGACCTCAACAACCTTGGTCAAATGCGCTTGTGAGCCCTG
M T S T T L V K C A C E P C

TCTCTGCAACGTCGATCCCAGCAAAGCGATCGATCGCAACGGTCTGTACTACTGCAGCGA
L C N V D P S K A I D R N G L Y Y C S E

AGCCTGTGCCGATGGCCACACCGGTGGTAGCAAAGGCTGCGGCCACACCGGCTGCAACTGN
A C A D G H T G G S K G C G H T G C N C

Figure 3.6 The sequencing strategy for the 1.8 k.b. genomic fragment showing restriction sites: *Hind* III (H), *Sal* I (S), *Pst* I (P), *Sac* I (Sa), *Nae* I (Na), *Nic* I (Nc). The sequenced fragments and the direction of sequencing are indicated with arrows.

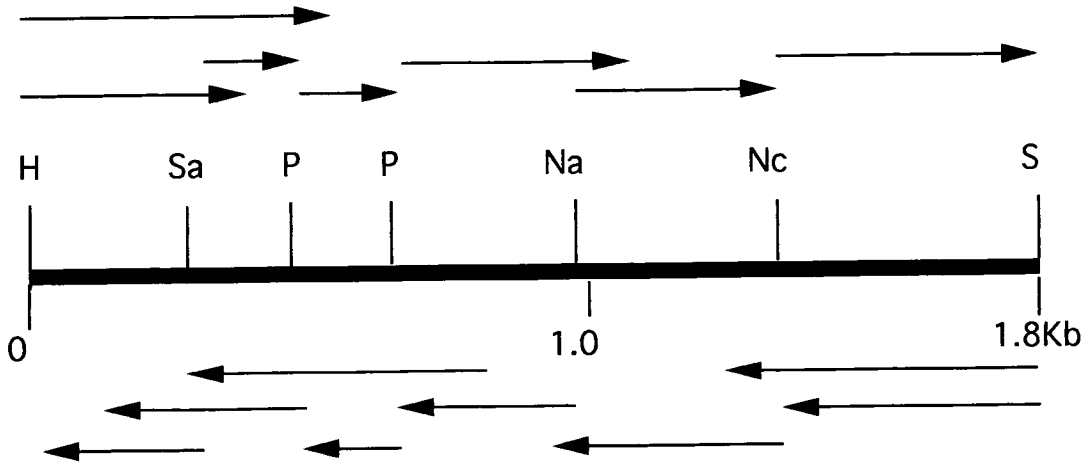

H
Sa
P
P
Na
Nc
S
0
1.0
1.8Kb

Figure 3.7 A 6 phase open reading frame map of the *smt* genomic fragment. The *smtA* and *smtB* open reading frames are indicated by arrows.

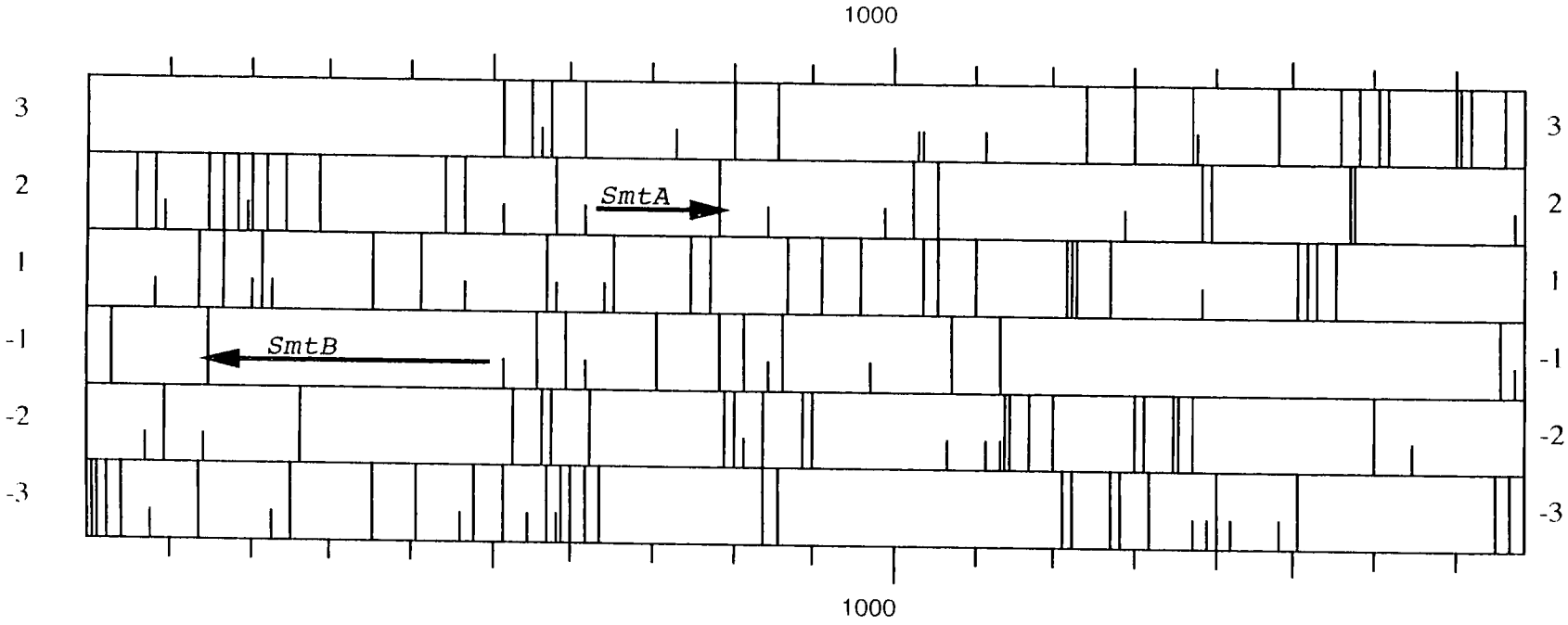
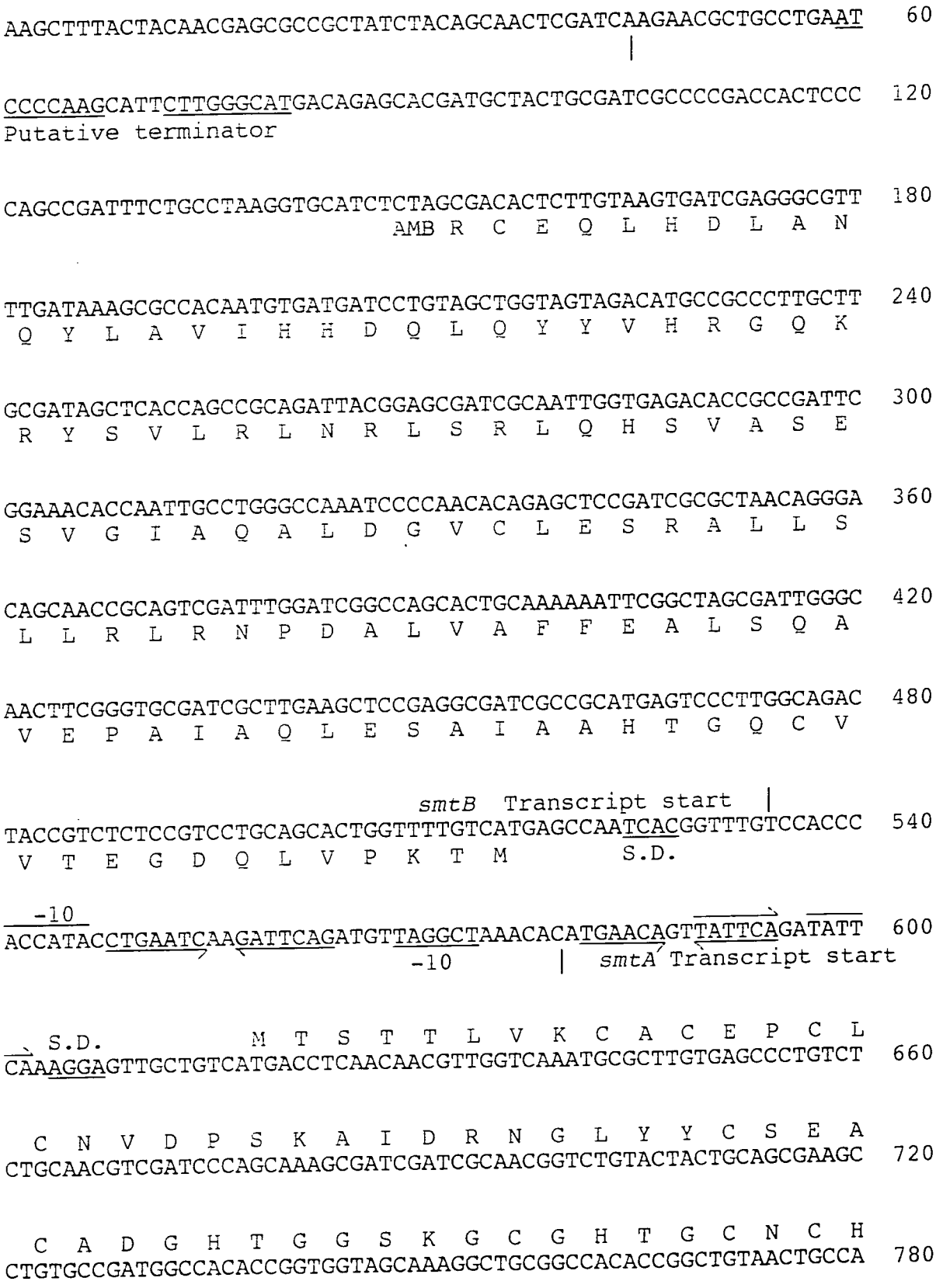

1000
3
2
1
-1
-2
-3
SmtA
SmtB
1000

Figure 3.8 Sequence of the genomic fragment (the insert of plasmid pJHNR49) containing the *smt* locus. The *smt* locus includes the *smtA* gene and a divergently transcribed *smtB* gene with encoded polypeptides. Note that the sequence of SmtB is written from carboxy- to amino-terminus due to the divergent nature of the genes. The transcriptional start sites are shown for both genes. The -10 consensus sequences (-10) and ribosome binding sites (S.D.) of the two genes are indicated. Repeat sequences within the *smt* operator/promoter region are indicated by arrows: CTGAATAAGATTCAG=inverted repeat1, TGAACAGTTATTCA=inverted repeat2, TATTCAGATATTACCATA=direct repeat. Sequences predicted to form stem-loop structures and act as transcriptional terminators are underlined. Predicted transcript termination points are marked with vertical lines (sequence features were identified with the aid of Dr. A.P. Morby).


AAGCTTTACTACAACGAGCGCCGCTATCTACAGCAACTCGATCAAGAACGCTGCCTGAAT 60
CCCCAAGCATTCTTGGGCATGACAGAGCACGATGCTACTGCGATCGCCCCGACCACTCCC 120
Putative terminator
CAGCCGATTTCTGCCTAAGGTGCATCTCTAGCGACACTCTTGTAAGTGATCGAGGGCGTT 180
AMB R C E Q L H D L A N
TTGATAAAGCGCCACAATGTGATGATCCTGTAGCTGGTAGTAGACATGCCGCCCTTGCTT 240
Q Y L A V I H H D Q L Q Y Y V H R G Q K
GCGATAGCTCACCAGCCGCAGATTACGGAGCGATCGCAATTGGTGAGACACCGCCGATTC 300
R Y S V L R L N R L S R L Q H S V A S E
GGAAACACCAATTGCCTGGGCCAAATCCCCAACACAGAGCTCCGATCGCGCTAACAGGGA 360
S V G I A Q A L D G V C L E S R A L L S
CAGCAACCGCAGTCGATTTGGATCGGCCAGCACTGCAAAAAATTCGGCTAGCGATTGGGC 420
L L R L R N P D A L V A F F E A L S Q A
AACTTCGGGTGCGATCGCTTGAAGCTCCGAGGCGATCGCCGCATGAGTCCCTTGGCAGAC 480
V E P A I A Q L E S A I A A H T G Q C V
smtB Transcript start
TACCGTCTCTCCGTCCTGCAGCACTGGTTTTGTCATGAGCCAATCACGGTTTGTCCACCC 540
V T E G D Q L V P K T M S.D.
-10
ACCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGATATT 600
-10 smtA Transcript start
S.D. M T S T T L V K C A C E P C L
CAAAGGAGTTGCTGTCATGACCTCAACAACGTTGGTCAAATGCGCTTGTGAGCCCTGTCT 660
C N V D P S K A I D R N G L Y Y C S E A
CTGCAACGTCGATCCCAGCAAAGCGATCGATCGCAACGGTCTGTACTACTGCAGCGAAGC 720
C A D G H T G G S K G C G H T G C N C H
CTGTGCCGATGGCCACACCGGTGGTAGCAAAGGCTGCGGCCACACCGGCTGTAACTGCCA 780

G OCH

CGGCTAATCAACTGTTTCCCTGCTAATCCCCCATCAATCGAAAACCGCTGGCTCCTCAAT 840

Putative

CATGGGCCAGCGGTTGATTATTTATAGGAGGTGCGATCGCGCAGCTTTACAACCCCTACT 900

terminator

CGCCGGTGATCGAGAGACCTTCGACCCAAACAGCCGGCGAAATCCCACCGGGCGTGATCT 960

GGGCGATCGCATCGACTTGGACGATATGTCGCAACAGTTCCCGAAAATCGCCTGCAACGG 1020

TTGCTGATTCGATGATGACGCGATCGCGTTTGATTGACCAACCAGCCATCAAAGGGCAGC 1080

GAGAACGAGCCTTGCAAGGACTGAACACCCGCATGGAGCGCTTGCAGATCATCGATCAAG 1140

ATCACAGACTCGGCCGTATCGAGGCTCAGTTCCGATCGTGCTTCCGCGGCTTCGACTACC 1200

CCAAGTGGGGGCTAACGGTGACTTTAGCCCCAAGGTTGGCGTGACCGGTCGGGCTGGCAC 1260

CCAGTCGCTTAGCCGTCCCCGCACTATGCAAGAATTTTTAAGTACACCGGCTTCAATCAA 1320

CGGGAGCCGGCGAGTCGGAGTCCCTTCACCGTCAAAAGCTGTCGCCCCCACATTAGATGG 1380

ATGACGGGCATCGTCGTAGAGATTCAACAGCGGCGAGGCATCGCCGTTTCCCAAGGATTC 1440

TGGCGTCGAGAGGCTCTGGCGATCGAGCACACTCTGGGCATTGAACAAGTTGGAAAAGGC 1500

TCCTAGCAGTTGTAGAAACGCTTCTGGTAAGAAGCAGACCAAGTATTTCCCTGAAGTGAT 1560

CGGGCGGTAGTCGAGGTGACTGATCGTTTTCTCTGCTGTCTCAGTAACGCAGCCACTGAA 1620

ATCGAGAATCGGCCAAGCGGTGCGCCATCCGAAACGCCCCCGCACTGCGCGGCTTGCGAT 1680

CGGCCTGCTCGGTCTTGCTGTAGAGGTAGAGCGAAGCGTAGCTGCGTTTCTCCGATCGCA 1740

AGGCCCCAGCGCTATTCAGATAGAAACCCTCATGCGTCGAC 1781

Figure 3.9 A comparison of the *smtA* encoded protein from *Synechococcus* PCC 7942 and the protein sequence of Olafson *et al.* (1988). The differences between the two sequences are indicated in bold in the *smtA* sequence.

Olafson *et al.*, 1988 T S T T L V K C A C E P C L C N V

SmtA **M** T S T T L V K C A C E P C L C N V

D P S K A I D R N G L Y Y C C E A C A D G H T G G S K G

D P S K A I D R N G L Y Y C **S** E A C A D G H T G G S K G

C G H T G C N C

C G H T G C N C **H G**

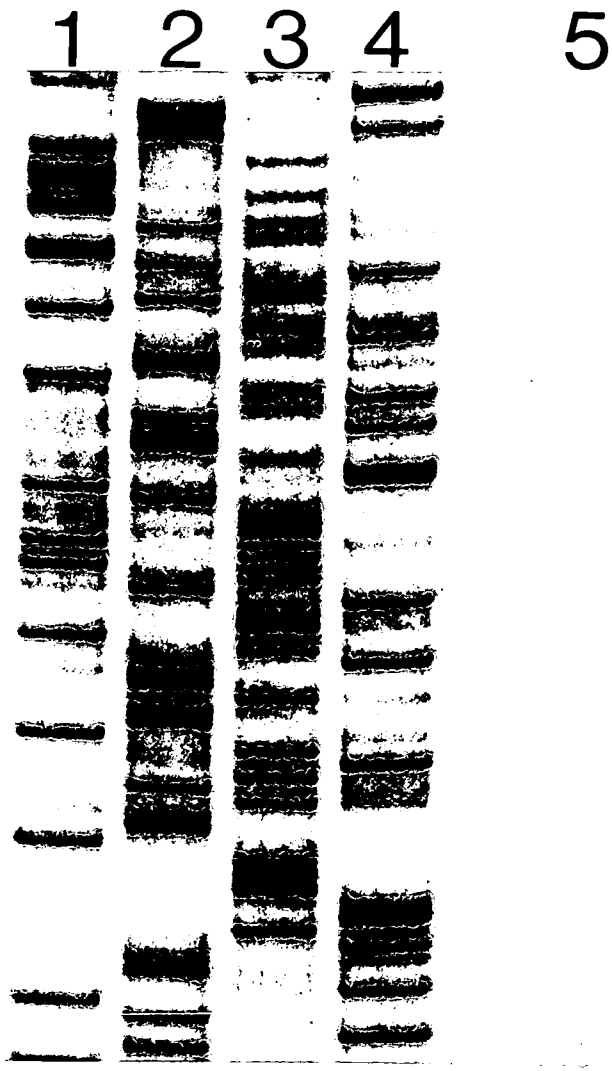
Figure 3.10 Multiple alignment of SmtB (from Huckle *et al.*, 1993). Primary amino acid sequences included are SmtB, 267ArsR (*Staphylococcus xylosus* plasmid pSX267), 773ArsR (*Escherichia coli* plasmid R773), 258ArsR (*Staphylococcus aureus* plasmid pI258), 258CadC (*Staphylococcus aureus* plasmid pI258) and OF4CadC (*Bacillus firmus* strain OF4). A consensus sequence was constructed with a plurality of 5. The underlined region of the SmtB sequence delineates a predicted helix-turn-helix motif.

```
           1                                                  50
   SmtB    ...MTKPVLQ DGETVVCQGT HAAIASE... LQAIAPEVAQ SLAEFFAVLA
267ArsR    .......... .......... .......... MSY......K ELSTILKVLS
773ArsR    .......... .......... .......... .....MLQLT PL.QLFKNLS
258ArsR    .......... .......... .......... MSY......K ELSTILKILS
258CadC    .......... MKKKDTCEIF CYDEEKVNRI QGDLQTVDIS GVSQILKAIA
OF4CadC    .......... VNKKDTCEIF CYDEEKVNRI QGDLKTIDIV SVAQMLKAIA
Consensus  ---------- ---------- ---------- ---------- ------K---

           51                                                 100
   SmtB    DPNRLRLLSL LAR.SELCVG DLAQAIGVSE SAVSHQLRSL RNLRLVSYRK
267ArsR    DPSRLEILDL L.SCGELCAC DLLEHFQFSQ PTLSHHMKSL VDNELVTTRK
773ArsR    DETRLGIVLL LREMGELCVC DLCMALDQSQ PKISRHLAML RESGILLDRK
258ArsR    DSSRLEILDL L.SCGELCAC DLLEHFQFSQ PTLSHHMKSL VDNELVTTRK
258CadC    DENRAKITYA LCQDEELCVC DIANILGVTI ANASHHLRTL YKQGVVNFRK
OF4CadC    DENRAKITYA LCQDEESCVC DIANIIGITA ANASHHLRTL HKQGIVRYRK
Consensus  D--R--I--- L----ELC-C D--------- ---SHHL--L -----V--RK

           101                                                150
   SmtB    QGRHVYYQLQ DHHIVALYQ. .........N ALDHLQ.... ..........
267ArsR    NGNKHMYQL. NH......EF LD....YINQ NLDIINTSDQ RCACKNMKSG
773ArsR    QGKWVHYRLS PHIPSWAAQI IEQAWLSQQD DVQVIARKLA SVNCSGSSKA
258ArsR    DGNKHWYQLN HAILDD.... .......IIQ NLNIINTSNQ RCVCKNVKSG
258CadC    EGKLALYSLG DEHIRQIMMI ALAHKKEVKV NV........ ..........
OF4CadC    EGKLAFYSLD DEHIRQIMMI VLEHKKEVNV NV........ ..........
Consensus  -G----Y-L- ---------- ---------- ---------- ----------

           150
   SmtB    ECR
267ArsR    EC.
773ArsR    VCI
258ArsR    DC.
258CadC    ...
OF4CadC    ...
Consensus  ---
```



5

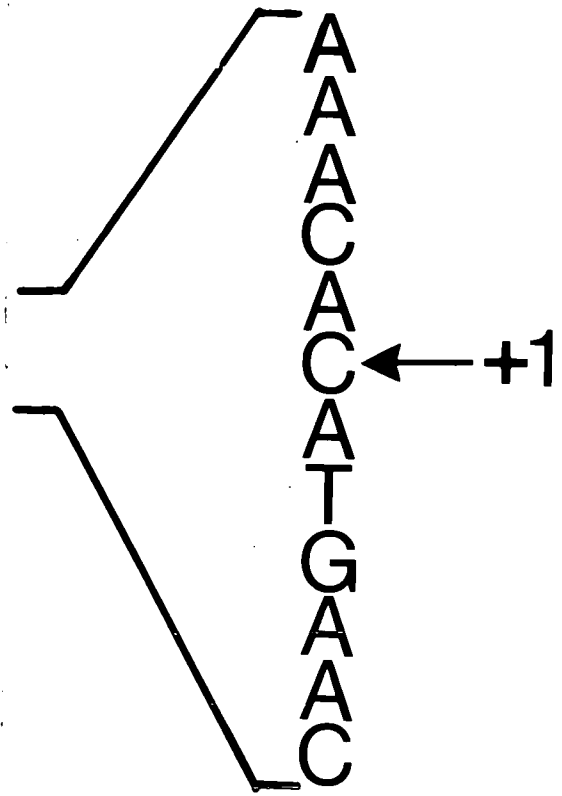


Figure 3.11 Designation of the transcript start site of *smtA* by primer extension analysis. The sequencing ladder was generated using the same primer (5'CA$^{A}/_{G}$TT$^{A}/_{G}$CAICCIGT$^{A}/_{G}$TGICC3') as in the primer extension reaction.

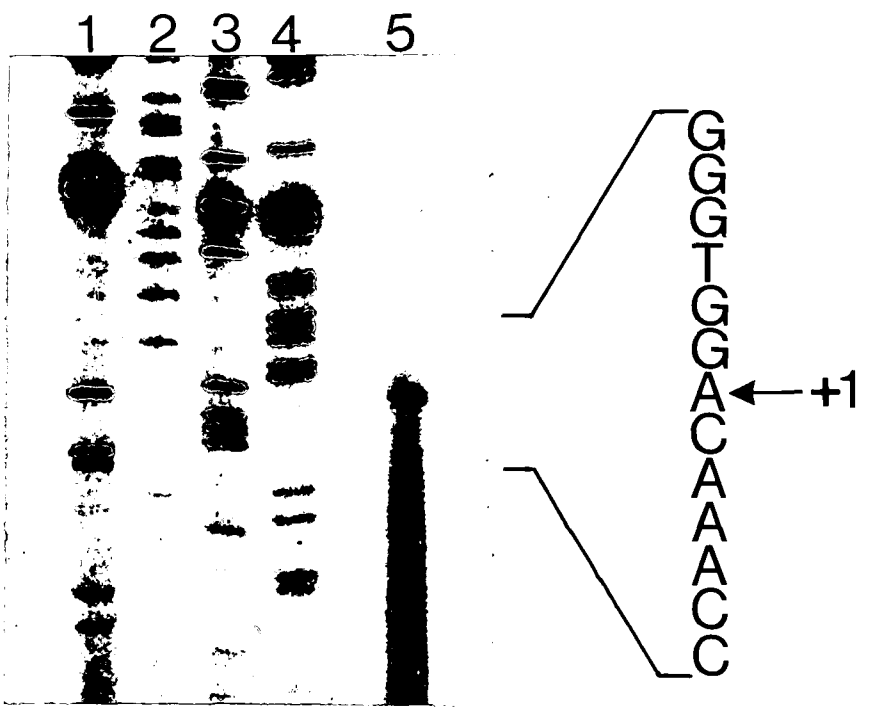

1
2
3
4
5
GGGTGGACAAAACC
+1

Figure 3.12 Designation of the transcript start site of *smtB* by primer extension analysis. The sequencing ladder was generated using the same primer (5'GTCCCTTGGCAGACTACCGTCT3') as in the primer extension reaction.

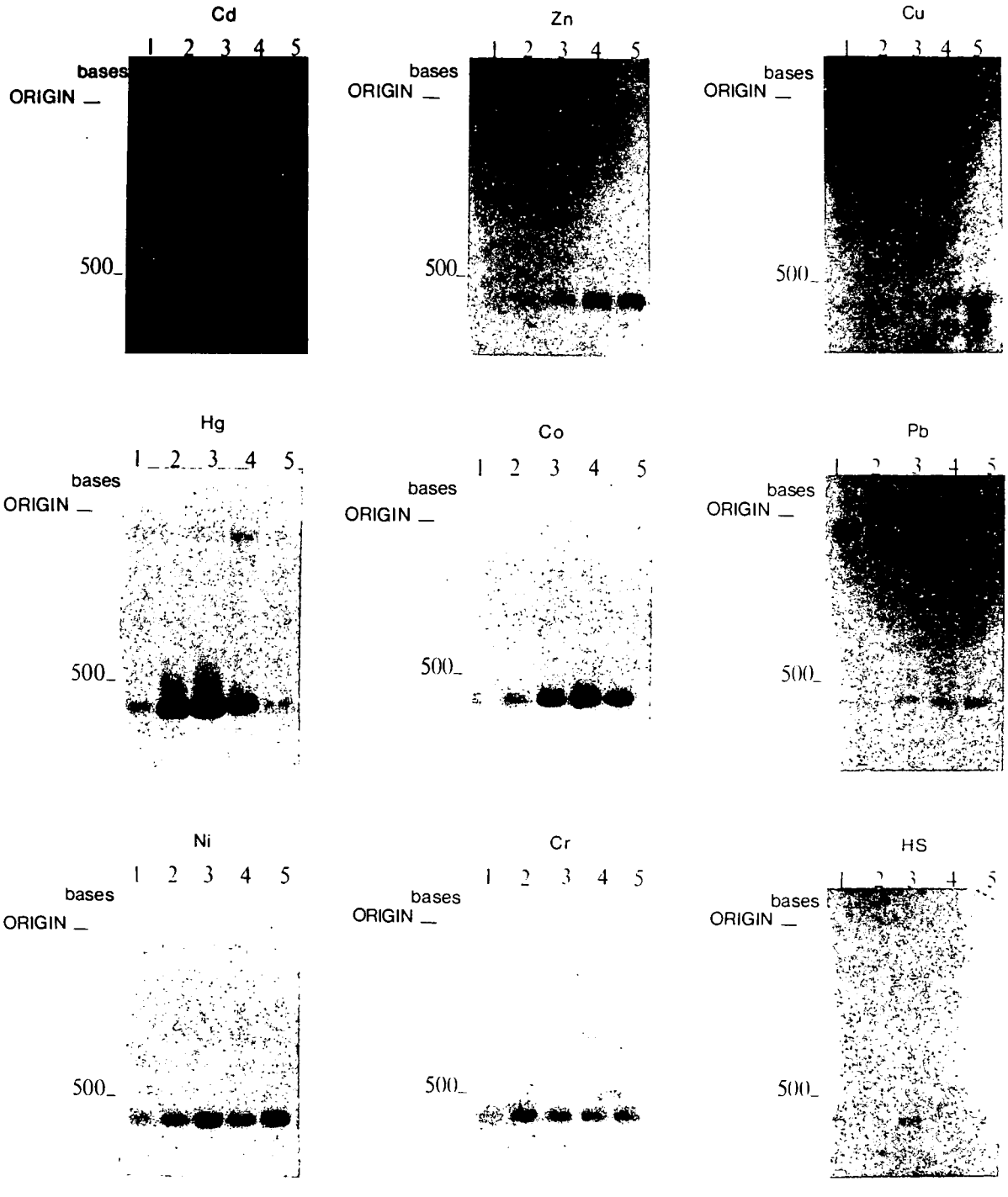

Cd
1 2 3 4 5
bases
ORIGIN
500
Zn
1 2 3 4 5
bases
ORIGIN
500
Cu
1 2 3 4 5
bases
ORIGIN
500
Hg
1 2 3 4 5
bases
ORIGIN
500
Co
1 2 3 4 5
bases
ORIGIN
500
Pb
1 2 3 4 5
bases
ORIGIN
500
Ni
1 2 3 4 5
bases
ORIGIN
500
Cr
1 2 3 4 5
bases
ORIGIN
500
HS
1 2 3 4 5
bases
ORIGIN
500

Figure 3.13 A northern blot of RNA from *Synechococcus* PCC 6301 showing a time course of induction of *smtA* transcripts in response to 2.5 μM $CdCl_2$. Lane 1, 0 min; lane 2, 5 min; lane 3, 10 min; lane 4, 15 min; lane 5, 20 min; lane 6, 30 min and lane 7, 60 min of exposure prior to RNA extraction.

Figure 3.14 Northern blots of RNA from *Synechococcus* PCC 6301 cultures showing the increase in *smtA* transcript abundance in response to 2 h exposures to metal salts ($CdCl_2$, $CoCl_2$, $CrCl_2$, CuCl, $HgCl_2$, $NiCl_2$, $PbCl_2$ and $ZnCl_2$) at various concentrations (lane 1 = 0 μM; 2 = 1 μM; 3 = 2.5 μM; 4 = 5 μM; 5 = 10 μM). A heat shock (HS) control is also included (lanes 1 = 32 °C; 2 = 45 °C for 10 min; 3 = 45 °C for 20 min; 4 = 55 °C for 10 min; 5 = 55 °C for 20 min).

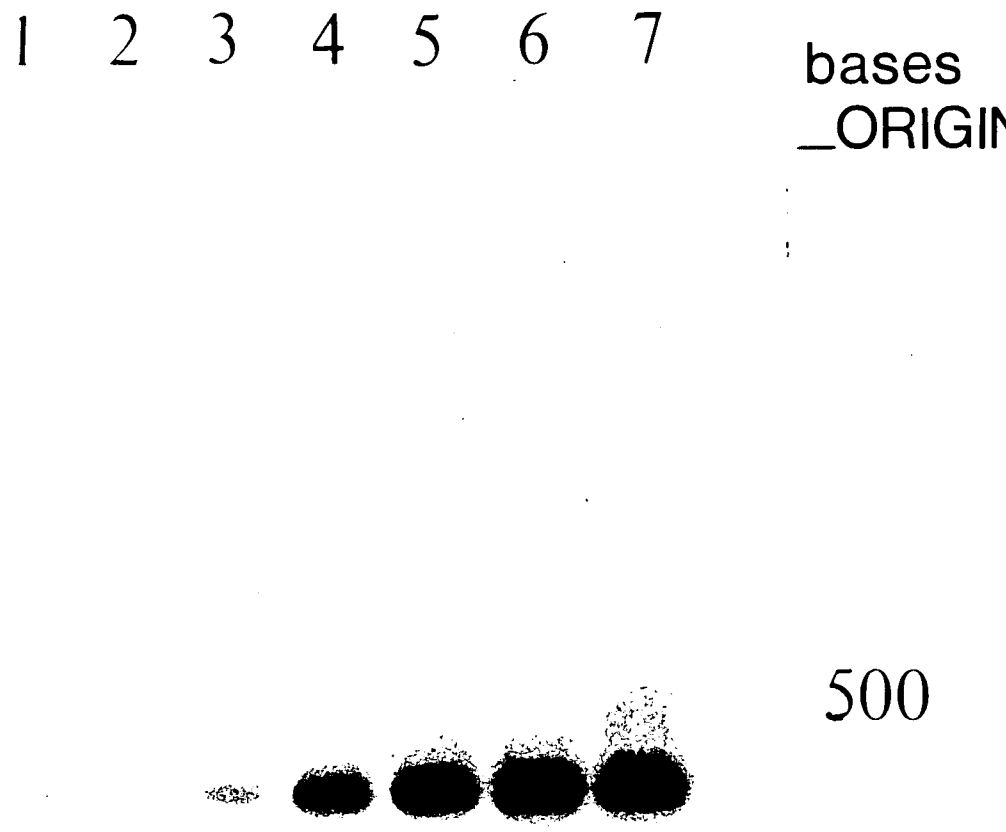
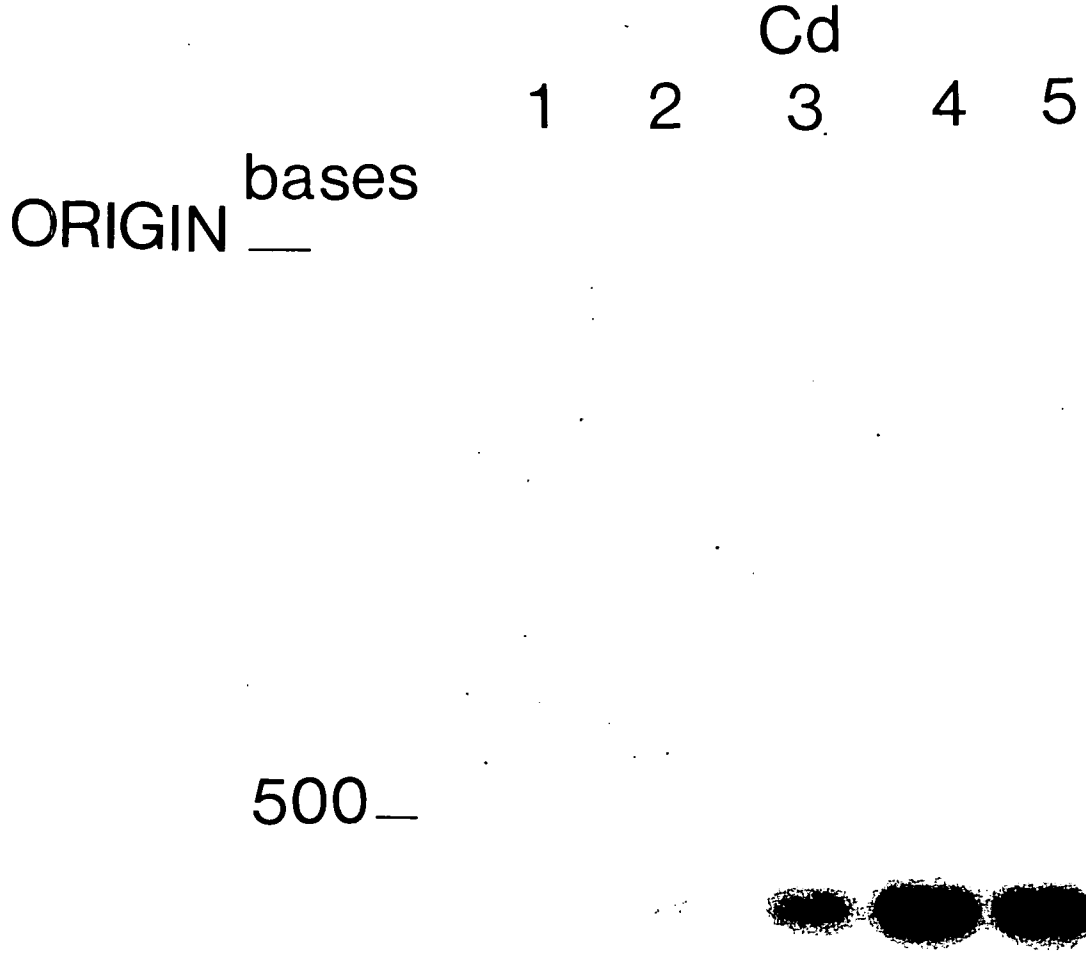

1 2 3 4 5 6 7
bases
_ORIGIN
500

Figure 3.15 A northern blot of RNA from *Synechococcus* PCC 7942 (PIM8) cultures showing the increase in *smtA* transcript abundance in response to a 2 h exposure to $CdCl_2$ at various concentration (lane 1 = 0 μM; 2 = 1 μM; 3 = 2.5 μM; 4 = 5 μM; 5 = 10 μM).

Figure 3.16 A northern blot of RNA from *Synechococcus* PCC 7942 (PIM8) cultures showing the increase in *smtB* transcript abundance in response to a 2 h exposure to $CdCl_2$ at various concentrations (lane 1 = 0 μM; 2 = 1 μM; 3 = 2.5 μM; 4 = 5 μM; 5 = 10 μM).


Cd
1 2 3 4 5
bases
ORIGIN
500

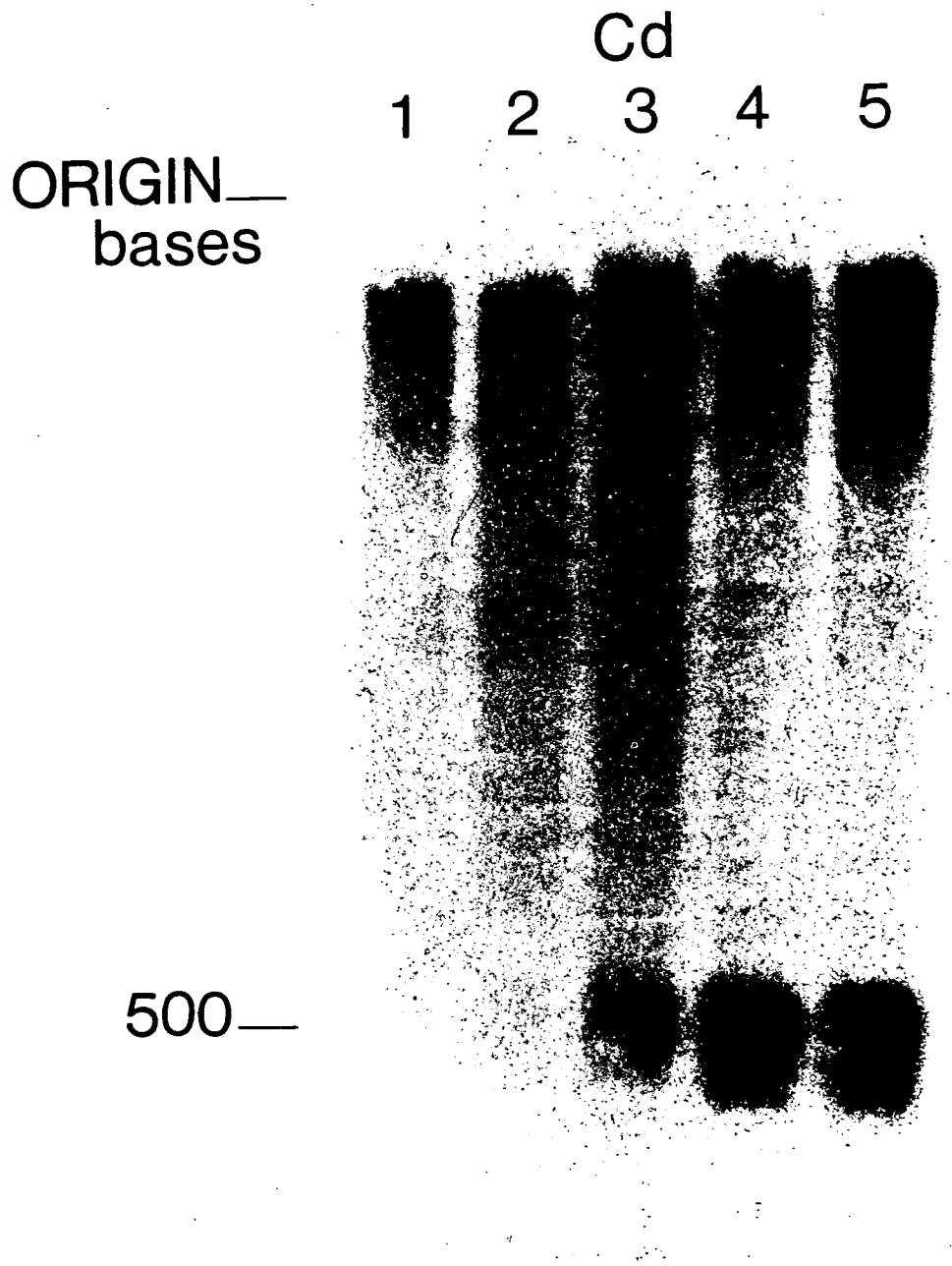
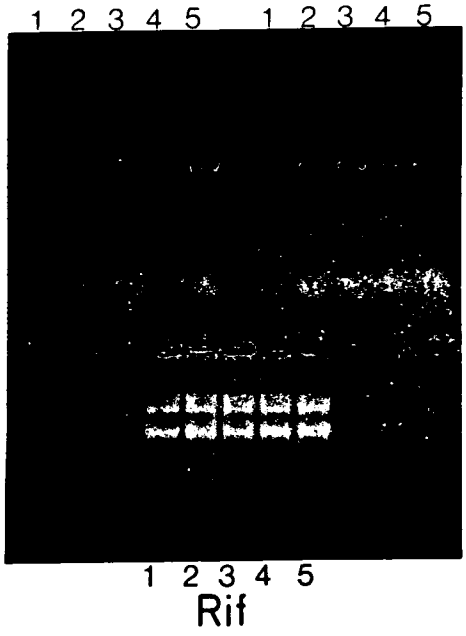

Cd
1
2
3
4
5
ORIGIN
bases
500

Figure 3.17 The use of rifampicin to analyse *smtA* transcript stability. Equal amounts of RNA were loaded in each track of the gel as shown in the photograph of the ethidium bromide stained gel (panel A). *Synechococcus* PCC 7942 (PIM8) cultures were treated with $CdCl_2$ 2.5 μM (Cd), $CdCl_2$ 2.5 μM and rifampicin 400 μgml^{-1} (Cd+Rif) and to rifampicin 400 μgml^{-1} (Rif). RNA samples were extracted every 15 mins over a 1 h time period. *smtA* transcript abundance is shown for a 4 day exposure, panel B and for a prolonged exposure, panel C.

A

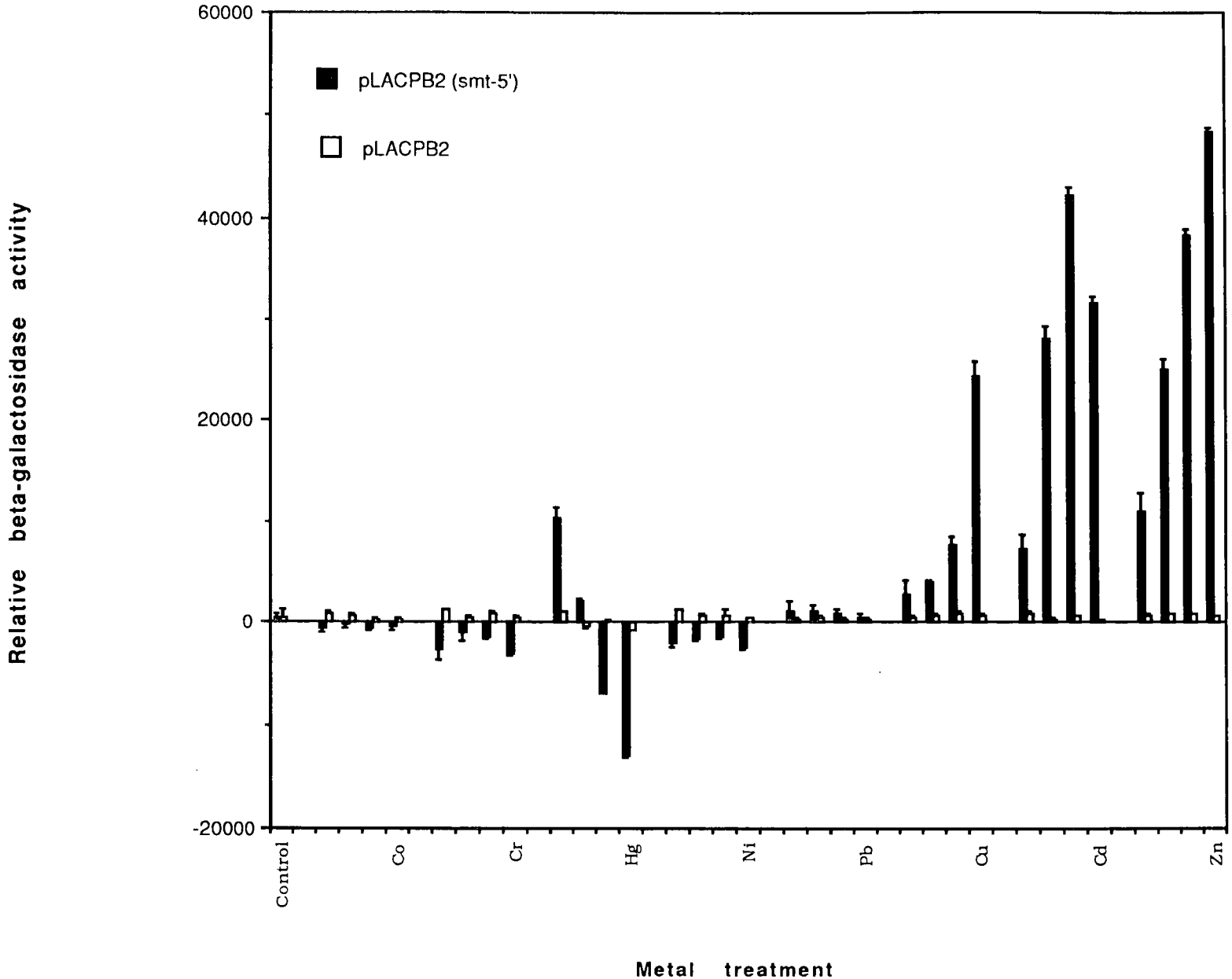


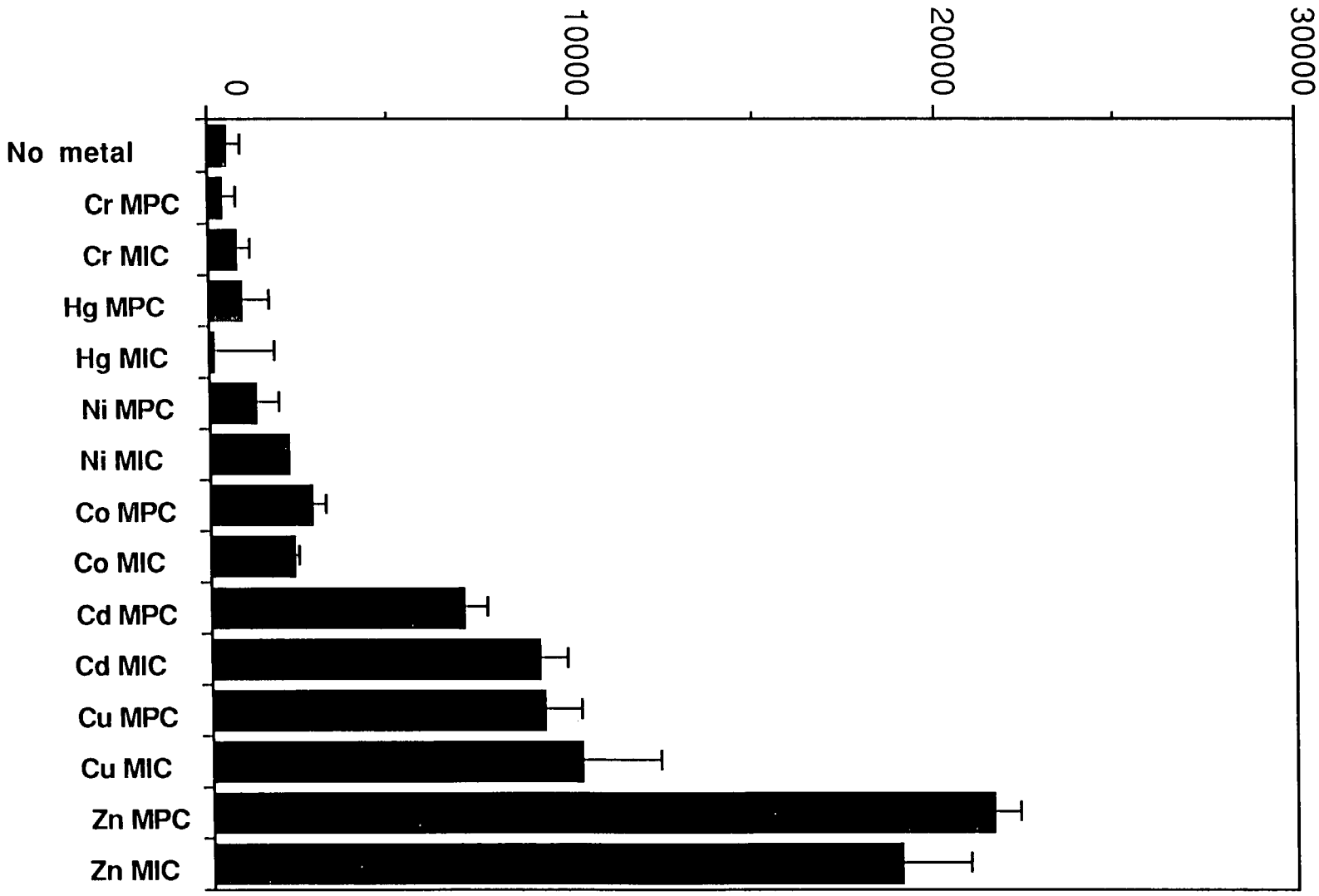
B

C

Figure 3.18 Metal induced expression of β-galactosidase in cells containing pLACBB2(*smt*-5'). Cultures of transformants were exposed to the same metal salts, ($CdCl_2$, $CoCl_2$, $CrCl_2$, CuCl, $HgCl_2$, $NiCl_2$,$PbCl_2$ and $ZnCl_2$) at the same concentration as for northern analysis (0 μM=control, 1 μM, 2.5 μM, 5 μM, 10 μM are the 4 values given for each metal) 2 h prior to the β-galactosidase assay. The graph shows the means of three separate determinations with standard deviations. pLACPB2 transformed cultures were also included as a control. The activity (in Miller units) was relative to the *Synechoccus* transformant not exposed to metal ions.

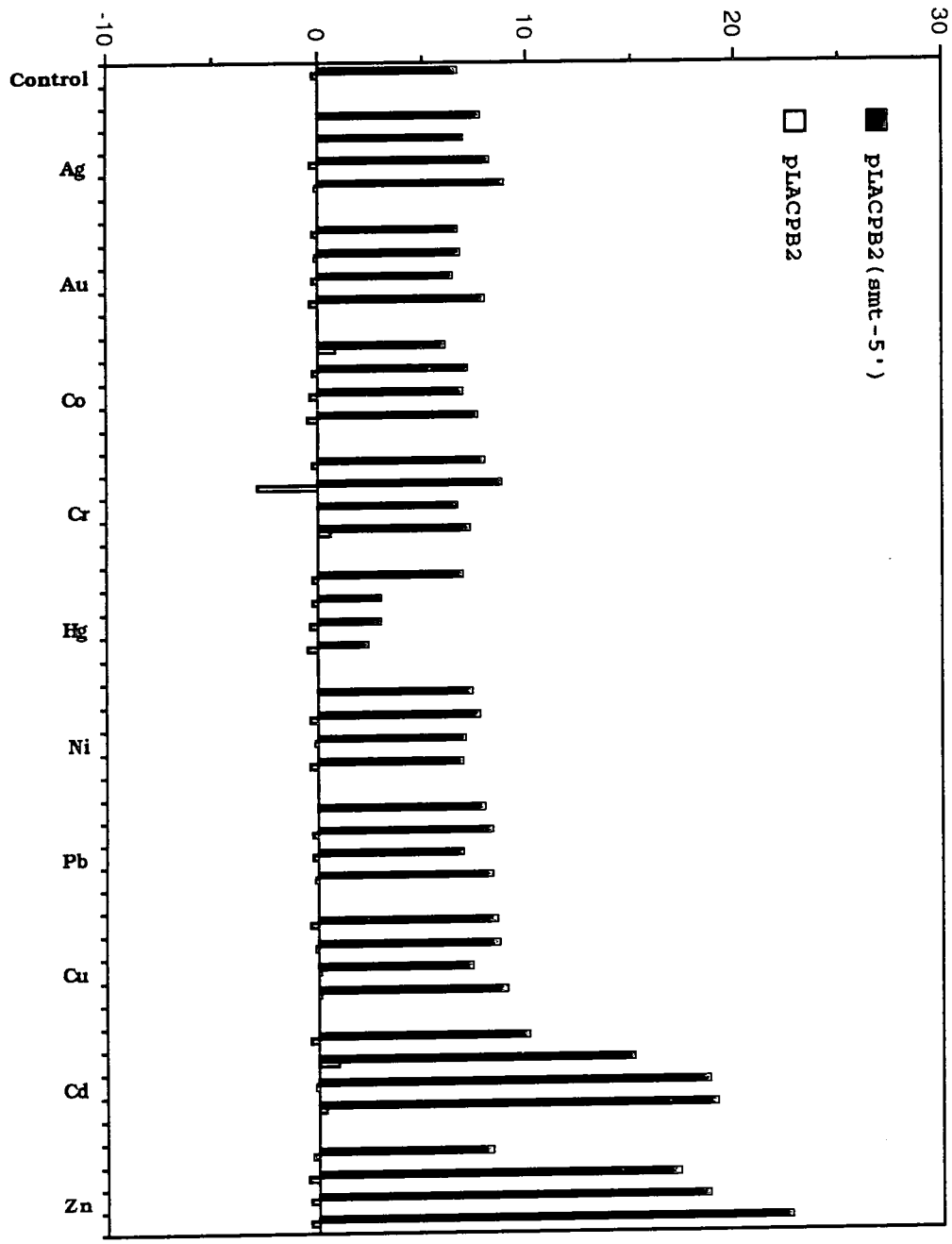
Figure 3.19 β-galactosidase activity in cells containing pLACPB2(*smt*-5') exposed to maximum permissive concentration (MPC) and minimum inhibitory concentration (MIC) of each metal (see table 3.1). Data are means of three separate determinations with standard deviations. The activity (in Miller units) was relative to the *Synechoccus* transformant not exposed to metal ions.


60000
40000
20000
0
-20000
pLACPB2 (smt-5')
pLACPB2
Relative beta-galactosidase activity
Control
Co
Cr
Hg
Ni
Pb
Cu
Cd
Zn
Metal treatment


Relative beta-galactosidase activity
0
10000
20000
30000
No metal
Cr MPC
Cr MIC
Hg MPC
Hg MIC
Ni MPC
Ni MIC
Co MPC
Co MIC
Cd MPC
Cd MIC
Cu MPC
Cu MIC
Zn MPC
Zn MIC

Figures 3.20a and 3.20b Metal induced expression of β-galactosidase in cells containing pLACBB2(*smt*-5'). Cultures of transformants were exposed to metal salts, ($CdCl_2$, $CoCl_2$, $CrCl_2$, CuCl, $HgCl_2$, $NiCl_2$,$PbCl_2$, $ZnCl_2$, $Ag(N0_3)$, and $Na(AuCl_4)$) at the same concentration as for northern blots (0 μM=control, 1 μM, 2.5 μM, 5 μM, 10 μM are the 4 values given for each metal) 2 h prior to the β-galactosidase assay. The assay used was a modified version of that described by Miller (1972). Figures 3.20a and 3.20b represent two sets of data, which were obtained on separate occasions using independent cultures of transformants. pLACPB2 transformed cultures were also included as a control.

Beta-galactosidase activity
(nmoles o-nitrophenol/min/mg protein)



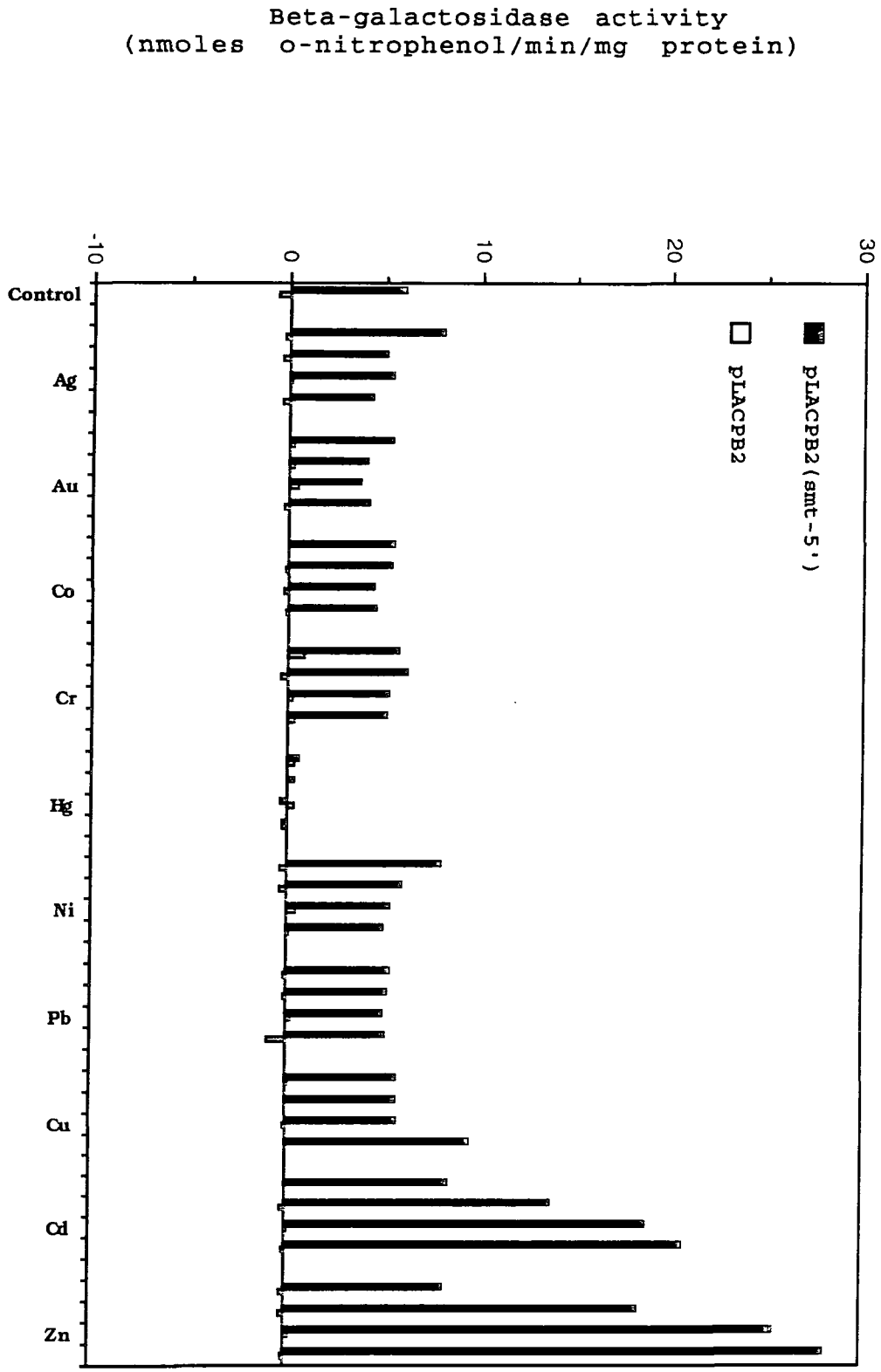
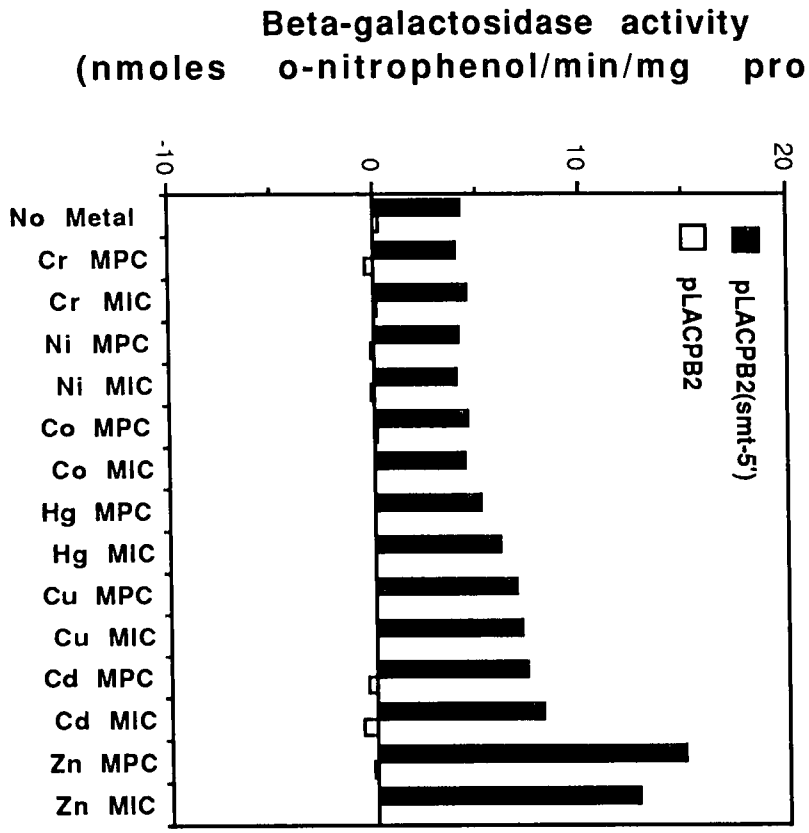

Beta-galactosidase activity
(nmoles o-nitrophenol/min/mg protein)
-10
0
10
20
30
Control
Ag
Au
Co
Cr
Hg
Ni
Pb
Cu
Cd
Zn
pLACPB2(smt-5')
pLACPB2
Metal Treatment

Figure 3.21 β-galactosidase activity in cells containing pLACPB2(*smt*-5') exposed to maximum permissive concentration (MPC) and minimum inhibitory concentration (MIC) of each metal (see table 3.1). Two sets of data are shown, which were obtained on separate occasions using independent cultures of transformants.


Beta-galactosidase activity
(nmoles o-nitrophenol/min/mg protein)
-10
0
10
20
No Metal
Cr MPC
Cr MIC
Ni MPC
Ni MIC
Co MPC
Co MIC
Hg MPC
Hg MIC
Cu MPC
Cu MIC
Cd MPC
Cd MIC
Zn MPC
Zn MIC
pLACPB2(smt-5')
pLACPB2

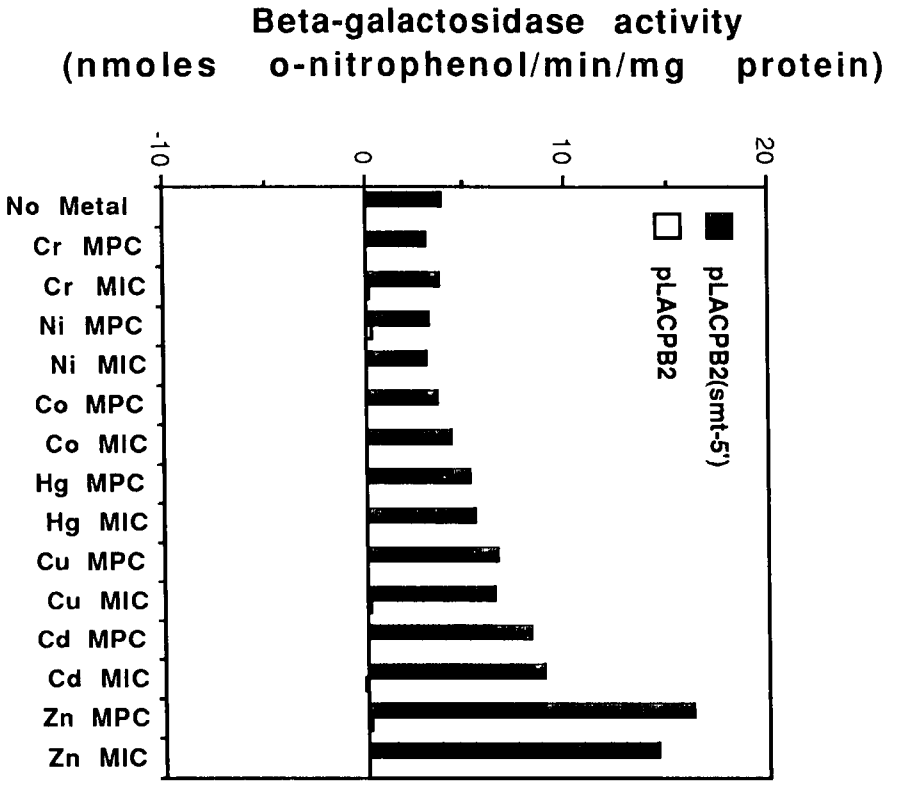
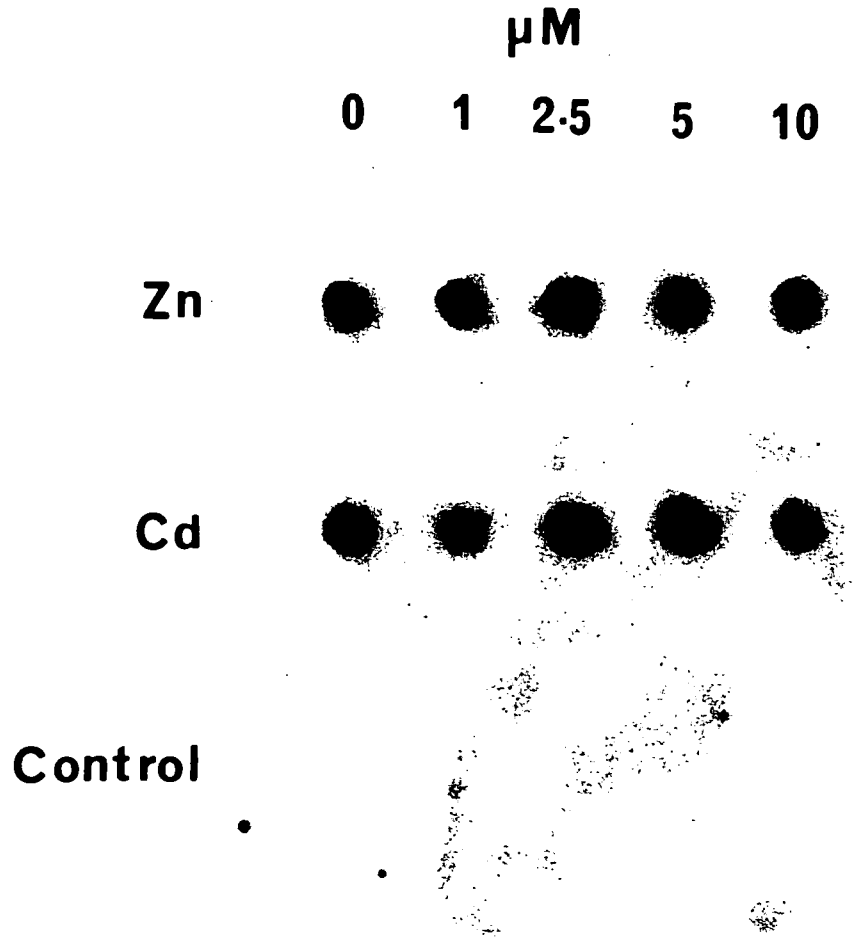
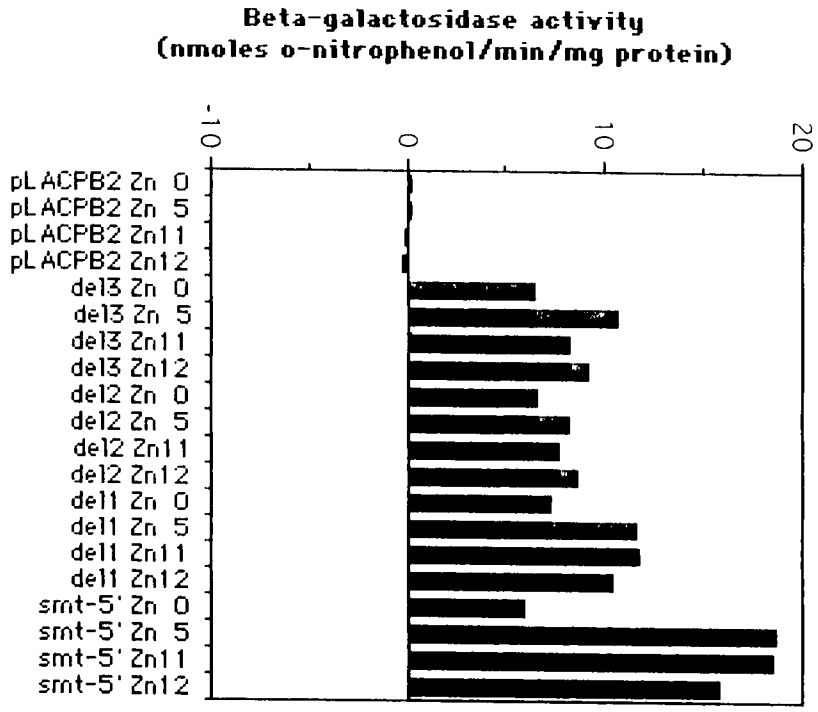

Beta-galactosidase activity
(nmoles o-nitrophenol/min/mg protein)
-10
0
10
20
No Metal
Cr MPC
Cr MIC
Ni MPC
Ni MIC
Co MPC
Co MIC
Hg MPC
Hg MIC
Cu MPC
Cu MIC
Cd MPC
Cd MIC
Zn MPC
Zn MIC
pLACPB2(smt-5')
pLACPB2

Figure 3.22 *In situ* hybridisation of *Synechococcus* PCC 7942 transformed with pLACPB2(*smt*-5'). Transformants were exposed to 0 μM, 1 μM, 2.5 μM, 5 μM, 10 μM of $ZnCl_2$ or $CdCl_2$ 2 h prior to in situ hybridisation using a probe produced from an *Eco*R1 fragment of pLACPB2. A control using untransformed *Synechococcus* PCC 7942 with no added metal was also included.

Figure 3.23 β-galactosidase activity in *Synechococcus* cells containing pLACPB2(*smt*-5') (*smt*-5'), pLACPB2(*smt*-5'del1) (del1), pLACPB2(*smt*-5'del2) (del2), pLACPB2(*smt*-5'del3) (del3) and pLACPB2 exposed to $ZnCl_2$ at 0, 5, 11, 12μM for 2 h prior to the β-galactosidase assay. Two sets of data are shown, which were obtained on separate occasions using independent cultures of transformants.


μM
0
1
2·5
5
10
Zn
Cd
Control


Beta-galactosidase activity
(nmoles o-nitrophenol/min/mg protein)
-10
0
10
20
pLACPB2 Zn 0
pLACPB2 Zn 5
pLACPB2 Zn11
pLACPB2 Zn12
del3 Zn 0
del3 Zn 5
del3 Zn11
del3 Zn12
del2 Zn 0
del2 Zn 5
del2 Zn11
del2 Zn12
del1 Zn 0
del1 Zn 5
del1 Zn11
del1 Zn12
smt-5' Zn 0
smt-5' Zn 5
smt-5' Zn11
smt-5' Zn12

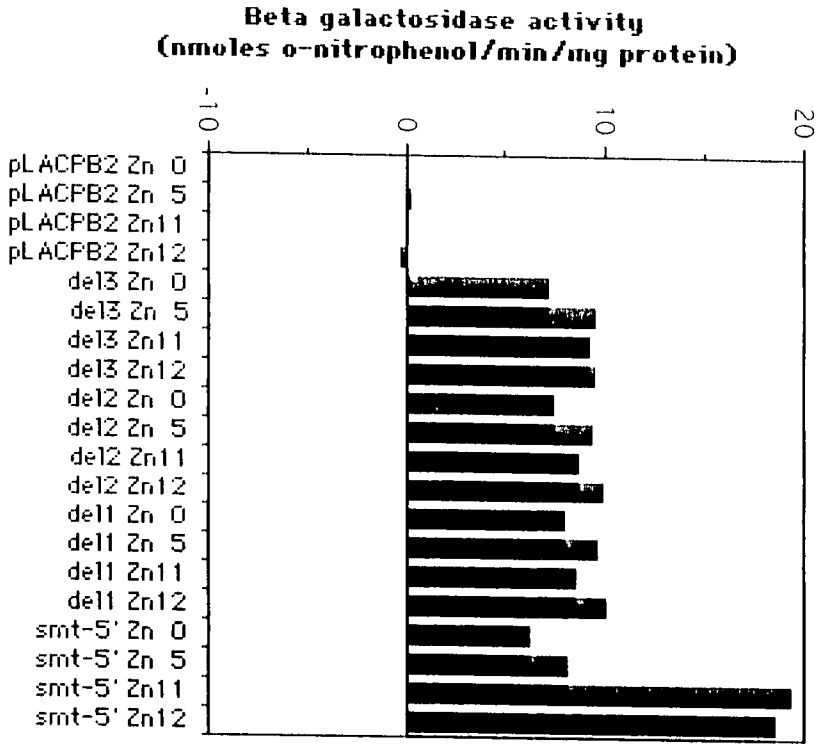
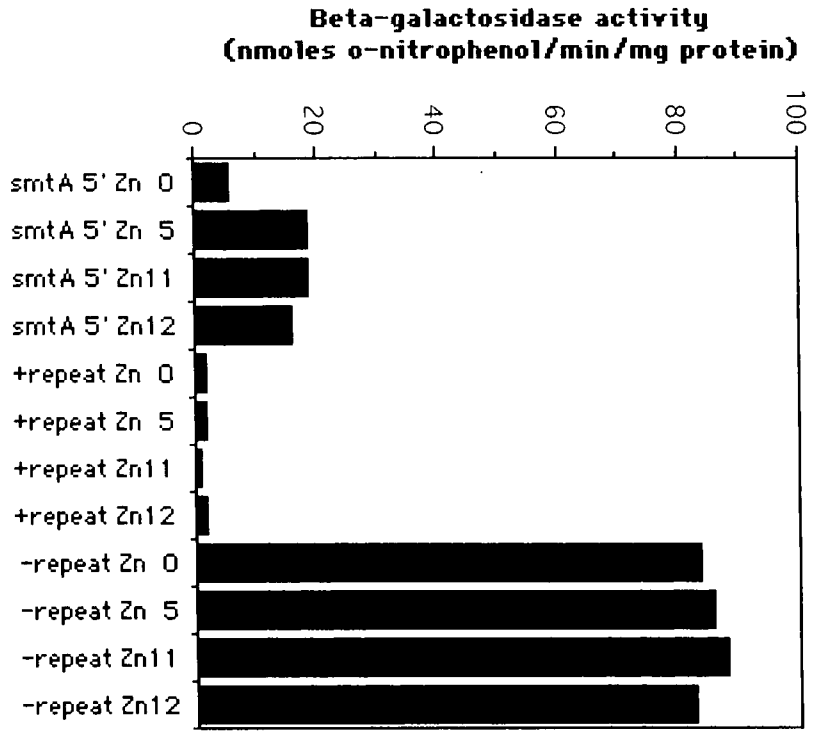
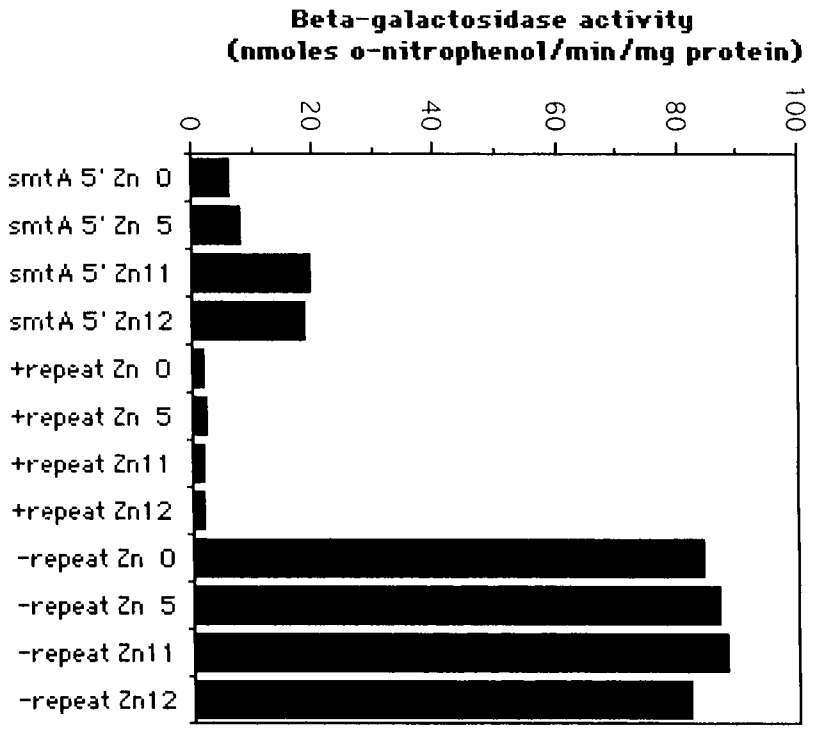
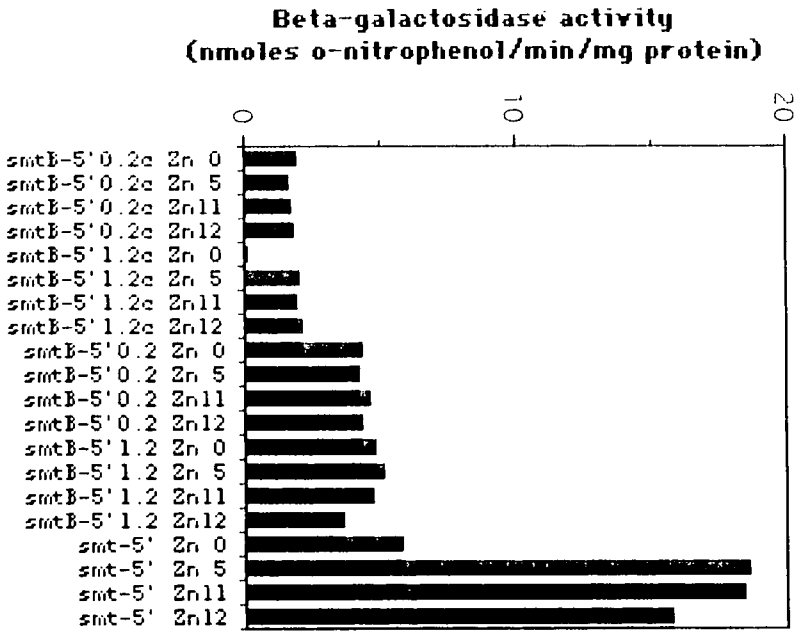

Beta galactosidase activity
(nmoles o-nitrophenol/min/mg protein)
-10
0
10
20
pLACPB2 Zn 0
pLACPB2 Zn 5
pLACPB2 Zn11
pLACPB2 Zn12
del3 Zn 0
del3 Zn 5
del3 Zn11
del3 Zn12
del2 Zn 0
del2 Zn 5
del2 Zn11
del2 Zn12
del1 Zn 0
del1 Zn 5
del1 Zn11
del1 Zn12
smt-5' Zn 0
smt-5' Zn 5
smt-5' Zn11
smt-5' Zn12

Figure 3.24 β-galactosidase activity in *Synechococcus* cells containing pLACPB2(*smt*-5') (*smt*A 5'), the hybrid promoter with inverted repeat1 (+repeat) and the hybrid promoter without inverted repeat1 (-repeat) exposed to $ZnCl_2$ at 0, 5, 11, 12μM for 2 h prior to the β-galactosidase assay. Two sets of data are shown, which were obtained on separate occasions using independent cultures of transformants.


Beta-galactosidase activity
(nmoles o-nitrophenol/min/mg protein)
0
20
40
60
80
100
smtA 5' Zn 0
smtA 5' Zn 5
smtA 5' Zn11
smtA 5' Zn12
+repeat Zn 0
+repeat Zn 5
+repeat Zn11
+repeat Zn12
-repeat Zn 0
-repeat Zn 5
-repeat Zn11
-repeat Zn12


Beta-galactosidase activity
(nmoles o-nitrophenol/min/mg protein)
0
20
40
60
80
100
smtA 5' Zn 0
smtA 5' Zn 5
smtA 5' Zn11
smtA 5' Zn12
+repeat Zn 0
+repeat Zn 5
+repeat Zn11
+repeat Zn12
-repeat Zn 0
-repeat Zn 5
-repeat Zn11
-repeat Zn12


Beta-galactosidase activity
(nmoles o-nitrophenol/min/mg protein)
0
10
20
smtB-5'0.2c Zn 0
smtB-5'0.2c Zn 5
smtB-5'0.2c Zn11
smtB-5'0.2c Zn12
smtB-5'1.2c Zn 0
smtB-5'1.2c Zn 5
smtB-5'1.2c Zn11
smtB-5'1.2c Zn12
smtB-5'0.2 Zn 0
smtB-5'0.2 Zn 5
smtB-5'0.2 Zn11
smtB-5'0.2 Zn12
smtB-5'1.2 Zn 0
smtB-5'1.2 Zn 5
smtB-5'1.2 Zn11
smtB-5'1.2 Zn12
smt-5' Zn 0
smt-5' Zn 5
smt-5' Zn11
smt-5' Zn12

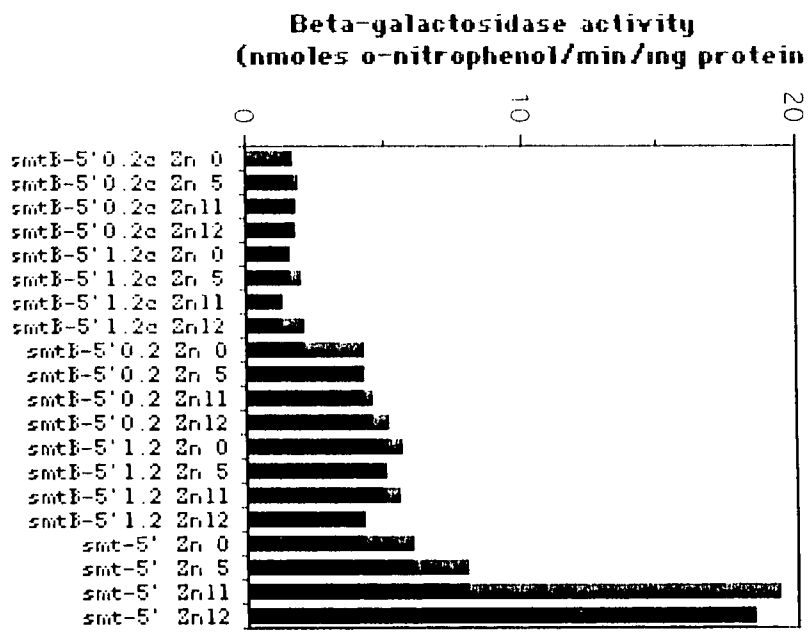

Beta-galactosidase activity
(nmoles o-nitrophenol/min/mg protein
0
10
20
smtB-5'0.2c Zn 0
smtB-5'0.2c Zn 5
smtB-5'0.2c Zn11
smtB-5'0.2c Zn12
smtB-5'1.2c Zn 0
smtB-5'1.2c Zn 5
smtB-5'1.2c Zn11
smtB-5'1.2c Zn12
smtB-5'0.2 Zn 0
smtB-5'0.2 Zn 5
smtB-5'0.2 Zn11
smtB-5'0.2 Zn12
smtB-5'1.2 Zn 0
smtB-5'1.2 Zn 5
smtB-5'1.2 Zn11
smtB-5'1.2 Zn12
smt-5' Zn 0
smt-5' Zn 5
smt-5' Zn11
smt-5' Zn12

Figure 3.26 β-galactosidase activity in *Synechococcus* cells containing pLACPB2(*smt*-5') (*smt*-5'), pLACPB2(*smtB*-5'1.2) (*smtB*-5'1.2), pLACPB2(*smtB*-5'0.2) (*smtB*-5'0.2) pLACPB2(*smtB*-5'1.2c) (*smtB*-5'1.2c), pLACPB2(*smtB*-5'0.2c) (*smtB*-5'0.2c) exposed to $ZnCl_2$ at 0, 5, 11, 12μM for 2 h prior to the β-galactosidase assay. Two sets of data are shown, which were obtained on separate occasions using independent cultures of transformants.

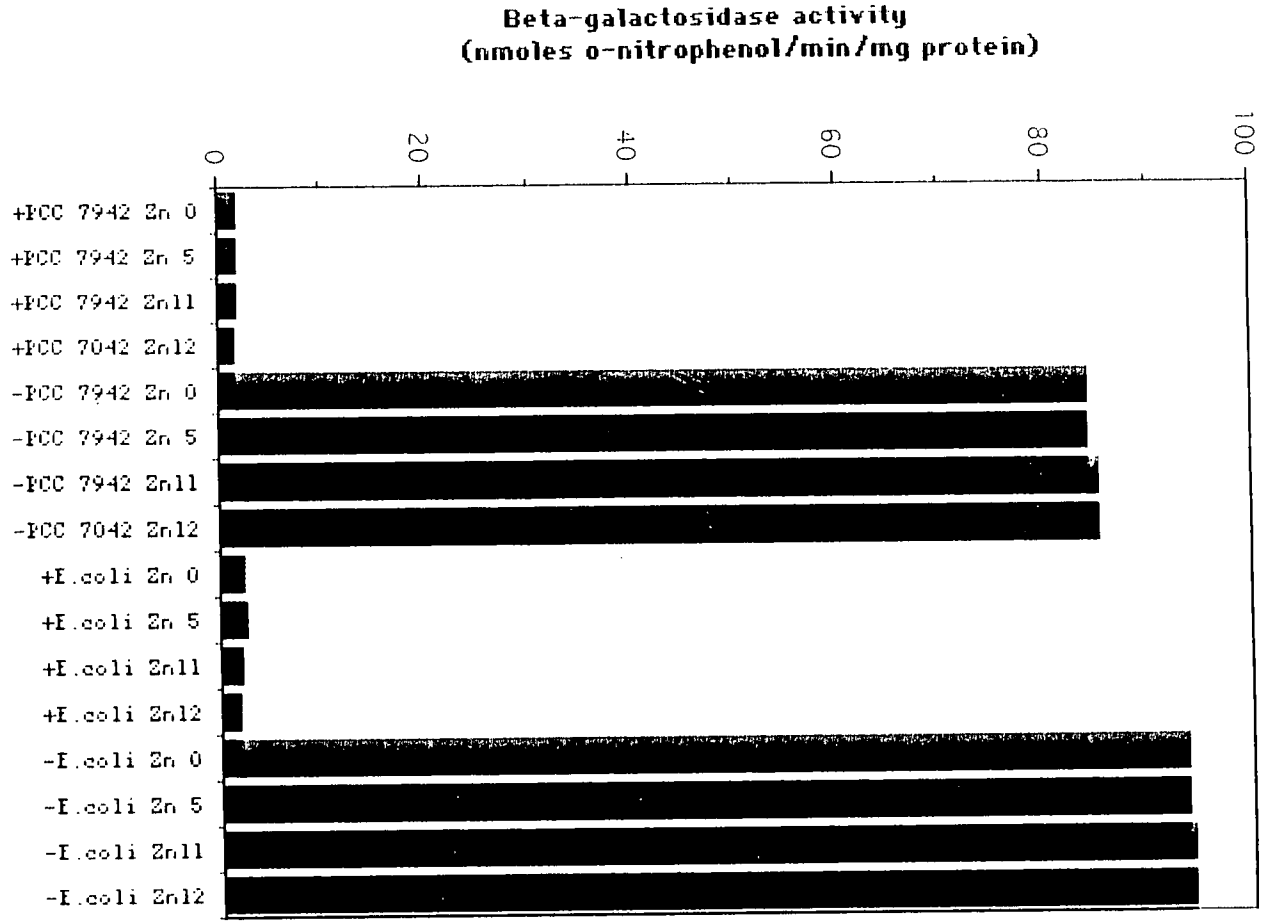

Beta-galactosidase activity
(nmoles o-nitrophenol/min/mg protein)
0
20
40
60
80
100
+PCC 7942 Zn 0
+PCC 7942 Zn 5
+PCC 7942 Zn11
+PCC 7042 Zn12
-PCC 7942 Zn 0
-PCC 7942 Zn 5
-PCC 7942 Zn11
-PCC 7042 Zn12
+E.coli Zn 0
+E.coli Zn 5
+E.coli Zn11
+E.coli Zn12
-E.coli Zn 0
-E.coli Zn 5
-E.coli Zn11
-E.coli Zn12

Figure 3.25 β-galactosidase activity in *Synechococcus* and *Escherichia coli* cells containing the hybrid promoter with inverted repeat1 (+) and the hybrid promoter without inverted repeat1 (-) exposed to $ZnCl_2$ at 0, 5, 11, 12μM for 2 h prior to the β-galactosidase assay.

Chapter 4

DISCUSSION.

Prior to the work described in this thesis, MT genes had only been characterised in eukaryotes, although reports had suggested that similar genes may exist in prokaryotes. In this chapter the structure and metal dependent expression of the *smtA* gene, the first (characterised) prokaryotic MT gene, are described. A gene encoding a putative regulator of *smtA*, *smtB*, has been identified and its possible roles in the regulation of *smtA* and in trace metal metabolism are discussed. The regulation of *smtA* is discussed in the context of metal-responsive gene expression. The isolation of the *smt* locus detailed herein has enabled subsequent studies which further analyse the function and regulation of the prokaryotic MT locus, the results of which are also included. SmtB is one of the first characterised cyanobacterial transcription factors. The regulation of cyanobacterial genes is also included in this discussion.

4.1 Characterisation of the *smt* locus.

4.1.1 Characterisation of *smtA* via PCR.

PCR was initially used to amplify two products, refer to section 3.1.1. (figure 3.1). The nucleotide and encoded amino acid sequence of the 144 b.p. product is shown in figure 3.2. The encoded amino acid sequence corresponded to that of the class II MT from *Synechococcus* TX-20 (Olafson *et al.*, 1988), inclusive of the primer binding regions shown in figure 2.1, with the exception of cysteine$_{32}$ being substituted for serine. The gene was therefore designated *smtA*, for *Synechococcus* metallothionein (MT). Locations corresponding to inosine (I) in the N-terminal primer contained G in the PCR product whereas locations corresponding to I in the C-terminal primer

contained C in the PCR product (figure 3.2). This is due to preferential I/C base pairing (Ohtsuka *et al.*, 1985). These six bases are shown in bold type face as they may not correspond to the nucleotides present in the template DNA.

The nucleotide sequence of the 116 b.p. fragment showed that it was a product of primer mismatch due to the presence of an internal region of *smtA* that had homology to the C-terminal primer-binding site giving rise to a truncated product (refer section 3.1.1).

The 5' end of the *smtA* gene was unsuitable for use as a primer binding site due to primer redundancy, causing the sequence (figure 3.2) to be missing 15 bases of the coding region at the 5' end of the *smtA* gene. Therefore, APCR was used to amplify the remainder of the coding region of *smtA* and the 3' and 5' flanking regions as described in section 2.3.4.13.3. The sum of the 3' and 5' products, 0.9 + 2.1 = 3.0 k.b., is equivalent to the size of the *Hind* III (2.8 k.b.) fragment indicated from the genomic Southern blot (figure 3.3). An additional 0.2 k.b. was amplified due to double amplification of the 144 b.p. coding region and an additional 50 b.p. of amplified pUC19 sequences. The sequence of the 5' APCR product (figure 3.5) includes the diagnostic *smtA* sequence, 15 b.p. of coding sequence upstream of the N-terminal primer site which were missing from the previous *smtA* clones, and the sequence of the 5' flanking region to the first 5' *Pst* I restriction site (refer to section 3.1.2).

During this study a strategy that combines several PCR methods was employed to amplify and clone a prokaryotic MT gene, designated *smtA*. The same inosine-containing primers used for the amplification of part of the coding region of *smtA*, were subsequently applied to chromosome crawl into the 5' flanking region of the *smtA* gene via APCR (refer to section 3.1.2).

In vitro nucleic acid amplification by PCR has rapidly become established as a powerful technique for both gene analysis and cloning (Saiki *et al.*, 1988). It has become possible to obtain specific DNA amplification using redundant primers designed from known protein sequences (Lee *et al.*, 1988; Gould *et al.*, 1989).

Additionally, the base analogue inosine can be incorporated into primers which correspond to polypeptide regions that would otherwise require complex oligonucleotide mixtures (Fordham-Skelton *et al.*, 1990). Furthermore, inverse PCR (IPCR) has been used to "chromosome crawl" amplifying regions flanking known genes (Ochman *et al.*, 1988; Trigilia *et al.*, 1988). Shyamala and Ames (1989) tested the feasibility of using single-specific-primer PCR, or anchored, PCR (APCR) as an alternative to IPCR for chromosome crawling.

PCR generated clones are of limited use, since PCR introduces errors in replication cycles and subsequently amplifies those errors. This results in an accumulation of mutations (0.3 - 0.8% after 30 cycles) (Karlovsky, 1990). Hence, when using PCR to determine DNA sequences, several independent clones from independent reactions have to be sequenced. When using these clones in subsequent studies, the fact that there may be a mutation in a critical position should be taken into consideration. Therefore, the above mentioned PCR fragments were used as probes to screen a *Synechococcus* PCC 7942 genomic library.

4.1.2 Isolation of a genomic clone encoding *smtA*.

Genomic DNA isolated from *Synechococcus* PCC 7942 was used to produce a genomic library. A homologue of *smtA* was identified in this strain via Southern analysis and PCR (Robinson *et al.*, 1990). This related strain of *Synechococcus* (refer to section 2.1.2) was used for the isolation of a genomic clone because it is readily transformable and would therefore be more amenable to the analysis of gene regulation. *Synechococcus* PCC 6301 was used in the original PCR characterisation, since the prokaryotic MT was originally isolated from this strain (Olafson *et al.*, 1988).

The sequence of the genomic clone is shown in figure 3.8. The *smtA* gene encodes a polypeptide of 56 amino acids and corresponds to the amino acid sequence of the prokaryotic MT (Olafson *et al.*, 1988) with three modifications. There are two additional amino acid residues histidine and glycine, encoded at the C-terminal end of

the predicted amino acid sequence of *smtA* and there is a serine substitution for cysteine$_{32}$ (serine$_{33}$ in *smtA* sequence) (figure 3.9).

6 b.p. upstream of the *smtA* transcription start site there is a sequence similar to a *Escherichia coli* -10 consensus sequence (refer to 3.8). This sequence was originally identified by Pribnow (1975) as the conserved DNA sequence to which RNA polymerase binds. The conserved motif (TATPuATG) has been designated the "Pribnow box". Subsequently, it was shown that sequences approximately 35 bases upstream from the transcript start site were also necessary for initial recognition by RNA polymerase. Since the initial characterisation, comparison of sequences known to be involved in transcription initiation in *Escherichia coli* have revealed the consensus sequences TTGACA (-35 consensus sequence) and TATAAT (-10 consensus sequence). The spacing between these regions is usually 16-18 b.p. and the transcript start site is usually 6 - 8 b.p from the -10 sequence (Hawley and McClure, 1983; Harley and Reynolds, 1987). The distance between the -10 and -35 regions is particularly important in determining the promoter strength. For a synthetic promoter with perfect consensus -35 and -10 regions it has been demonstrated that a 17 b.p. spacing gives the maximum strength (Ayoama and Takanami, 1988). However, *in vivo* a 16 b.p. space has been shown to be the optimum for promoter strength (Brosius *et al.*, 1985). The DNA structure in the spacer region is also important since base substitutions in the spacer region, which change the structural characteristics of the DNA can also affect promoter strength (Auble *et al.*, 1986).

smtA has no region corresponding to an *Escherichia coli* -35 consensus sequence. Some promoters can function with no apparent -35 elements (Keilty and Rosenberg, 1987; Harley and Reynolds, 1987). These promoters usually have weak constitutive activities and are activated by positively acting proteins which seem to substitute for the -35 element (Raibaud and Schwartz, 1984). However, the structure of the RNA polymerase varies depending on whether a -35 region is present. When the -35 region is absent, the RNA polymerase, on failing to make contact in the -35 region, makes upstream compensatory contacts that involve distortion of the DNA (Chan *et al.*,

1990). The activators bind to a region between -35 and -80, suggesting that activation requires a direct interaction between the activator and RNA polymerase (Gralla, 1990). Furthermore, it has been speculated that the interaction of an activator with RNA polymerase may be required for stable promoter recognition and binding (Reznikoff, 1992). It has recently been shown that the sequence 5'-TGN-3' in an extended -10 region can substitute for the -35 region (Minchin and Busby, 1993). This extended sequence is present in the *smtA* promoter which lacks a -35 consensus region (figure 3.8).

Schneider *et al.* (1987) purified the principal RNA polymerase from *Anabaena* 7120. This RNA polymerase, in common with the other characterised cyanobacterial RNA polymerases contained a γ subunit in addition to the $\alpha 2\beta\beta'$ core enzyme common to prokaryotes (Schneider *et al.*, 1987). The holoenzyme also contains the σ-subunit, which confers promoter-specific activity on the core enzyme (Schneider *et al.*, 1997). Schneider *et al.* (1991), compared the activity and sequence of several promoters from the cyanobacterium *Anabaena* 7120. The promoters of most of the *Anabaena* genes characterised to date were found to depart significantly from the consensus *Escherichia coli* promoter, particularly around the -35 sequence. However, in vitro transcription assays using the *Anabaena* 7120 holoenzyme showed that promoters most similar to the consensus *Escherichia coli* promoter gave the greatest rates of transcription. It was concluded that most of the *Anabaena* promoters were "weak" unless they were transcribed by the minor forms of RNA polymerase, or that they require activators (Schneider *et al.*, 1987; Schneider *et al.*, 1991).

The *smtA* -10 consensus sequence is spanned by repeat sequences. An inverted repeat sequence is present upstream, whilst an inverted repeat sequence and a direct repeat sequence are present downstream. One of the inverted repeats (inverted repeat1, figure 3.8) is a region of perfect dyad symmetry 5' CTGAATC-AA-GATTCAG 3'; the other inverted repeat (inverted repeat2, figure 3.8) has a single base difference between the repeat sequences, 5' TGAACA-GT-TATTCA 3'. The direct repeat, 5' TATTCA-GA-TATTCA overlaps inverted repeat2 (figure 3.8). These

sequences are putative sites for the interaction of DNA-binding regulatory proteins, discussed in section 4.2.4.

The *smt* locus contains the *smtA* and *smtB* genes arranged in a divergent orientation about the *smt* operator/promoter region (refer to figures 3.7 and 3.8). The translational start codons of both genes are separated by 100 b.p of DNA, designated the *smt* operator/promoter region. The transcriptional start sites of both genes have been mapped within the *smt* operator/promoter. This divergent, overlapping orientation has precedent in some prokaryotic systems. Closely spaced promoters are found in three orientations: they may be arranged in tandem and transcribe the same gene or operon, they may face opposite directions and transcribe either gene in a divergent orientation, or they may transcribe both strands of the same DNA in a convergent orientation (Goodrich and McClure, 1991).

Divergent promoters (divergons) are found in many prokaryotic systems. This promoter architecture represents a general type of gene organisation (Beck and Warren, 1988). The advantages of divergent transcription units include: the interaction of different RNA polymerase molecules on the same site of DNA, either via direct protein-protein interactions or indirectly by altering the topology of the neighbouring promoter; the regulation for both genes may be provided by the binding of regulatory proteins to regions within the divergent promoters; the tight control of structural gene expression due to co-ordinate expression of the regulator and effector; the prevention of gene activation by transcriptional read through, and that these autonomous DNA regions may be more stable than other units of genes in the course of recombination and translocation (Beck and Warren, 1988). Similar promoter architecture has been characterised in prokaryotic metal resistance determinants, where the divergent gene encodes the regulator of the system (discussed in section 1.2).

The divergent *smtB* open reading frame encodes a protein of 122 amino acids and contains 3 cysteine residues, none of which are arranged in Cys-Xaa-Cys, Cys-Xaa-Xaa-Cys or Cys-Cys motifs. The polypeptide sequence contains a 21 amino acid region (62-82) which scores highly (5.5) on a Dodd and Egan helix-turn-helix

distinction matrix, refer to section 4.2.3 (Huckle *et al.*, 1993). The OWL 14.0 data base was searched for sequences similar to SmtB, and ArsR and CadC sequences were found to be most significant (figure 3.10) (analysis carried out by Dr. A.P. Morby). 7 b.p. upstream of the *smtB* transcription start site there is a sequence similar to an *Escherichia coli* -10 consensus sequence. Both of the *smt* genes have sequences predicted to form stem-loop structures and act as transcriptional terminators (figure 3.8).

The isolation of a homologue of *smtA* has recently been reported (Shimizu *et al.*, 1992). This prokaryotic MT gene was isolated from *Synechococcus vulcanus* and has been designated *mtnA*. The predicted amino acid sequence of MtnA is one amino acid longer than SmtA from *Synechococcus* PCC 7942 and shows 52% identity. Further analysis of the reported sequence revealed that there is a divergent open reading frame with similarity to *smtB*, although the sequence in the data base terminates before the end of the open reading frame. The sequence shows 36% identity to SmtB at the amino acid level. The *mtnA* promoter region contains an inverted repeat (5' TGAACA-GT-TGTTCA 3') 2 b.p. upstream of the -10 sequence (A.P.Morby, unpublished observations). This inverted repeat is similar to inverted repeat2 of the *smt* locus (figure 3.8).

In summary, the genomic clone pJHNR49 contains a region designated the *smt* locus which consists of two open reading frames arranged in a divergent orientation (refer to figures 3.7 and 3.8). These divergent open reading frames are separated by the *smt* operator/promoter region which has three regions that are candidate sites for protein-DNA interaction. The smaller open reading frame, designated *smtA*, has a sequence similar to an *Escherichia coli* -10 promoter consensus sequence. The divergent open reading frame, *smtB*, also has a sequence similar to an *Escherichia coli* -10 promoter consensus sequence and encodes a region that is a candidate for DNA binding (figure 3.10). The function of these open reading frames and their putative control elements requires further study.

4.2 Metallo-regulation of the *smt* locus.

4.2.1 The expression of *smtA*.

Olafson *et al.*, (1980) reported that levels of *Synechococcus* MT were highly induced by supplementation of the growth media with either Zn^{2+} or Cd^{2+} but not copper ions. The observation that MT induction is decreased by actinomycin D suggested that regulation is at the level of transcription.

The relative abundance of *smtA* transcripts (figure 3.14) confirms that there is an increase in *smtA* transcripts in response to several metals ions. An increase in transcript abundance was observed upon exposure to $HgCl_2$, $CoCl_2$, $CdCl_2$, $ZnCl_2$ and CuCl with maximum induction observed at 2.5, 5, 2.5, 5, 5 μM respectively. Exposure to $NiCl_2$ and $CrCl_2$ gave similar levels of transcript at all concentrations used. The transcript abundance declined at higher concentrations with some metals, which is assumed to correlate with loss of cell viability. Induction in response to $PbCl_2$ was slight, which may reflect the insolubility of lead salts in aqueous solution. There was no increase in the relative abundance in *smtA* transcripts in response to heat shock, suggesting that these responses were (somewhat) metal specific, and not general stress related responses. A similar pattern in the relative abundance of *smtA* transcripts was observed for *Synechococcus* PCC 7942 (PIM8) as for *Synechococcus* PCC 6301 in response to $CdCl_2$ exposure, figure 3.15. This suggests the control of *smtA* transcription is regulated in the same manner in these two strains of *Synechococcus*.

The induction by such a broad range of metals has not yet been reported for a metal responsive prokaryotic gene. The response is rapid with an increase in *smtA* transcripts observed in cultures that have been exposed to 2.5 μM $CdCl_2$ for 10 minutes, with maximum induction after 30 minutes (figure 3.13). Metal induction is repressed in cells exposed to the transcriptional inhibitor rifampicin. *smtA* transcripts decayed at similar rates in rifampicin treated cells regardless of exposure to $CdCl_2$

(figure 3.17), indicating that there is no effect of Cd^{2+} on *smtA* transcript stability. These data are consistent with Olafson's observations, in that *smtA* is regulated at the level of transcription. Interestingly, the iron-regulated expression of ferredoxin I is mediated via differential mRNA stability in *Synechococcus* PCC 7942 (Bovy *et al.*, 1993).

Sequences upstream of *smtA* can confer metal dependent expression upon a promoterless *lacZ* gene (refer to sections 3.3.3.1.1 and 3.3.3.1.2). The increase in β-galactosidase activity was not due to metal ions increasing plasmid copy number (figure 3.22). Dependent upon the method of measurement of β-galactosidase activity, there are a different number of metals which appear to induce activity. Using the method described by Miller, (1972) exposure to $ZnCl_2$, $CdCl_2$, CuCl and $HgCl_2$ gave an increase in β-galactosidase activity at concentrations used for northern analysis (0, 1, 2.5, 5, 10 μM). At MPC and MIC levels, exposure to $NiCl_2$, $CoCl_2$, $CdCl_2$, CuCl and $ZnCl_2$ gave an increase in β-galactosidase activity using Miller's method. This method was modified because the addition of metal salts to the cultures changed the cellular pigmentation, see section 2.3.3.2. Using the modified assay, exposure to CuCl, $CdCl_2$and $ZnCl_2$ gave an induction of β-galactosidase at concentrations used for northern analysis (0, 1, 2.5, 5, 10 μM). At MPC and MIC levels, exposure to CuCl, $CdCl_2$, and $ZnCl_2$ gave an increase in β-galactosidase activity. It can be concluded from these results that the *smt* locus is induced upon exposure to CuCl, $CdCl_2$ and $ZnCl_2$, which stimulated β-galactosidase activity regardless of the method of measurement. However, the results for other metals are not conclusive; exposure to $NiCl_2$, $CoCl_2$ and $HgCl_2$ may also give a response.

Using northern analysis, exposure to $HgCl_2$ gave the most potent induction followed by exposure to $CdCl_2$, $CoCl_2$, $ZnCl_2$ and CuCl. With the β-galactosidase assay, exposure to $ZnCl_2$ gave the most potent response followed by exposure to $CdCl_2$ and CuCl. The differences in these observations may be a function of differential toxicity upon the transcriptional and translational machinery of the *Synechococcus* cells or perhaps a result of metal mediated β-galactosidase inactivation.

4.2.2 The expression of *smtB.*

Northern analysis indicates that the relative abundance of *smtB* transcripts increases with increased exposure to $CdCl_2$, refer to section 3.3.1.3 (figure 3.15). The gel used for the northern blot had to be very heavily loaded with RNA before the *smtB* transcripts could be detected, suggesting that *smtB* transcripts were of lower abundance than *smtA* transcripts. The *lacZ* fusion with the *smtB* promoter suggests that *smtB* transcription is not induced upon exposure to $ZnCl_2$ (section 3.3.3.3, figure 3.26).

4.2.3 Similarity of SmtB, ArsR, CadC, MerR and NolR.

SmtB shows similarity at the amino acid level to the ArsR protein of the *Escherichia coli* and *Staphylococcus ars* operons (described in section 1.2.1.2) and to the CadC proteins of *Staphylococcus aureus* and *Bacillus firmus* (discussed in 1.2.2.4), figure 3.10. Similarity scores show that SmtB is most similar to the two *Staphylococcus* ArsR proteins (Huckle *et al.*, 1993). ArsR proteins are repressors of *Escherichia coli* (San Francisco *et al.*, 1990), *Staphylococcus xylosus* (Rosenstein *et al.*, 1992) and *Staphylococcus aureus* (Ji and Silver, 1992a) *ars* operons. The CadC proteins of *Staphylococcus aureus* (Yoon and Silver, 1991) and *Bacillus firmus* OF4 (Mac Ivey *et al.*, 1992) are encoded by *cadC*, a gene located upstream of *cadA*, which encodes a ATP-dependent Cd^{2+} efflux system. Morby *et al.* (1993) also report the similarity of SmtB to MerR from *Streptomyces lividans* and NolR, a regulator of *nod* gene expression in *Rhizobium meliloti.*

4.2.4 The function of SmtB.

There are several features that suggest that SmtB encodes a repressor of the expression of *smtA*. SmtB shows homology to a known repressor, ArsR (described in section 4.2.3), at the amino acid level, furthermore, the divergent orientation of *smtA* and *smtB* (discussed in section 4.1.2.), supports this hypothesis. SmtB also contains a putative DNA-binding helix-turn-helix that scores very highly (5.5) on the Dodd and Egan matrix (Dodd and Egan, 1990). X-ray crystallography of several proteins and protein-DNA complexes have elucidated these structures in detail (Struhl, 1989).

The crucial structure in this class of proteins consists of two α-helices separated by a β-turn. Despite considerable sequence variability, the structural geometry of the helix-turn-helix motifs is highly conserved, so much so that a matrix has been compiled describing the sequence conservation at each position in the motif. This is used to predict the presence of such motifs in other proteins (Dodd and Egan, 1990). This matrix was compiled from a reference set of 91 presumed helix-turn-helix motifs and calibrated against a protein data base. All the protein domains with matrix scores of 4.5 or above were found to be helix-turn-helix proteins. Dodd and Egan also concluded that if a protein is known to be DNA binding, a score of at least 2.5 indicates that it is almost certain to be a helix-turn-helix protein.

The structure of the two helices, related by the two-fold symmetry of the dimeric protein, is in the correct orientation (34 Angstroms apart, which is equivalent to one turn of the DNA double helix) to mediate DNA-protein interaction. One of the helices lies in the major groove of the DNA, providing sequence-specific DNA interactions and is termed the recognition helix. The other helix lies across the major grove and makes non-specific contacts with DNA. The N-terminal regions of both helices point towards the phosphate backbone. The amino acid side chains of the recognition helix are presumed to make specific interactions with exposed functional groups in the major grove of the DNA (Freemont *et al.*, 1991). The prokaryotic helix-turn-helix proteins bind as dimers to DNA sequences that have dyad symmetric character

(Schleif, 1988). The *smt* operator/promoter region contains 3 repeat sequences that are potential sites for interaction with the helix-turn-helix motif of SmtB. Deletion of the *smtB* gene from the 5' flanking region of *smtA* in reporter gene constructs lead to an increase in the basal level (no added metal) of β-galactosidase activity, suggesting a role for SmtB as a repressor of *smtA* transcription (figure 3.23).

Subsequent to the work described in this thesis, other workers in the laboratory have obtained further evidence to support the proposal that SmtB is a *trans*-acting metallo-responsive repressor of transcription from the *smt* operator/promoter region. In *smt* deficient mutants there is a greater than 20 fold increase in the basal expression and loss of metal dependency from the *smtA* operator/promoter. The level of basal expression of the *smtA* operator/promoter region in these cells was greater than 4 times that of maximally induced cells containing *smtB*. These results suggest that the loss of the SmtB repressor gives rise to the increase in the basal level of expression (Huckle *et al.*, 1993).

Gel retardation assays comparing the binding of protein extracts to the *smt* operator/promoter suggest that SmtB either binds to or causes another protein to bind to a region of DNA in the *smt* operator/promoter region, forming a complex designated MAC1. The site of interaction includes the direct repeat and inverted repeat2 of the *smt* locus. SmtB has not been shown to bind to or give rise to binding to inverted repeat1 (Morby *et al* ., 1993). MAC1 is absent in extracts from *smt* mutants. In R2 -PIM8 (wild-type, plasmid cured PCC 7942) extracts the DNA-protein complex is present in the absence of Zn^{2+} ions and is diminished in their presence, an effect that can be reversed by the addition of chelating agents *in vitro*. This suggests that SmtB is a repressor and may act to give rise to signal responsive derepression of *smtA* expression (discussed in section 4.2.5). Complementation of over-expression from the *smt* operator/promoter in *smt* mutants with plasmid borne *smtB* demonstrates that SmtB acts in *trans* as a repressor of *smtA* transcription (Huckle *et al.*, 1993).

Synechococcus PCC 6301 cells that were selected for Cd^{2+} tolerance showed amplification and rearrangement of the *smt* locus (Gupta *et al.*, 1992). The rearrangements were shown to be a deletion within *smtB* (Gupta *et al.*, 1993). The functional deletion of *smtB* in cells selected for Cd^{2+}-tolerance is consistent with the observation that *smtB* encodes a transcriptional repressor of *smtA*. Derepressed expression of *smtA* may be beneficial for cells continuously challenged by metal ions, although amplification of the *smtA* gene was also observed along with increased expression of *smtA*. The increase in metal resistance may have been due to the increase in the copy number of the *smtA* gene and not solely due to the absence of SmtB, or due to a mutation in another gene not analysed in this study .

4.2.5 Metal ion dependent gene regulation.

The formation of transcriptionally competent complexes (open complexes) between RNA polymerase and the promoter region has been modelled as a two step process. The first step involves RNA polymerase recognition, binding to the promoter and the formation of the closed complex. The next stage involves isomerisation of the closed complex to form an open complex, which involves unwinding of the DNA strands around the -10 region. This gives rise to transcription from the open complex. It is the rate of initiation of transcription that controls RNA synthesis and not the rate of RNA elongation (Goodrich and McClure, 1991).

There are two forms of negative control, signal responsive repression and signal responsive derepression. In metallo-regulated systems, in the case of signal responsive repression, RNA polymerase transcribes the target gene with maximal efficiency in the absence of the metal. The metal, when available, complexes with the transcriptional regulator, which then performs as a repressor. This binds to a specific region of the DNA that overlaps the promoter sequence, resulting in the repression of transcription. In the case of signal responsive derepression the regulatory protein blocks transcription of a specific gene until the metal ion complexes with it. This causes a

conformational change in the structure of the regulatory protein, allowing RNA polymerase to initiate transcription (O'Halloran, 1989; Hennecke, 1990).

In the case of positive control, the RNA polymerase would transcribe the regulated gene with maximal efficiency in the presence of the metal. This involves the binding of a metal-activated gene regulator to a specific region of the DNA, or by the regulator binding to or modifying other proteins in the transcription complex causing the formation of the RNA polymerase open complex. The transcriptional activator enhances the rate of transcriptional initiation. (O'Halloran, 1989; Hennecke, 1990). Present data support the proposal that SmtB acts as a metal-responsive-derepressor of *smtA* transcription.

4.2.6 Other regulatory genes isolated from *Synechococcus* species.

A gene thought to encode a global nitrogen regulator *ntcA* was isolated from *Synechococcus* PCC 7942 by Vega-Palas *et al.* (1992). *ntcA* is proposed to represent a positive-acting element required for the expression of a number of genes involved in nitrogen assimilation. Insertional inactivation of *ntcA* resulted in a nitrogen-assimilation-minus phenotype. NtcA is a protein of 24817 Da that belongs to a family of bacterial activators that includes the Crp (regulator of catabolite-sensitive genes) and Fnr *Escherichia coli* regulators (section 1.3). A region of NtcA is thought to contain a helix-turn-helix motif and it is speculated that interaction of this motif with DNA favours the expression of nitrogen-regulated genes (Vega-Palas *et al.*, 1992).

The genes involved in sulphate transport have also been isolated from *Synechococcus* PCC 7942. A sulphur-regulated gene (*cysA*) encoding a membrane-associated ATP-binding protein was identified by Green *et al.* (1989). Adjacent to *cysA*, and transcribed in the opposite direction is a gene involved in sulphate binding (*sbpA*). Two other genes, *cysT* and *cysW*, encode proteins that are thought to form a channel for the transport of sulphate ions across the cytoplasmic membrane are located upstream of *sbpA* (Laudenbach and Grossman, 1991). A fourth gene *cysR* is located

between *cysT* and *cysW*. *cysR* encodes a protein of 206 amino acids that has sequence similarity to the DNA binding regulatory proteins Fnr (section 1.3) and Crp from *Escherichia coli* and FixK from *Rhizobium meliloti* (regulator of genes involved in nitrogen fixation). A 200 b.p region separates the transcript start site of the *cysA* and *sbpA* genes. This DNA region includes a 12 b.p palindrome, thought to be involved in regulation (cited in Silver *et al.*, 1992).

4.3 The function of *smtA*.

The isolation of the *smtA* gene described in this study has subsequently enabled other workers in the laboratory to examine the function of SmtA.

4.3.1 The metal-binding properties of SmtA.

Olafson *et al.*, (1988) reported that *Synechococcus* MT was predominantly isolated with Zn^{2+} and Cd^{2+}, dependent upon the metal administered to the cells. In addition, a small amount of copper ions was detected associated with the protein when cells were induced with either Cd^{2+} or Zn^{2+}. In order to examine the metal binding properties of SmtA, the protein has been expressed in *Escherichia coli* as a carboxyterminal extension of glutathione-S-transferase using the expression vector, pGEX3X (Shi, *et al.*, 1992). The pH of half dissociation of Zn^{2+}, Cd^{2+} and copper ions from the expressed protein was found to be 4.10, 3.50 and 2.35 respectively. Mercury ions could not be displaced from the protein even at low pH suggesting a high affinity for mercury. In comparison with mammalian MT, the SmtA fusion protein had high apparent affinity for Zn^{2+}. Accumulation of Cd^{2+}, copper and Zn^{2+} ions was measured *in vivo* for *Escherichia coli* constitutively expressing the fusion protein, Zn^{2+} ions were found to be over-accumulated suggesting Zn^{2+}-binding *in vivo*.

4.3.2 Characterisation of *smt* deficient mutants.

The function of the *smt* locus in *Synechococcus* cells was studied via generation of *smt* deficient mutants using homologous recombination giving rise to insertional inactivation (Turner *et al.*, 1993). *smt* deficient cells were found to be hypersensitive to Zn^{2+} and showed a reduced tolerance to Cd^{2+}, although no difference in copper tolerance was observed between *smt* mutants and wild type cells. Restoration of Zn^{2+} tolerance has subsequently been used as a selectable marker for transformation of these mutants (Turner *et al.*, 1993). These studies suggest that the *smt* locus predominantly has a role in Zn^{2+} homoeostasis/detoxification and allows growth in elevated concentrations of Cd^{2+}.

4.3.3 Amplification and rearrangement of the *smt* locus in cells selected for Cd^{2+} tolerance.

Exposure of *Synechococcus* cells to elevated concentrations of Cd^{2+} resulted in a prolonged lag phase of growth. The resumption of growth coincided with an increase in the production of cellular MT (Olafson, 1984; Olafson, 1986). When these cells were transferred to fresh medium in the absence of Cd^{2+}, MT production dropped to basal levels. Upon retransfer to Cd^{2+} containing media the cells no longer exhibited a prolonged lag phase. This acquisition of Cd^{2+} tolerance was speculated to have arisen from the amplification of an extrachromosomal MT gene (Olafson, 1986). *Synechococcus* strains PCC 6301 and PCC 7942 retain two plasmids (Laudenbach *et al.*, 1983). However, the *smt* locus is not located on either of these plasmids (Turner *et al.*, 1993).

Synechococcus cell lines that were selected for Cd^{2+} tolerance via stepwise adaptation showed amplification and rearrangement of the *smt* locus. These cell lines were phenotypically distinct, displaying enhanced Cd^{2+} tolerance when compared to wild-type cell lines. It is proposed that the increased expression of *smtA* gives rise to

increased internal metal ion sequestration via the SmtA protein and facilitates Cd^{2+} resistance in these cell lines (Gupta *et al.*, 1993; Gupta *et al.*, 1992).

4.3.4 SmtA may play a role in Zn^{2+} homoeostasis.

There is evidence to support a primary role of SmtA in Zn^{2+} homoeostasis. The 5' region of *smtA* confers metal dependent expression of *lacZ* and Zn^{2+} is the most potent inducer. At MPC there is maximum induction by Zn^{2+} and the induction is lower at the MIC of Zn^{2+} (figures 3.19 and 3.21). There is an increase in the induction of β-galactosidase activity between the MPC and MIC of the other metals that give an induction response, suggesting that the *smt* system is more sensitive to Zn^{2+}, at levels in which *Synechococcus* can survive. β-galactosidase activity in *smt* deficient mutants, R2-PIM8(*smt*), containing pLACPB2 (*smt*-5') was maximally induced by 2.5 μM Zn^{2+}. This corresponds to the MPC of Zn^{2+} for these mutant cells (Huckle *et al.*, 1993). When *smtA* was expressed in *Escherichia coli* it was found to have a lower pH of half dissociation for Zn^{2+} than equine renal MT, with preferential binding of Zn^{2+} over copper and Cd^{2+} ions *in vivo* (Shi *et al.*, 1992). Mutants deficient in *smt* are Zn^{2+} hypersensitive, suggesting the major role of SmtA is in Zn^{2+} metabolism/homoeostasis.

This hypothesis is of particular interest because it has been speculated that higher eukaryotic MTs may be involved in the co-ordinate regulation of a large subset of genes whose transcription depends on Zn^{2+} requiring transcription factors, via an alteration in the supply of Zn^{2+} (Zeng *et al.*, 1991). However, *smt* deficient mutants are still viable, therefore *smtA* does not have a "vital" role in the donation of Zn^{2+} to apoproteins in these prokaryotic cells. Zn^{2+} requiring transcription factors have been characterised in eukaryotic systems (reviewed by Vallee *et al.*, 1991; Luisi, 1992). The donation of Zn^{2+} to apoproteins requires the maintenance of a precise Zn^{2+} homoeostasis that presents the metal at the right place and time. Such homoeostasis would require tight control, as it would directly affect the machinery of gene

regulation. Zn^{2+} bearing transcription factors have yet to be identified in prokaryotes. Luisi, (1992) speculates that prokaryotes may have avoided a hidden cost of maintaining regulation by Zn^{2+} requiring transcription factors. However, cyanobacteria may still have a requirement for intracellular Zn^{2+} buffering.

4.4 The regulation of *smtA* expression.

The *Escherichia coli* hybrid promoter was very active in *Synechococcus* PCC 7942 (figure 3.24). This result is consistent with the work of Schneider *et al.* (1991), discussed in section 4.1.2. The activity of the hybrid promoter with inverted repeat1 was compared to the hybrid promoter without inverted repeat1 (figure 3.24). The result suggested that inverted repeat1 may have been the binding site for a repressor protein in *Synechococcus.* This hypothesis was tested by comparing the activity of the two promoters with and without inverted repeat1 in *Escherichia coli* and *Synechococcus* (figure 3.25). The two promoters showed similar activity in *Escherichia coli* as in *Synechococcus* suggesting that if this were the binding site for a repressor protein, the repressor would exist in both hosts, which is unlikely as no equivalent *smt* system has been characterised in *Escherichia coli.* An explanation for this result may be that the dyad may have formed a secondary structure in the "artificial promoter" that caused the promoter to have low activity. Moreover, Morby *et al.* (1993) speculated that inverted repeat1 may play a role in the regulation of *smtB* alone, and/or only slightly modify *smtA* expression (see below).

Subsequent to the results described in this thesis, the *smt* truncated constructs described in section 2.3.4.13.7 have been used to investigate the regulation of *smtA* expression (Morby *et al.*, 1993) in R2-PIM8 (wild type small plasmid cured) and *smt*- mutants. There was a significant loss of inducible reporter gene activity using pLACPB2 (*smt*-5'*del*1), when compared to pLACPB2(*smt*-5'). This is in agreement with results observed in this study, figure 3.23. This decrease was not observed in the mutant background. It was concluded that this observation reflected the loss of a

"remote" *cis*-acting element essential for maximal expression, or the loss of plasmid born *smtB* and hence inequity between the number of copies of *smtB* and the number of copies of the *smt* operator-promoter. Further deletion, pLACPB2(*smt*-5'del2) resulted in loss of induction that was only apparent in the *smt*- mutant background. The deleted region was shown to correspond to a site of protein-DNA interaction (seen in gel retardation studies). The region was speculated to be a *cis*-acting activatory region (CCACC) found immediately upstream of inverted repeat1, and the DNA-protein complex was designated MAC3. Deletion of inverted repeat1 pLACPB2(*smt*-5'del3) did not alter the induction in wild type or *smtB*$^-$ mutants (Morby *et al.*, 1993).

Morby *et al.* (1993) found that inverted repeat1 was also a site for protein DNA interaction using gel retardation assays. The protein DNA complex was designated MAC2. It was speculated that MAC2 may play a role in the regulation of *smtB* alone, and/or only slightly modify *smtA* expression.

Another site for DNA-protein interaction, MAC1, was identified within the *smt* operator/promoter region using gel retardation assays. This complex was competed out using the inserts of pLACPB2(*smt*-5'del2) and pLACPB2(*smt*-5'del3). This demonstrates that MAC1 forms with the region lying between inverted repeat1 and the Shine-Dalgarno sequence of *smtA*. MAC2 was diminished in reactions containing the insert of pLACPB2(*smt*-5'del2) but not pLACPB2(*smt*-5'del3). This suggests MAC2 associates with inverted repeat1. MAC3 was not diminished by either competitor and is therefore proposed to bind upstream of inverted repeat1. Only one major complex corresponding to MAC1 was formed with the insert of pLACPB2(*smt*-5'del3). MAC1 was found to be absent in *smt* mutants lacking functional *smtA* and *smtB* genes, although MAC2 and MAC3 are present. MAC1 formation is restored upon reintroduction of plasmid borne *smtB*, hence MAC1 formation requires *smtB*. It was also proven that MAC1 formation was diminished by the presence of Zn^{2+} and that this effect could be reduced by the addition of chelating agents *in vitro*. This is consistent with the fact that MAC1 formation is *smtB* dependent and that *smtB* is a

repressor of *smtA* expression. Morby *et al.* (1993) concluded that inverted repeat2 is the likely site for the formation of MAC1.

In conclusion, the first prokaryotic MT locus has been isolated, although at present the precise function of this *smt* locus is unclear. The relative induction responses *in vivo* suggest a function for SmtA in Zn^{2+} homoeostasis. This is consistent with the previous observations by Olafson *et al.* (1988), showing that *in vivo Synechococcus* MT is induced by, and associated with, Zn^{2+}. Other data indicate that the *smt* locus can also play a role in the detoxification of Cd^{2+}.

Metal ion induced expression of the *smtA* gene is directed by an operator/promoter under the control of metal responsive factors with no detectable effect of metal ions on transcript stability. The divergent and overlapping orientation of the *smtA* and *smtB* promoters has precedent in some prokaryotic systems, where the divergent gene often encodes a regulatory protein. Several observations: gene architecture, similarity to known transcriptional regulators, the presence of a putative DNA-binding motif and the increase in the basal level of activity in the *smt* promoter lacking *smtB* are consistent with a role for SmtB as a transcriptional repressor. Subsequent studies using *smt* mutants have confirmed that SmtB is a *trans*-acting repressor of *smtA* transcription.

Further work is required to assign more precise functions to the *smtA* and *smtB* products and to understand the mechanism of the *smt* signal transduction pathway. While *smtA* is thought to play a role in Zn^{2+}-homoeostasis and Cd^{2+} detoxification, the mechanism of action is not yet proven. By analogy to eukaryotic MT it may sequester excess metal within cells, however a more dynamic role (for example part of a mechanism of metal efflux) in metal-tolerance cannot be eliminated.

4.5 Summary.

The following is a summary of the main findings of the research presented in previous chapters.

4.5.1 Characterisation of the *smt* locus.

1) *Synechococcus* strains PCC 6301 and PCC 7942 contain a gene encoding the first characterised prokaryotic MT, designated *smtA*.

2) The *smt* locus contains two open reading frames *smtA* and *smtB*, which are arranged in a divergent orientation.

3) These divergent open reading frames are separated by a region of DNA, designated the *smt* operator/promoter region.

4) The operator/promoter region has three regions which are candidate sites for protein DNA interaction.

5) The transcript start sites for both *smt* genes have been mapped to (within) the *smt* operator/promoter region.

6) The *smtA* transcript is 285 bases in length and the *smtB* transcript is 491 bases in length.

7) The divergent open reading frame designated *smtB* encodes a region that is a candidate for DNA binding.

8) SmtB shows similarity to a family of related proteins including several transcriptional regulators and a number of proteins involved in metal metabolism.

4.5.2 Expression of the *smt* genes.

1) Northern analysis indicates that there is an increase in *smtA* transcript abundance in response to exposure to $HgCl_2$, $CoCl_2$, $CdCl_2$, $ZnCl_2$, and CuCl.

2) An equivalent response was observed in *Synechococcus* PCC 7942 upon exposure to $CdCl_2$, suggesting the regulation of *smtA* is similar in these related strains.

3) This response is rapid, an increase in *smtA* transcripts can be observed in cell cultures that have been exposed to $CdCl_2$ for 10 minutes, with maximum induction after 30 minutes.

4) There is no increase in *smtA* transcript stability in response to metal ions suggesting that the regulation of *smtA* is controlled at the level of transcription.

5) The *smtA* 5' region can confer metal dependent expression upon a promoter-less *lacZ* in *Synechococcus* PCC 7942.

6) The *smtA* 5' region is most responsive to Zn^{2+} at biologically significant levels (MPC and MIC).

7) Deletion of the *smtB* gene from the 5' flanking region of *smtA* causes an increase in the basal level of expression from the *smt* promoter suggesting a role of *smtB* as a repressor of *smtA*.

8) A northern blot indicated that *smtB* transcripts also increase in abundance in response to $CdCl_2$ exposure.

9) The activity of the *smtB*5' reporter gene fusions suggest that *smtB* transcription is not induced upon exposure to MPC and MIC levels of $ZnCl_2$.

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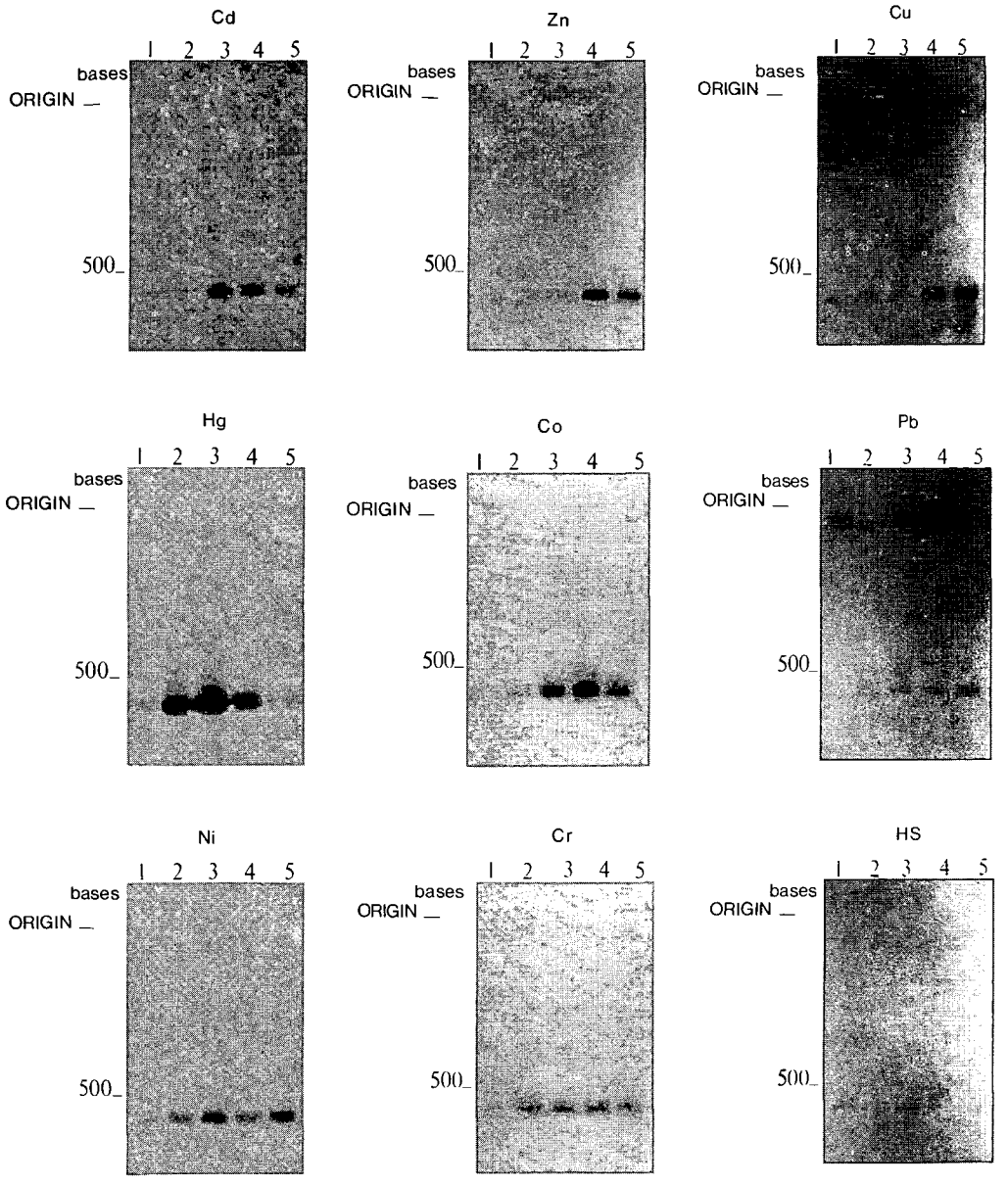


Fig. 4. Metal induction of *smtA* transcripts. Northern blots of RNA from *Synechococcus* PCC 6301 showing the increase in *smtA* transcript abundance in response to 2 h of exposure to exogenous metal ions (Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn) at various concentrations. Lane 1, 0 μM; 2, 1 μM; 3, 2.5 μM; 4, 5 μM; 5, 10 μM. A heat-shock (HS) control is also shown (lanes: 1, 32°C; 2, 45°C for 10 min; 3, 45°C for 20 min; 4, 55°C for 10 min; 5, 55°C for 20 min).

Analysis of metal-dependent β*-galactosidase activity in an* smt *mutant*

R2-PIM8 cells containing pLACPB2(*smt*-5') showed maximal induction of β-galactosidase at the maximum permissive concentration of Zn for growth (11 μM) (Fig. 8). Metal dependency, with an elevated basal level of expression (in media containing no metal supplements), was observed in R2-PIM8 cells containing a deletion of pLACPB2(*smt*-5'), designated pLACPB2(*smtB*$^-$) (Fig. 6A), which lacks a functional plasmid-borne *smtB* (Fig. 8). Beta-galactosidase activity in *smt*-deleted mutants, R2-PIM8(*smt*), containing pLACPB2(*smt*-5') was maximally induced at 2.5 μM Zn. This corresponds to the maximum permissive concentration of Zn for these mutant cells. There was also elevated basal expression in R2-PIM8(*smt*) containing pLACPB2(*smt*-5') (although it is noted that elevated basal expression in R2-PIM8(*smt*) containing pLACPB2(*smt*-5') was less apparent in subsequent experiments using modified protocols; data not

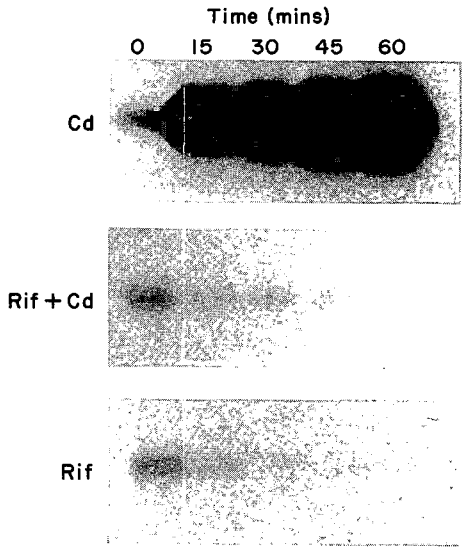


Fig. 5. Stability of the *smtA* transcript. Northern blots of *smtA* transcripts extracted from *Synechococcus* PCC 7942 after exposure to 2.5 μM Cd (Cd), 2.5 μM Cd + 400 μg ml^{-1} rifampicin (Rif + Cd) and to 400 μg ml^{-1} rifampicin alone (Rif). Samples were extracted every 15 min over a 1 h period and separated on agarose gels.

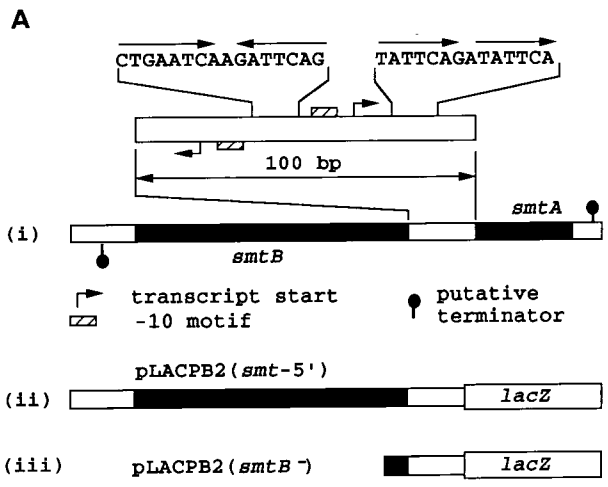
shown). Most significantly, there was highly elevated basal expression of β-galactosidase and loss of metal dependency in the *smt* mutant strain, R2-PIM8(*smt*), containing pLACPB2(*smtB*$^-$). These cells are devoid of a functional plasmid or genomic *smtB*.

Discussion

In this report we describe the isolation and subsequent characterization of a prokaryotic MT locus, *smt*. The product of a second ORF in the *smt* region, designated *smtB*, shows significant sequence similarity to ArsR and CadC proteins involved in metal tolerance. Similarity scores show that SmtB is most similar to the two *Staphylococcus* ArsR proteins. ArsR proteins are known to regulate the transcription of the *E. coli* (San Francisco *et al.*, 1990), *Staphylococcus xylosus* (Rosenstein *et al.*, 1992) and *S. aureus* (Ji and Silver, 1992) *ars* operons, which encode arsenite, arsenate and antimonite efflux systems. Efflux of these metal-oxyanions confers resistance. In the absence of inducers, the *E. coli ars* operon is repressed by ArsR. *ars* operon induction is mediated by arsenite, arsenate, antimonite and gratuitously by bismuthite (Silver *et al.*, 1981; San Francisco *et al.*, 1990; Wu and Rosen, 1991). The CadC protein in *S. aureus* is encoded by *cadC* which lies upstream of *cadA*, a gene encoding an ATP-dependent Cd(II) efflux system in *S. aureus* (Yoon and Silver, 1991). The mechanism of action of CadC is unknown but it is essential for high-level Cd-resistance. CadC is not required for Cd-dependent expression of the *cad* operon (Yoon and Silver, 1991). The *cadC* gene from *Bacillus firmus* OF4 (Mack Ivey *et al.*, 1992) partially complements sodium sensitivity in an *nhaA* mutant of *E. coli*, and a role in sodium/proton antiport is proposed in this system.

smtA transcript abundance appears to increase upon exposure to a range of metals shown to induce animal MT genes. As a negative control, *smtA* transcript abundance has also been examined in response to conditions known to induce the synthesis of heat-shock proteins in this organism. No increase was observed. Induction of *smtA* by a range of metals is in contrast to the yeast MT gene, *CUP1*, which is induced by exposure to Cu and Ag only (Butt *et al.*, 1984; Karin *et al.*, 1984). There was no detectable effect of Cd on *smtA* transcript stability. *smtA* transcripts did not increase in abundance in cells exposed to rifampicin and similar rates of transcript decay were observed in both the presence and absence of Cd. Metallo-regulation was also examined by fusion of the *smtA* 5' flanking region to a promoterless *lacZ* gene. Zn was the most potent inducer of β-galactosidase activity at maximum permissive concentrations. At maximum permissive concentrations, there was no significant induction of β-galactosidase activity in response to Hg (Fig. 7), while Northern blots indicate gratuitous increases in transcript abundance in response to 2 h of exposure to higher (lethal) Hg concentrations (Fig. 4). The magnitude of increase in β-galactosidase activity in response to 3 and 4 μM Ni and Co (Fig. 7) appeared to be relatively less than observed changes in *smtA* transcript abundance in response to 2.5 and 5.0 μM of the same metals (Fig. 4). This may be a reflection of differential toxicity towards translational rather than transcriptional machinery or a result of metal-mediated β-galactosidase inactivation. No significant increase in activity was observed in response to Cr. The *in vivo* response of the *smtA* operator–promoter to maximum permissive concentrations of different metal ions provides an indication of the relative importance of the locus to the metabolism of each of these ions. It is noted that the relative potency of metals as inducers may differ *in vitro*.

The divergent and overlapping orientation of the *smtA* and *smtB* promoters is an architecture which has precedent in some prokaryotic systems (dubbed divergons), where one gene often encodes a regulatory protein (Beck and Warren, 1988). Several observations (gene architecture, similarity to a known transcriptional regulator and also the presence of a putative DNA-binding motif) are consistent with a role for SmtB as a transcriptional repressor, while similarity to CadC could suggest alternative functions. In *smt*-deficient mutants there is a >20-fold increase in basal expression from the *smtA* operator–promoter which exceeds (by *c.* fourfold) maximal Zn-induced



expression in R2-PIM8 (Fig. 8). Complementation by plasmid-borne *smtB* demonstrates that SmtB acts in *trans* as a repressor of *smtA* transcription. Any putative direct interaction between SmtB and DNA could be mediated by residues 62 to 81, inclusive, which score highly on a prediction matrix for the helix-turn-helix DNA-binding motif. It is noted that in R2-PIM8 basal expression from pLACPB2(*smtB*⁻) is greater than from pLACPB2(*smt*-5'). This correlates with inequity between the number of copies of the *smt* operator-promoter and the number of copies of *smtB* which may result in titration of the repressor, and possibly the loss of remote *cis*-acting sequences. In addition, maximal expression of pLACPB2(*smt*-5') in R2-PIM8(*smt*) occurs at a lower concentration of Zn than in R2-PIM8 (2.5 and 11 μM respectively). In both cases maximal induction occurs at the maximum permissive

B (i) pLACPB2

```
CTGCAGGTCGACGGATCCCCGGGAATTCATCGAGCAACATATTAATGAGCCAGAGAAATGCTGGCGGCACTG
 PstI  SalI BamHI SmaI EcoRI

AAAGTTTTTGTACAAGCCGATGAAAGCGGCGACGCGCAGTTAATCCCACAGCCGCCAGTTCCGCTGGCGGCA

                  Shine-Dalgarno
TTTTAACTTTCTTTATCACACAGGAAACAGCT ATG ACC ATG ATT ACG GAT TCA CTG GCC GTC
                                 Met Thr Met Ile Thr Asp Ser Leu Ala Val
                                                lacZ
```

(ii) *smt*

```
                                                    Primer              BamHI
                                                    GTCAATAAGTCTATAAGTCCTAGGTTG
CCACCCACCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGATATTCAAGGAGT
             Inverted repeat       -10                 Direct repeat

GCTGTC ATG ACC TCA ACA
       Met Thr Ser Thr
           smtA
```

(iii) *smt-lacZ* fusion

```
                                                                    BamHI
CCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGATATTCAGGATCCCCGGGAA

TTCATCGAGCAACATATTAATGAGCCAGAGAAATGCTGGCGGCACTGAAAGTTTTTGTACAAGCCGATGAAAGC

GGCGACGCGCAGTTAATCCCACAGCCGCCAGTTCCGCTGGCGGCATTTTAACTTTCTTTATCACACAGGAAACA

GCT ATG ACC ATG ATT ACG GAT TCA CTG GCC GTC
    Met Thr Met Ile Thr Asp Ser Leu Ala Val
                   lacZ
```

Fig. 6. Fusion of the *smtA* operator–promoter to *lacZ*.
A. Schematic representations of: (i) the *smt* locus with an expanded operator–promoter region showing the positions and sequences of an inverted repeat and a direct repeat, transcript starts and corresponding consensus promoter structures; (ii) the upstream region of *smtA* (including *smtB*) transcriptionally fused to *lacZ*, pLACPB2(*smt*-5'); and (iii) the truncated derivative of pLACPB2(*smt*-5') lacking a functional *smtB*. The deleted region lies between a *Hin*dIII site (Fig. 1 nucleotides 1–6) and a *Pst*I site (Fig. 1, nucleotides 496–501).
B. Nucleotide sequences of pLACPB2, the *smtA* operator–promoter and the *smt–lacZ* fusion. (i) pLACPB2, polylinker and *lacZ* N-terminal coding sequence. (ii) 5' flanking region of smtA. The PCR primer used for amplification of a fragment which was fused to *lacZ* is shown above its complement. (iii) Fusion of the *smtA* operator–promoter to *lacZ*. The junction lies at a *Bam*HI site.

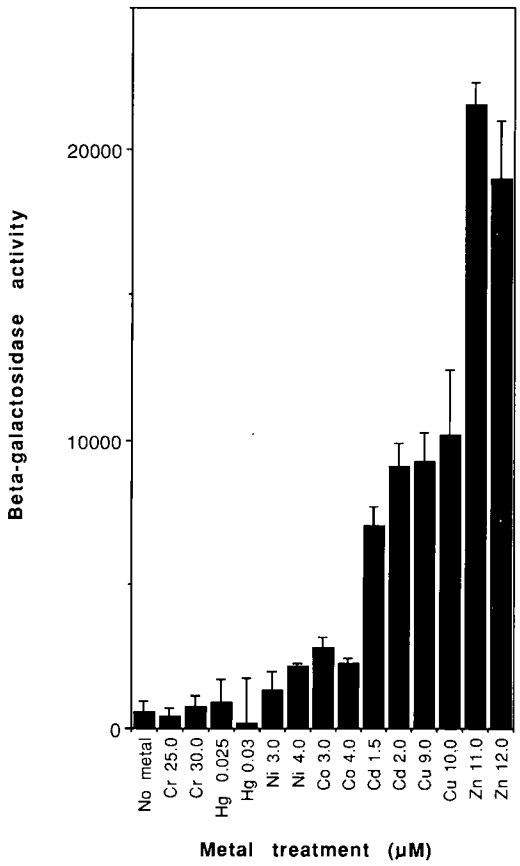


Fig. 7. Metal-induced expression of β-galactosidase.
Beta-galactosidase activity in cells containing pLACPB2(*smt*-5') exposed to maximum permissive concentrations (first bar) and to minimum inhibitory concentrations (second bar) of each metal. Data are the means of three separate determinations with standard deviations.

concentration (MPC) of Zn. To our knowledge, cyanobacterial genes encoding transcriptional regulators have not previously been reported.

At this time the precise function(s) of the *smt* locus is (are) unclear. It is thought to play a role in essential metal ion homoeostasis and resistance to certain non-essential metal ions, analogous to eukaryotic MT. A relatively lower pH of half-displacement of Zn (and hence higher Zn affinity) than that estimated for equine renal MT was determined for the SmtA protein expressed in *E. coli* (Shi *et al.*, 1992). The relative induction responses *in vivo* (highest for Zn at maximum permissive concentrations) and metal affinities (relative to equine MT) strongly support a function for SmtA in Zn homoeostasis. This is also in agreement with previous observations showing that MT *in vivo* in *Synechococcus* sp. is induced by, and associated with, Zn (Olafson *et al.*, 1988). A role in the detoxification of a non-essential metal ion (Cd) is reported in the following paper (Gupta *et al.*, 1993, accompanying paper).

In conclusion, we have isolated a prokaryotic metallothionein locus which includes the structural gene *smtA* and a divergent gene, *smtB*. Metal-ion-induced expression of the *smtA* gene is directed by an operator–promoter under the control of metal responsive factors with no detectable effect of metal ions on *smtA* transcript stability. The divergent gene *smtB* encodes a *trans*-acting repressor of *smtA* transcription. Further work is required to assign more precise functions to the *smtA* and *smtB* products and to understand the mechanism of signal transduction.

Experimental procedures

Cyanobacterial culture and transformation

Synechococcus PCC 6301 and PCC 7942 were cultured in AC medium (Kratz and Meyers, 1955) as modified by Shehata and Whitton (1982). *Synechococcus* PCC 7942 (R2-PIM8), a methionine auxotroph also lacking the 8 kb plasmid, was cultured in Allen's medium (Allen, 1968) supplemented with DL-methionine (30 μg ml^{-1}) and streptomycin (5 μg ml^{-1}). An *smt*

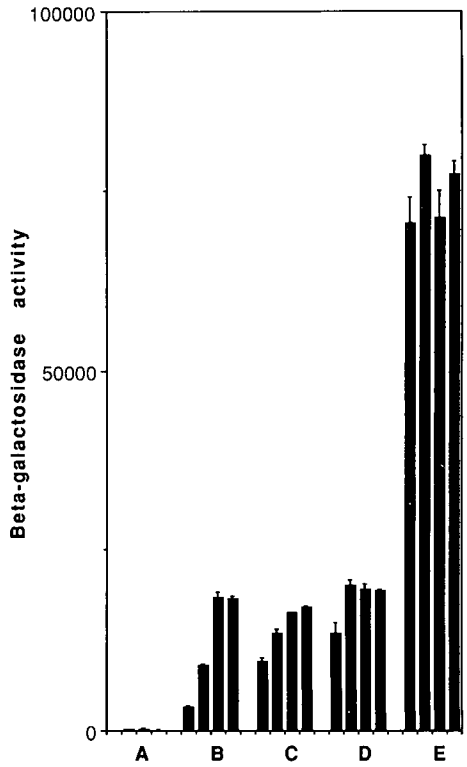


Fig. 8. Beta-galactosidase activity in R2-PIM8 and the *smt*-deleted mutant, R2-PIM8(*smt*). Beta-galactosidase activity of: A, R2-PIM8 containing pLACPB2 only as a control; B, R2-PIM8-containing pLACPB2(*smt*-5'), which includes plasmid and chromosomal *smtB*; C, R2-PIM8 containing pLACPB2(*smtB*$^-$), lacking a plasmid-encoded, but including chromosomal, *smtB*; D, R2-PIM8(*smt*) containing pLACPB2(*smt*-5'), which only carries a copy of *smtB* on the plasmid; and E, R2-PIM8(*smt*) containing pLACPB2(*smtB*$^-$), devoid of *smtB*. Each strain was exposed to increasing concentrations (0, 2.5 μM, 11μM and 12μM) of $ZnCl_2$ (left to right) prior to assay.

mutant strain, R2-PIM8(*smt*), was constructed (J. S. Turner *et al.*, in preparation) in which the *smt* operator–promoter and 5' regions of both genes were replaced with the plasmid pSU19(Cm^R), a derivative of pSU2719 (Martinez *et al.*, 1988). These mutants were cultured in media supplemented with chloramphenicol (7.5 μg ml^{-1}) and have been confirmed to be deficient in functional *smtA* and *smtB* (J. S. Turner *et al.*, in preparation). R2-PIM8(*smt*) containing pLACPB2 plasmids were cultured in Allen's medium (as above) further supplemented with carbenicillin (50 μg ml^{-1}). Culture conditions were as described by Robinson *et al.* (1990).

Transformation of R2-PIM8 was performed according to Kuhlemeier *et al.* (1983). Transformants were plated onto Allen's medium containing 1.5% (w/v) bacto agar, as described by Scanlan *et al.* (1990), supplemented with appropriate antibiotics.

In vitro *DNA manipulation; cloning and characterization of the* smt *locus*

Restriction endonucleases and other DNA-modifying enzymes were supplied by Northumbria Biologicals Limited and Boehringer Mannheim. *Taq* polymerase was supplied by Stratagene or Perkin–Elmer/Cetus. [α-^{32}P]-dCTP (14.8 TBq $mmol^{-1}$) was supplied by Amersham International. Sequencing was performed by the dideoxy-sequencing method of Sanger *et al.* (1977), and reaction products analysed using an Applied Biosystems 370A DNA Sequencer.

Genomic DNA was isolated from cyanobacteria using a protocol described previously for the isolation of nucleic acids from plant cell cultures (Robinson *et al.*, 1988), but excluding caesium chloride gradients.

To prepare a size-fractionated genomic library, DNA from *Synechococcus* PCC 7942 was digested with *Sal*I and *Hin*dIII, fractionated by sucrose density-gradient centrifugation (25–50%) and the fraction containing *smtA* identified by dot-blot hybridization to *smtA* probe prepared from gel-purified fragments of pJHNR11 (a pUC19 clone containing PCR fragments corresponding to part of *smtA*; Robinson *et al.*, 1990). DNA was radiolabelled with [α-^{32}P]-dCTP according to the procedure of Feinberg and Vogelstein (1983). DNA within the identified fraction was ligated to *Sal*I/*Hin*dIII-digested pGEM4Z (Promega), used to transform *E. coli* JM101 competent cells (Alexander *et al.*, 1984), and the resulting library screened with *smtA* probe, described above, to isolate clone pJHNR49.

RNA isolation, analysis and primer extension

Total RNA was isolated from *Synechococcus* PCC 7942 and PCC 6301 using standard techniques (Dzelzkalns *et al.*, 1988). Cells were grown to mid-logarithmic phase and treated with metal salts at a range of concentrations for 2 h. All of the metal salts were introduced as the divalent chloride, except for Pb which was nitrate. Equivalent amounts of RNA (20 μg) from different cell extracts were glyoxylated, resolved on 1.5% agarose gels, and transferred to nylon membranes (Hybond-N, Amersham International) (Sambrook *et al.*, 1989). Blots were hybridized to *smtA* probe, washed with 0.5 × SSC, 0.1% SDS at 65°C. Primer extension used a protocol described by San Francisco *et al.* (1990), the RNA template being isolated from cultures exposed to 2.5 μM $CdCl_2$ for 2 h prior to extraction.

The use of rifampicin to inhibit transcription

Rifampicin was used to inhibit transcription in *Synechococcus* cultures as described by Wealand *et al.* (1989). Cultures were exposed to rifampicin alone, rifampicin and Cd (2.5 μM), and to Cd alone. RNA was then extracted from each culture every 15 min over a 1 h period and used for northern blots as described above.

Construction of smt-lacZ *fusions*

PCR was used to generate approximately 600 bp of *smtA* 5' flanking region (which also contains *smtB*), introducing a *Bam*HI site at the 3' end of the PCR product (Fig. 6). PCR was essentially as described previously (Robinson *et al.*, 1990) but using 1 μg of pJHNR49 as template, an M13 reverse primer as the second PCR primer, and only 12 cycles of amplification to minimize PCR-mediated errors. Fragments were ligated to pGEM4Z and sequenced prior to subcloning into the promoter probe vector pLACPB2 (Scanlan *et al.*, 1990), creating a transcriptional fusion with *lacZ*, and the ligation used to transform *E. coli* JM101 competent cells. Following subsequent transformation of R2-PIM8, the plasmid, designated pLACPB2(*smt*-5'), was recovered from R2-PIM8, used to transform *E. coli*, purified and restriction mapped. A derivative, designated pLACPB2($smtB^-$), deficient in functional *smtB*, was constructed by ligating a *c.* 100 bp *Pst*I–*Bam*HI fragment from pLACPB2(*smt*-5'), carrying the *smtA* operator–promoter, into pKS (Stratagene), cut with *Pst*I-*Bam*HI to create pKS($smtB^-$). Subsequently, a similar fragment was excised on a *Sal*I–*Bam*HI fragment from pKS($smtB^-$) and ligated to pLACPB2 to create pLACPB2($smtB^-$) (Fig. 6).

Determination of β*-galactosidase activity*

Beta-galactosidase activity was assayed using *o*-nitrophenyl–D-galactopyranoside (ONPG) (SigmaK) as the substrate, as described by Miller (1972). Cells were lysed with chloroform/SDS. Activity was calculated relative to one of three values obtained for R2-PIM8 containing pLACPB2 alone, with no added metal (the mean and standard deviation of three replicate control values is also shown).

Acknowledgements

This work was supported by Research Grant GR3/7883 from the UK Natural Environment Research Council (NERC). J.W.H. is supported by an NERC Research Studentship. The authors acknowledge the support and advice given by Dr Brian A. Whitton and thank Dr Dave Scanlan for the donation of pLACPB2. N.J.R. is a Royal Society University Research Fellow.

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stringency of 0.5 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate) at 65 °C for 15 min and autoradiographed at −80 °C by using pre-flashed X-ray film (Fuji RX) and intensifying screen.

(*i*) *Ligation mediated, or anchored,* PCR *(APCR)*

Genomic DNA (10 μg), restricted with *Hind* III, was ligated to 4 μg of pUC19 plasmid DNA, previously restricted with *Hind* III and *Pst* I. Aliquots (0.35 μg) of the ligation were used as template DNA for four separate APCR's using combinations of *smt*A gene primers, as specified in figure 1, and universal M13 forward and reverse sequencing primers, which hybridize to sites on pUC19, as described below. Reaction conditions were similar to those described previously for standard PCR by Fordham-Skelton *et al.* (1990), with the exception that 10 μg of *smt*A specific primer and 0.5 μg of generic primer were used in a 100 μl reaction, and the reactions were subjected to 32 cycles of the following series of temperatures and times 92 °C for 1.5 min; 55 °C for 2 min; 72 °C for 5 min.

(*j*) *RNA isolation and analysis*

Total RNA isolated from *Synechococcus* PCC 6301 by using standard techniques (Dzelkalns *et al.* 1988) was analysed on 15 g l^{-1} agarose gels and visualized with ethidium bromide to allow quantitation. Equivalent amounts of RNA (c.a. 20 μg) from different cell extracts were glyoxalated, resolved on 15 g l^{-1} agarose gels, and Northern blotted onto nylon membranes (Hybond-N, Amersham International, Aylesbury, U.K.) by standard techniques (Sambrook *et al.* 1989). Blots were hybridized to *smt*A probe, washed to 0.5 × SSC, 1 g l^{-1} SDS at 65 °C, and autoradiographed as before. All Northern blots were hybridized concurrently with the same batch of probe and subsequently exposed simultaneously to X-ray film.

3. RESULTS AND DISCUSSION

(*a*) *Amplification and cloning of part of the coding region of smt*A

Inosine-containing oligonucleotide primers were synthesized corresponding to the two peptide regions shown in figure 1. These regions were selected to minimize primer redundancy and maximize the proportion of the coding region that would be amplified. Inosines were incorporated at base positions with greater than twofold redundancy, and restriction endonuclease recognition sites were included at the 5′ ends, as shown, to facilitate cloning. The analysis of the PCR products obtained using these primers and *Synechococcus* PCC 6301 template DNA is shown in figure 2. Two amplification products of 144 b.p. and 116 b.p. were obtained. The nucleotide, and encoded amino acid, sequence of the insert in a plasmid containing the larger fragment, is shown in figure 3. This amino acid sequence corresponds to the known sequence (Olafson *et al.* 1988) of the class II MT in *Synechococcus* PCC 6301 inclusive of regions 1 and 2 shown in figure 1, with the exception of Cys_{32} being

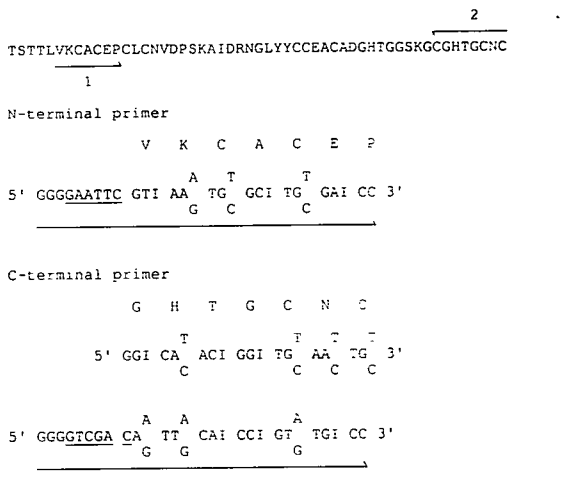


Figure 1. Amino acid sequence of *Synechococcus* MT and the deduced primer sequences used in DNA amplification reactions. The arrows show the relative location and orientation of the two primers. Restriction endonuclease recognition sites, added to the 5′ ends of the primers, are underlined.

Figure 2. Visualization of amplified fragments on an agarose gel. Amplification conditions (using *Synechococcus* PCC 6301 genomic DNA) were as described in the Materials and Methods.

substituted for Ser. It is noted that this Cys was one of only two amino acids, in the published MT amino acid sequence, which was not confirmed in two independent sequence analyses. The observed homology confirms that part of the MT gene has been amplified and hence designated *smt*A.

It is noted that sequence errors will arise due to the use of the base analogue inosine in the primer region or the low frequency of base misincorporation by *Taq* DNA polymerase, although three independent clones

```
GTG AAA TGC GCG TGT GAG CCC TGT CTC TGC AAC GTC GAT CCC AGC
Val Lys Cys Ala Cys Glu Pro Cys Leu Cys Asn Val Asp Pro Ser

AAA GCG ATC GAT CGC AAC GGT CTG TAC TAC TGC AGC GAA GCC TGT
Lys Ala Ile Asp Arg Asn Gly Leu Tyr Tyr Cys Ser Glu Ala Cys

GCC GAT GGC CAC ACC GGT GGT AGC AAA GGC TGC GGC CAC ACC GGC
Ala Asp Gly His Thr Gly Gly Ser Lys Gly Cys Gly His Thr Gly

TGC AAC TGN
Cys Asn Cys
```

Figure 3. Nucleotide, and encoded amino acid, sequence of the 144 b.p. fragment shown in figure 2. This corresponds to part of the known amino acid sequence of *Synechococcus* MT with the exception of the underlined Ser. A region of internal homology to the C-terminal primer site (underlined) corresponded to the 3′ end of the truncated 116 b.p. product shown in figure 2. Bases corresponding to the positions of inosines in the primers may be incorrectly assigned and are shown in bold.

(pJHNR11, pJHNR12, pAGNR11) were sequenced to detect such errors. Locations corresponding to I in the N-terminal primer contain G in the PCR product and locations corresponding to I in the C-terminal primer contain C in the PCR product (figure 3). This is because of preferential I/C base pairing (Ohtsuka *et al.* 1985). These six bases are shown in bold type-face (figure 3) as they may not correspond to the nucleotides present in the template DNA.

The nucleotide sequence of the 116 b.p. fragment (figure 2) showed that it is a product of a primer mismatch to an internal region of homology to the C-terminal primer-binding site within *smt*A, shown on figure 3, to produce a truncated product.

The sequence obtained for part of the *smt*A gene and shown in figure 3 was missing 15 bases of *smt*A coding region at its 5′ end. Therefore, APCR was employed to isolate and sequence this region and also associated translational signal sequences.

(*b*) *Chromosome crawling by* APCR

A genomic Southern blot of restricted DNA from *Synechococcus* PCC 6301, probed with labelled *smt*A insert from a clone containing the 144 b.p. PCR product, shows a single major hybridizing band for each digest (figure 4). *Hind* III was selected for APCR because of the relatively small size (2.8 kb) of the *smt*A *Hind* III fragment. pUC19 was digested with *Pst* I, the linear form recovered from an agarose gel and then digested with *Hind* III in order to obtain plasmid DNA with only one *Hind* III compatible end which could subsequently ligate to *Hind* III digested genomic DNA. *Hind* III digested genomic DNA (10 μg) was ligated to 4 μg of digested pUC19. Four separate APCR's were set up using combinations of the two *smt*A specific primers, and two generic M13 universal primers which hybridize to the vector. A negative control reaction was also set up containing the two *smt*A specific primers but without template DNA. Diverse *Hind* III fragments will ligate to pUC19 and both universal M13 primers will hybridize to all of these ligation products. However, only fragments containing the *smt*A gene will amplify exponentially when the ligation mix is used as a template for PCR because of the specificity of the

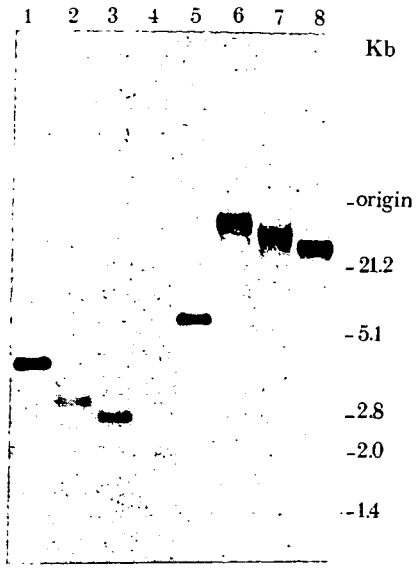


Figure 4. *Synechococcus* PCC 6301 genomic Southern blot hybridized with ^{32}P-labelled *smt*A probe prepared from the insert in plasmid pJHNR11, which contains the 144 b.p. fragment shown in figure 2. DNA was digested to completion with the following restriction endonucleases: lane 1, *Sal* I & *Nco* I; lane 2, *Sal* I & *Bgl* II; lane 3, *Hind* III; lane 4, *Pst* I; lane 5, *Sal* I; lane 6, *Bam*H I; lane 7, *Kpn* I; lane 8, *Eco*R I.

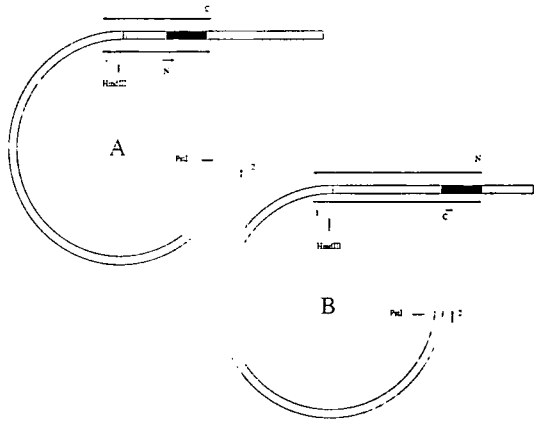


Figure 5. Diagrammatic representation of ligation mediated, or anchored, PCR. Total genomic DNA, digested with *Hind* III, was ligated to pUC19 digested with *Hind* III and *Pst* I (represented as partial circle). The universal M13 reverse primer (1) will prime unidirectional single strand synthesis of all ligated fragments but only *Hind* III fragments containing the *smt*A gene will be exponentially amplified (long arrows). Fragments containing *smt*A will be ligated in both orientations A and B. In reactions containing the *smt*A N-terminal primer (N) there will be exponential amplification of fragments ligated in orientation B of part of the *smt*A coding region (black box) and 3′ flanking sequence (open box) to the first 3′ *Hind* III site. In reactions containing the C-terminal primer (C) there will be exponential amplification of fragments ligated in orientation A of the *smt*A coding region and 5′ flanking sequence (shaded box) to the first 5′ *Hind* III site. There will be no amplification of *smt*A fragments in reactions containing the M13 forward primer (2). Primers that will not give exponential amplification of *smt*A fragments in a particular ligation are shown as short arrows.

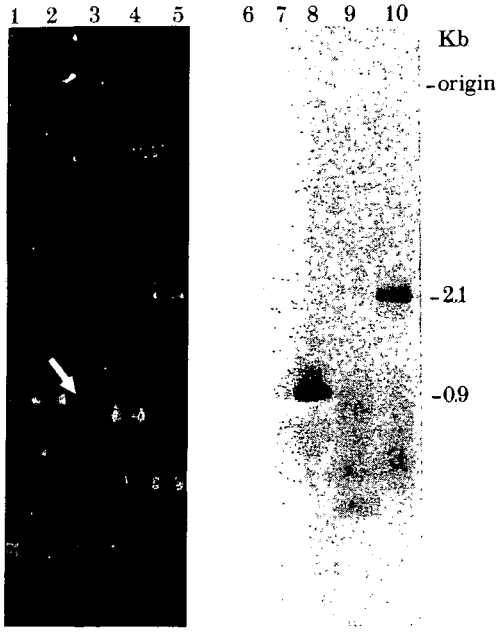


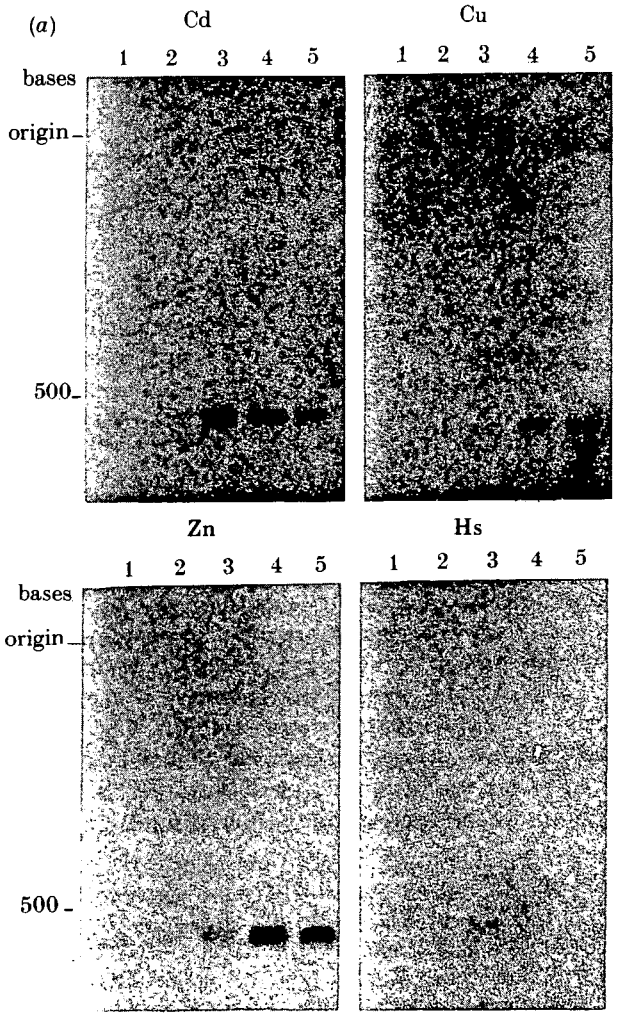
Figure 6. Visualization of APCR products on an agarose gel. The primers used were: lane 1, *smt*A N- and C-terminal primers, no template control; lane 2, M13 forward & *smt*A C-terminal; lane 3, M13 reverse & *smt*A C-terminal; lane 4, M13 forward & *smt*A N-terminal; lane 5, M13 reverse & *smt*A N-terminal. Lanes 6 to 10 are a Southern blot of lanes 1–5 probed with ^{32}P-labelled *smt*A probe. Two specific products are revealed in lanes 8 and 10. The 5′ amplification product which was subsequently cloned and sequenced is shown with an arrow in lane 3.

```
TCAAAGGAGTTGCTGTCATG ACC TCA ACA ACC TTG GTC AAA TGC GCG
                     Thr Ser Thr Thr Leu Val Lys Cys Ala

TGT GAG CCC
Cys Glu Pro
```

Figure 7. Nucleotide sequence of part of the APCR fragment produced from reactions containing the *smt*A C-terminal primer giving the correct sequence (corrected base in bold) of the complement to the N-terminal primer binding site, the 15 b.p. of *smt*A coding sequence missing from figure 3, and an additional 20 b.p. of 5′ region including AAGGAG and ATG sequences.

*smt*A primer in each reaction. Furthermore, only reactions containing the M13 reverse primer will lead to amplification of ligated fragments as only this primer is adjacent to the *Hind* III ligation site in pUC19, while the M13 forward primer is adjacent to the incompatible *Pst* I site (figure 5). APCR with the *smt*A C-terminal primer (figure 1) will amplify the upstream coding sequence of *smt*A and its 5′ flanking region to the first upstream *Hind* III site, while the *smt*A N-terminal primer will amplify the downstream *smt*A coding sequence and its 3′ flanking region to the first downstream *Hind* III site (figure 5). Both sets of amplification products will therefore contain the diagnostic coding sequence, shown in figure 3, at one end. Figure 6 shows that a variety of non-specific amplification products were produced; however specific *smt*A products were revealed on a Southern blot probed with the insert of pJHNR11, and the 5′ fragment which was subsequently cloned is denoted



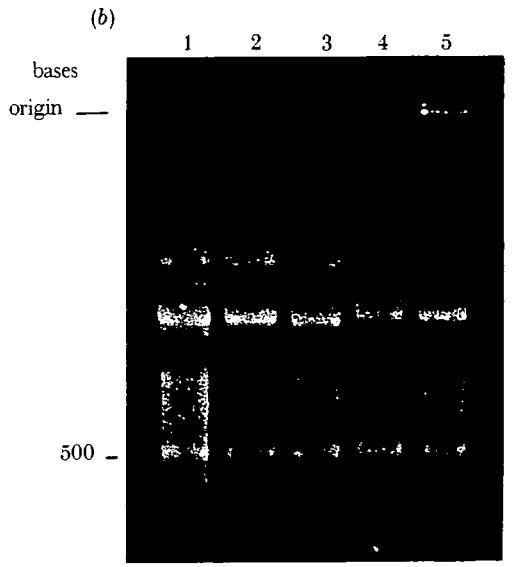


Figure 8. (*a*) Northern blots of RNA from *Synechococcus* PCC 6301 grown in the presence of different concentrations (lane 1 = 0 μM; 2 = 1 μM; 3 = 2.5 μM; 4 = 5 μM; 5 = 10 μM) of added metals (Zn; Cu; Cd) or elevated temperature (Hs) (lane 1 = 32 °C; lane 2 = 45 °C for 10 min; lane 3 = 45 °C for 20 min; lane 4 = 55 °C for 10 min; lane 5 = 55 °C for 20 min), and probed with *smt*A. (*b*) Equivalent amounts of RNA were loaded onto each track as shown by the visualization of rRNA bands for isolates from cells exposed to Cu.

with an arrow in the first panel of figure 6. The sum of the estimated sizes of the two specific *smt*A fragments, 0.9 + 2.1 = 3.0 kb, is equivalent to the size (2.8 kb) of the *Hind* III fragment indicated from genomic Southern blots (figure 4), plus an additional 0.2 kb (comprising 144 b.p. of *smt*A coding region, since this has been amplified twice, and $2 \times 25 = 50$ b.p. of amplified pUC19 sequences). The 0.9 kb APCR fragment was purified from agarose gels incubated with T4 DNA polymerase and blunt end ligated to pUC19. Part of the sequence of the inserts in plasmids (pJHNR21, pJHNR22, pJHNR23, pJHNR24) containing this fragment, is shown in figure 7. It is noted that this APCR strategy introduces a second M13 primer site (either forward or reverse depending upon the vector used in the initial ligation) and therefore only the alternative M13 site can be used for subsequent sequence analysis, unless the duplicate site is removed.

The sequence shown in figure 7 corresponds to the N-terminal primer site, 15 bases of coding region upstream from the N-terminal primer site which were missing from the previous *smt*A clones, and 20 bases of 5′ region flanking the coding sequence, which include the AAGGAG and ATG sequences for ribosome-binding and start of translation. The five codons absent from figure 3 encode the five amino acids at the N-terminus of *Synechococcus* MT as determined by Olafson *et al.* (1988) and this confirms the utility of APCR for chromosome crawling. One of the bases in the N-terminal primer region was incorrectly assigned as G in figure 3 but shown to be C in figure 7. This base corresponds to I in the N-terminal primer and will have arisen because of preferential I/C pairing as described above. Therefore, using APCR the correct sequence of the N-terminal primer region has been obtained.

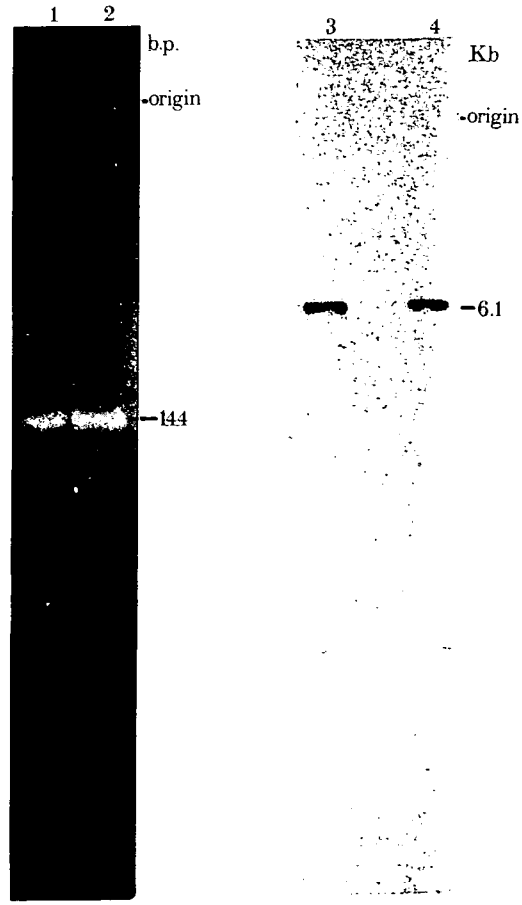


Figure 9. Identification of a *smt*A homologue in *Synechococcus* PCC 7942. Lanes 1 & 2 show visualization of amplified *smt*A fragments on an agarose gel by using template DNA from: lane 1, *Synechococcus* PCC 6301; lane 2, *Synechococcus* PCC 7942. Lanes 3 & 4 are a genomic Southern blot of *Sal* I digested genomic DNA from: lane 3, *Synechococcus* PCC 7942; lane 4, *Synechococcus* PCC 6301, probed with *smt*A.

(*c*) *Expression of smt*A *in Synechococcus PCC 6301 and identification of a homologue in Synechococcus PCC 7942*

The abundance of *smt*A transcripts in *Synechococcus* PCC 6301 increases following exposure to increased concentrations of Cd, Zn and Cu ions in the growth medium (figure 8*a*). Visualization of the rRNA bands showed that similar amounts of RNA were loaded on each track (figure 8*b*). In response to elevated concentrations of Cd, *smt*A transcripts are maximally abundant after exposure to 5 μM, and slightly less abundant following exposure to 10 μM. By contrast, *smt*A transcripts are more abundant following exposure to 10 μM compared to 5 μM Zn. There is an increase in *smt*A transcript abundance following exposure to elevated concentrations of Cu, although Cu appears to be a less potent inducer than either Zn or Cd. The transcripts did not increase in abundance following exposure to conditions which induce synthesis of heat-shock proteins (figure 8 and Borbely *et al.* (1985)).

Using the *smt*A primers (figure 1) a fragment (*ca.* 144 b.p.) was amplified from genomic DNA from *Synechococcus* PCC 7942 (= *Anacystis nidulans* R2) (figure 9). Furthermore, a *smt*A homologue was also identified on a genomic Southern blot of *Sal* I digested DNA from *Synechococcus* PCC 7942 (figure 9). Therefore *smt*A genes have been detected in both of these two strains which have been referred to as *Anacystis nidulans*.

In conclusion, it has been possible to characterize the first prokaryotic MT gene, *smt*A, without screening genomic DNA libraries, thus demonstrating the feasibility of the strategy described. This has allowed subsequent examination of the effect of elevated concentrations of certain trace metals on *smt*A transcript abundance. Exposure to elevated concentrations of Zn, Cd and Cu ions lead to an increase in *smt*A transcript abundance. It remains to be established whether these changes are due to transcriptional activation of the prokaryotic MT gene, analogous to eukaryotic MT genes (see Palmiter (1987) for a review), or changes in transcript stability. The possible role of these genes in mechanisms of metal tolerance, and in the metabolism of essential trace metals such as Zn, in cyanobacteria requires investigation.

N.J.R. is a Royal Society University Research Fellow. J.W.H. is supported by a N.E.R.C. research studentship and A.G. by a Nehru Memorial Trust scholarship. This work was partly financed by S.E.R.C. research grant GR/E 91257. The authors thank J. Bryden and J. Gilroy for technical

assistance, and gratefully acknowledge the work of R. W. Olafson *et al.*, on which this research was based.

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(*Received 3 September 1990: accepted 18 September 1990*)

Nucleic Acids Research, 1993, Vol. 21, No. 4 921–925

SmtB is a metal-dependent repressor of the cyanobacterial metallothionein gene *smtA*: identification of a Zn inhibited DNA-protein complex

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Received November 6, 1992; Revised and Accepted January 21, 1993 EMBL accession no. X64585

ABSTRACT

The *smt* locus of *Synechococcus* PCC 7942 contains a metal-regulated gene (*smtA*), which encodes a class II metallothionein, and a divergently transcribed gene, *smtB*, which encodes a repressor of *smtA* transcription. Regions containing *cis*-acting elements required for efficient induction, and required for *smtB*-dependent repression, of the *smtA* operator-promoter were identified. Specific interactions between proteins extracted from *Synechococcus* PCC 7942 and defined regions surrounding the *smtA* operator-promoter were detected by electrophoretic mobility shift assays. Three metallothionein operator-promoter associated complexes were identified, one of which (MAC1) showed Zn-dependent dissociation and involved a region of DNA immediately upstream of *smtA*. Treatment with Zn-chelators facilitated re-association of MAC1 *in vitro*. MAC1 was not observed in extracts from *smt* deficient mutants but was restored in extracts from mutants complemented with a plasmid borne *smtB*. SmtB is thus required for the formation of a Zn-responsive complex with the *smt* operator-promoter and based upon the predicted structure of SmtB we propose direct SmtB-DNA interaction exerting metal-ion inducible negative control.

INTRODUCTION

Metal-induced expression of metallothionein (MT) genes in different animals involves the association of metal-activated factors, MTF-1, ZAP, MEP-1 with defined metal responsive elements (MRE's) (cited in 1). Cu-induced expression of *CUP1*, which encodes yeast Cu-thionein (MT), is mediated by ACE1 (2). Cu-ACE1 binds to *CUP1* upstream activator sequences (UAS) and stimulates MT expression.

We have recently isolated a prokaryotic MT locus, *smt*, from the cyanobacteria *Synechococcus* PCC 7942 and PCC 6301 (3, 4). Deletion of the *smt* locus reduces Zn/Cd tolerance (5). The *smt* locus includes *smtA*, which encodes a class II MT (6, 7) and a divergently transcribed gene *smtB* which encodes a repressor of *smtA* transcription (4). Transcription from the *smt* operator-promoter is stimulated by certain trace metal ions (notably Zn, Cu and Cd)(4). A 100 bp operator-promoter region lies between the *smtA* and *smtB* protein coding regions and contains divergent promoters that border a 7-2-7 hyphenated inverted repeat. An imperfect 6-2-6 hyphenated inverted-repeat and a 6-2-6 hyphenated direct repeat are located between the sites of transcriptional and translational initiation in *smtA*. Complementation studies show that *smtB* encodes a transcriptional regulator of *smtA* (4). The deduced SmtB polypeptide contains a region that scores highly (5.5) on a prediction matrix for the helix-turn-helix DNA-binding motif (4, 8). SmtB also has sequence similarity to a family of prokaryotic metal oxyanion-responsive factors, ArsR, (4, 9, 10) and MerR from *Streptomyces lividans* (11). These observations are suggestive of direct SmtB/DNA interaction. SmtB also shares sequence similarity to CadC, a protein of unknown function that is essential for high level Cd-resistance in *Staphylococcus aureus* (4, 12) and to CadC from *Bacillus firmus* OF4.

We report interactions between the *smt* operator-promoter region and proteins from *Synechococcus* PCC 7942.

MATERIALS AND METHODS

Bacterial strains

Derivatives of *Synechococcus* PCC 7942 referred to as R2-PIM8 (15), and R2-PIM8(*smt*) were used for protein extractions and phenotypic analyses. R2-PIM8(*smt*) is an *smt* mutant strain (4), in which the *smt* operator-promoter and 5′ regions of both genes are deleted and is therefore deficient in functional *smtA* and *smtB*. R2-PIM8 strains were cultured in Allens media (16, 3). *E.coli* SURE (Stratagene Ltd) was used for all genetic manipulations. *E.coli* was cultured in L-broth and plated on L-agar (17). Where appropriate, streptomycin (5 μg/ml), chloramphenicol (7.5/34 μg/ml) carbenicillin (10/50 μg/ml) and zinc chloride (2.5, 10 and 11 μM) were added to the growth medium (the concentrations given are for *Synechococcus*/*E.coli*).

Construction of *smt-lacZ* fusions and promoter deletions

PCR was used to generate two *smt* operator-promoter deletions flanked by *Sal*I and *Bam*HI sites, these sites were used to clone the truncated fragments into pGEM4Z and, after nucleotide sequence analysis, into the *lacZ* fusion vector, pLACPB2 (17),

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as described in (4). Standard cloning techniques were employed (18). Both truncated fragments (δ1, δ2) included the region downstream of the hyphenated inverted repeat, one (δ1) also contained the hyphenated inverted repeat (Figure 1E, F). The primers used to generate the PCR products were 5'-GGCGTCGACCTGAATCAAGATTCAGATGTTAGG-3' for δ1 and 5'-GGCGTCGACATGTTAGGCTTAAACACAT-3' for δ2, in conjunction with primers detailed in (4).

Protein extraction

Synechococcus cultures (1L, O.D.$_{540}$ = 0.3) were harvested by centrifugation and resuspended in 1 ml of extraction buffer (10 mM Tris, 1 mM EDTA (or 10 μM $ZnCl_2$), 250 mM KCl, 0.5 mM DTT, 10% glycerol (v/v) and 1 mM PMSF). The cell suspension was frozen in liquid nitrogen and ground to a fine powder, suspended in 10 ml of extraction buffer, and sonicated. Cellular debris was pelleted (15,000×g, 20 mins) and protein was precipitated from the supernatant by the addition of $(NH_4)_2SO_4$ (0.4 g/ml). The protein precipitate was pelleted (15,000×g, 20 mins) and resolvated in 0.5 ml of extraction buffer without KCl. The protein extract was then dialysed for 12 h against 2L of extraction buffer without KCl. All manipulations were performed at 4°C.

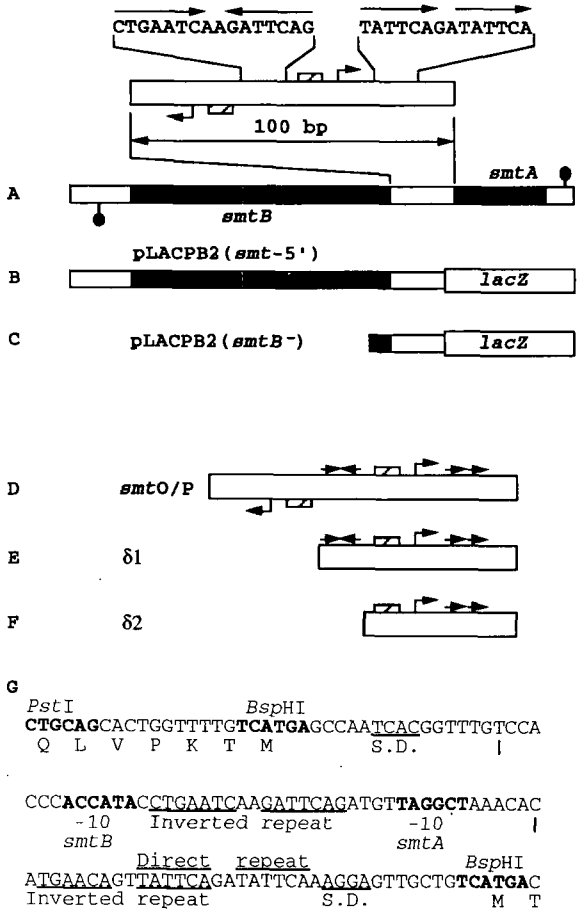

Figure 1. Organisation of the *smt* locus, reporter gene fusions and EMSA probes/competitors. A: *smt* locus. The divergent genes *smtA* and *smtB* are shown as black rectangles. The 100 bp operator-promoter region is expanded to show the sequence of the hyphenated inverted repeat (converging arrows) and hyphenated direct repeat (unidirectional arrows). Other features include determined transcript start sites (bent arrow), −10 motif (hatched box) and putative terminators (circle). B, C: Reporter gene fusions. *smtB* is again shown as a black rectangle within regions fused to a promoterless *lacZ* gene in the vector pLACPB2. D: *smt*O/P region used as a probe in EMSA. E, F: Deletion derivatives of the *smt*O/P region used as specific competitor DNA (δ1 and δ2), EMSA probe (δ2) and in reporter gene constructs. G: Sequence of the *smt* operator-promoter region showing partial amino acid sequence for SmtA and SmtB, *Bsp*HI and *Pst*I restriction enzyme sites (bold), −10 sequences (bold), Shine-Dalgarno sequences (underlined) and inverted/direct repeats (under/over-lined).

Electrophoretic mobility shift assays

This technique was performed as previously described (19) except the for the omission of EDTA from the binding buffer when using extracts enriched for Zn. Electrophoresis was for 150 mins at 140 V and the DNA-protein complexes were visualised by direct autoradiography. The probes used were *smt*O/P (100bp *Bsp*HI fragment, figure 1D, G), δ1 and δ2 (*Sal*I-*Bam*HI fragments, figure 1E, F).

β-galactosidase assays

These assays were performed using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma, Dorset, UK) as the substrate (20). Optical density (O.D.) readings at 420 and 600 nm were normalised against water and Allens media respectively. β-galactosidase activity was calculated using a modified equation Activity(nmoles *o*-nitrophenol/min/mg protein) = (O.D.$_{420(t)}$ − O.D.$_{420(0)}$)300/(t×v×O.D.$_{600}$)1.83 Cells were lysed with chloroform/SDS.

Computer analysis

All computer analyses used the GCG package on the SERC Daresbury facilities DLVH and SEQNET.(21)

RESULTS

Analysis of metal-dependent β-galactosidase activity in an *smt* mutant

Metal dependent β-galactosidase activity was detected in R2-PIM8 containing pLACPB2(*smt*-5') (Figure 2). Cells containing a 5' deletion derivative of this construct,

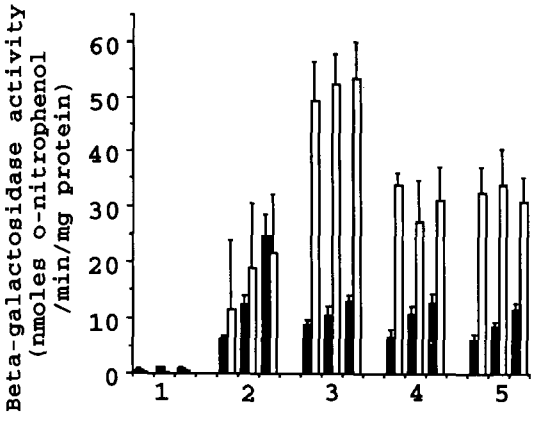

Figure 2. β-galactosidase activity measured in *Synechococcus* strains containin *smtA-lacZ* reporter gene fusions and deletions. Closed columns represent a R2-PIM8 background and open columns represent an R2-PIM8(*smt*) backgroun Each block of three values corresponds to 0, 2.5 and 11 μM Zn exposure fro right to left. The constructs are; pLACPB2 (1), pLACPB2(*smt*-5') (2 pLACPB2(*smtB*⁻) (3), pLACPB2(δ1) (4) and pLACPB2(δ2).(5)

was not significantly diminished by either competitor and is proposed to bind upstream of the 7-2-7 hyphenated inverted repeat. As anticipated, only one major complex (C1), corresponding to MAC1, forms with δ2 (Figure 4B).

MAC1 (Figure 4A), and hence C1 (Figure 4B), do not form when using extracts from R2-PIM8(*smt*), which lacks functional *smtA* and *smtB* genes, however, MAC2 and MAC3 are retained (Figure 4A). C1 is restored upon reintroduction of a plasmid borne *smtB* (Figure 4B) demonstrating that SmtB is necessary for C1 (MAC1) formation.

The stability of MAC1 is slightly diminished by treatment with Zn *in vivo* (Figure 3A). A more pronounced diminution of C1 (MAC1), in response to *in vivo* Zn, was observed using extracts prepared with EDTA-free buffers (Figure 5). In these extracts, C1 increased following *in vitro* addition of 1,10-phenanthroline. These data suggest a direct interaction of this factor with metals. To date it has not been possible to affect binding via the addition of Zn *in vitro* (data not shown). This is also apparent in Figure 5 where a difference in binding between extracts from *in vivo* Zn exposed and non-exposed cells is observed when both extracts have been extensively dialysed against buffers containing 5 μM Zn. This may indicate a requirement for other factors or conformational change in the ligand *in vitro* (such as oxidation of a metal-binding site). Zn-dependent dissociation is consistent with the observations that C1 (MAC1) is *smtB* dependent and that SmtB is a repressor of *smtA* expression. The association of this complex with the δ2 fragment (figure 1), containing only the *smtA* promoter and downstream regions, is consistent with a role in transcriptional repression. A 6-2-6 direct repeat (TATTCA-GA-TATTCA) is present in the region retained in the δ2 fragment and represents a candidate for DNA-protein interaction, however, prokaryotic proteins employing helix-turn-helix structure generally bind to inverted repeats rather than to directly repeated sequences. Another candidate site is a degenerate 6-2-6 inverted repeat, which incorporates the left half of the 6-2-6 direct repeat (TGAACA-GT-TATTCA)(figure 1G).

SmtB shows significant similarity to a range of proteins within the OWL 18.0 database. This family of related proteins includes several transcriptional regulators and a number of proteins involved in metal metabolism. Within this family only NolR is not involved in metal-ion metabolism.

All of the known metal responsive transcription factors involved in the regulation of eukaryotic MT genes directly bind metals and, as a result, are activated to bind specific promoter-DNA sequences to activate transcription (1). Similarly other eukaryotic transcription factors, Zn-fingers, Zn-clusters and Zn-twists (22), require Zn for DNA-'association'. By contrast, although the SmtB-dependent complex (C1, MAC1), also interacts directly with metals, this interaction mediates dissociation from specific promoter-DNA sequences rather than binding. This component of prokaryotic MT gene regulation imparts inducible negative control compared with the inducible positive control seen in eukaryotic MT genes. The presence of other factors (MAC2, MAC3) suggests additional regulatory mechanisms.

In summary, *smtB* encodes a transcriptional repressor of *smtA* and is required for the formation of a complex, MAC1, with a region immediately upstream of the *smtA* coding sequence. MAC1 shows Zn-dependent dissociation from its target sequence. Circumstantial evidence supports the proposal that MAC1 is formed by the interaction of SmtB with DNA. Two other DNA-protein complexes have been identified, MAC2 and MAC3. MAC3 forms with an upstream region which plays a positive regulatory role in expression from the *smtA* operator-promoter. To our knowledge *Synechococcus* genes encoding *trans*-acting DNA-binding proteins have not previously been characterised and the observed Zn-dependent dissociation is unique among known metal-responsive transcription factors.

ACKNOWLEDGEMENTS

This work was supported by research grant GR3/7883 from the U.K. Natural Environment Research Council. J.W.H. and J.S.T. were supported by studentships from the U.K. Natural Environment Research Council. The authors thank K. Elborough for unpublished EMSA protocols. N.J.R. is a Royal Society University Research Fellow.

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Volume 303, number 2,3, 159–163 FEBS 11081 June 1992

Cyanobacterial metallothionein gene expressed in *Escherichia coli*

Metal-binding properties of the expressed protein

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Received 7 April 1992

The recently isolated Synechococcus gene *smtA* encodes the only characterised prokaryotic protein designated to be a metallothionein (MT). To examine the metal-binding properties of its product the *smtA* gene was expressed in *Escherichia coli* as a carboxyterminal extension of glutathione-*S*-transferase. The pH of half dissociation of Zn, Cd and Cu ions from the expressed protein was determined to be 4.10, 3.50, 2.35, respectively, indicating a high affinity for these ions (in particular for Zn in comparison to mammalian MT). *E. coli* expressing this gene showed enhanced (ca. 3-fold) accumulation of Zn.

smtA; SmtA protein; Prokaryotic metallothionein; Cyanobacteria; Synechococcus; *Anacystis nidulans*; Metal-accumulation; Metal-tolerance

1. INTRODUCTION

Although metallothionein (MT)-'like' metal-ligands have been reported in several different prokaryotes (cited in [1]) only one such protein has been isolated (from a cyanobacteria by Olafson et al. [2]) and sequenced. There is no significant similarity between the amino acid sequence of this Synechococcus protein and any other sequenced MT. The amount of this protein in Synechococcus cells was found to increase following supplementation of the growth medium with either Zn or Cd, but not Cu [3]. The native protein was isolated associated predominantly with either Zn or Cd corresponding with the metal administered to the cells. In addition, a small amount of Cu was detected following induction with either Cd or Zn. CD spectral analysis of the native Zn-protein identified some characteristics resembling mammalian Zn-MT [2]. However, the conformation of the metal-binding site(s) has (have) not been determined and the metal affinities of this protein have not previously been reported. Whether or not this prokaryotic protein has high affinities for metals, equivalent to those of eukaryotic MTs, needs to be established.

We have recently isolated PCR fragments corresponding to the gene encoding this cyanobacterial protein [4]. Genomic clones have subsequently been isolated and the gene designated *smtA*. Several different eukaryotic MT genes have been expressed in *E. coli* and their proteins shown to form conformations which allow association with metal ions [5–10]. In some cases eukaryotic MTs have been demonstrated to bind metal ions when expressed as fusions with another protein [7,9]. In addition, *E. coli* cells expressing murine MT-1 over-accumulated Cd and showed elevated resistance to Hg, Ag, Cu, Cd and Zn [10]. In contrast *E. coli* expressing rainbow trout MT did not show any significant increase in resistance to trace metals but did show increased accumulation of Cd [8]. Expression of *Neurospora crassa* MT, and variants of this protein, in *E. coli* led to increased accumulation exclusively of Cd and Cu from a mixture of 16 different metal ions [9]. In this letter we describe the expression of *smtA* in *E. coli* in order to facilitate analysis of the metal-binding characteristics of the expressed protein. In addition, *E. coli* cells expressing this gene have been examined for phenotypic effects in terms of modified metal-tolerance or -accumulation.

2. MATERIALS AND METHODS

2.1. *In vitro amplification, cloning and sequence analysis of smtA coding region*

The following two oligonucleotide primers were synthesized using an Applied Biosystems 381A DNA synthesizer; 5'GGCGGATCCCCATGACCTCAACAACCTTGGTC 3'; 5'GGCGAATTCACTACAGTTGCAGCCGGTGTGGCC 3'. These primers facilitated amplification by the polymerase chain reaction (PCR) of the *smtA* protein coding region and incorporated *Bam*HI and *Eco*RI restriction endonuclease recognition sites at opposite ends. These sites allowed cloning (in frame) into the glutathione-*S*-transferase (GST) fusion protein expression vector, pGEX3X (Pharmacia).

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PCR was performed using 200 ng of plasmid pJHNR4.9 (a genomic clone in the vector pGEM4z containing a 1.8 kb *Hin*dIII–*Sal*I fragment of *Synechococcus* PCC7942 DNA which includes the *smtA* gene) as template and standard conditions as described previously [4]. Purified PCR products were digested with *Bam*HI and *Eco*RI and then ligated into the equivalent sites of pGEX3X.

The sequence and orientation of the cloned *smtA* fragment in pGEX3X (pGPMT1) was verified by sequencing in both directions using the PCR oligonucleotide primers. The determined sequence of the *smtA* coding region and part of GST is given in Fig. 1. DNA sequencing was performed by modified dideoxy termination (Dye Deoxy Termination: Applied Biosystems) and reaction products were analysed using an Applied Biosystems 370A DNA sequencer.

2.2. *Expression, purification and characterisation of the recombinant SmtA protein*

The recombinant protein was expressed in *E. coli* and purified using glutathione Sepharose 4B (Pharmacia) as described previously [11]. Protein content was determined using a Coomassie blue based reagent (Bio-Rad) and bovine serum albumin as a standard. Metal concentration of samples was determined by atomic absorption spectrophotometry. Proteins were resolved on 15% SDS-PAGE gels [12] and visualised following Coomassie brilliant blue staining.

The protein sequence between GST and SmtA includes a specific protease recognition sequence for blood coagulation factor Xa [13] which facilitated cleavage of SmtA protein from GST. SmtA protein (purified from cells grown in LB medium supplemented with 0.5 mM Zn) was cleaved from GST while the fusion protein was associated with glutathione Sepharose 4B [14]. Column eluant containing factor Xa and SmtA protein was subsequently fractionated on Sephadex G-50. Fractions (2.5 ml) were collected and analysed for protein and metal. The amino acid sequence of the protein in the Zn-peak fraction was determined as described previously [11].

2.3. *Analysis of metal-binding properties of the expressed protein*

Aliquots of GST-SmtA fusion protein (purified from cells supplemented with 2 mM Cu; 0.5 mM Zn; 0.5 mM Cd, or 20 μM Hg) were incubated in different pH buffers and the proportion of bound metal ions determined as previously described [11]. Preparations of Zn-GST-SmtA fusion protein were also incubated with a two fold molar excess (with respect to Zn) of Cu. Unbound metal was removed by gel filtration (PD-10, Pharmacia) and the pH of half-dissociation of in vitro associated metal ions determined. The experiment was also repeated with a commercial preparation of equine metallothionein (Sigma).

2.4. *Analysis of metal-resistance and -accumulation*

Overnight cultures containing pGEX3X, or pGPMT1 were diluted 90% (v/v) in fresh LB medium supplemented with 50 $\mu g \cdot ml^{-1}$ ampicillin, then grown for 1 h at 37°C. Cultures were diluted again into fresh LB media containing 50 $\mu g \cdot ml^{-1}$ ampicillin such that the final OD_{600} of all cultures was 0.025. After a further incubation of 1 h, production of GST, or GST-SmtA, was induced by the addition of IPTG to a final concentration of 1 mM and metal was added to inhibitory concentrations (1 mM $ZnSO_4$; 2 mM $CuSO_4$; 0.6 mM $CdSO_4$). Cells were incubated at 37°C and growth-monitored by OD_{600} after 1 h and at hourly intervals thereafter.

To determine the amount of metal accumulated by *E. coli* cells, standardised aliquots (equivalent absorption at 600 nm) were removed from cultures grown as described above. The cells were harvested by centrifugation, washed twice in LB medium and solubilised overnight by incubation in 70% (v/v) nitric acid/water. Metal content was determined by atomic absorption spectrophotometry and converted to amount of metal per 8×10^8 cells (assuming an optical density of 1 is equivalent to 8×10^8 cells ml^{-1}). Additionally, metal accumulation was analysed in a similar manner for cells grown in the presence of lower concentrations of each metal (0.5 mM $ZnSO_4$, 0.5 mM $CuSO_4$, 0.3 mM $CdSO_4$.

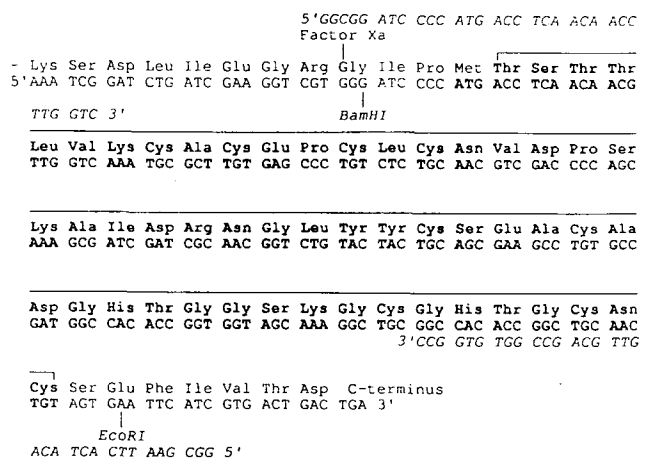


Fig. 1. The determined nucleotide sequences of part of the GST coding sequence from the expression vector pGEX3X and of the *smtA* coding region (shown in bold) generated from a genomic clone by PCR using oligonucleotide primers shown in italics. The PCR product was ligated using *Eco*RI and *Bam*HI recognition sites to produce an in-frame translational fusion which may be cleaved with factor Xa at the indicated site. The SmtA protein sequence is in bold type and is denoted with a line.

3. RESULTS AND DISCUSSION

Sequence analysis of pGPMT1 confirmed the orientation and frame of the *smtA* coding region fused to GST (Fig. 1). The predicted amino acid sequence of the product of *smtA* is in agreement with the reported sequence of the protein purified from *Synechococcus* sp. [2], with the exception of Ser^{32}, a discrepancy which we have previously observed [4]. Most recently we have also established that the *smtA* gene from *Synechococcus* PCC7942 contains an additional two codons adding a further ^{N}His-Gly^{C} to the C-terminus of the SmtA protein in this organism. These two amino acids are absent from the protein expressed here.

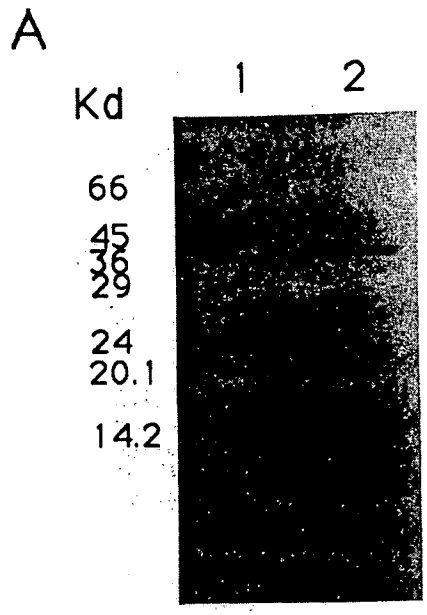
A protein corresponding to the predicted size (M_r 35,500) of the GST-SmtA fusion protein was detected in GSH-affinity purified lysates of induced JM101 cells containing plasmid pGPMT1 following growth in media supplemented with 0.5 mM Zn (Fig. 2A). A protein corresponding to the predicted size (M_r 26,500) of

Table I

Metal content of GST and GST-SmtA, purified from lysates of cells grown in media supplemented with Cd, Cu, Hg or Zn.

	Metal ion/polypeptide			
Protein	Cd	Cu	Hg	Zn
GST	0.87 ± 0.14	0.35 ± 0.32	0.80 ± 0.18	0.89 ± 0.25
GST-SmtA	5.89 ± 1.35	1.72 ± 1.03	6.78 ± 0.25	4.34 ± 0.27

The data shows the estimated number of moles of metal associated with each mol of protein. Mean values are given for extracts from three replicate cultures with standard deviations.



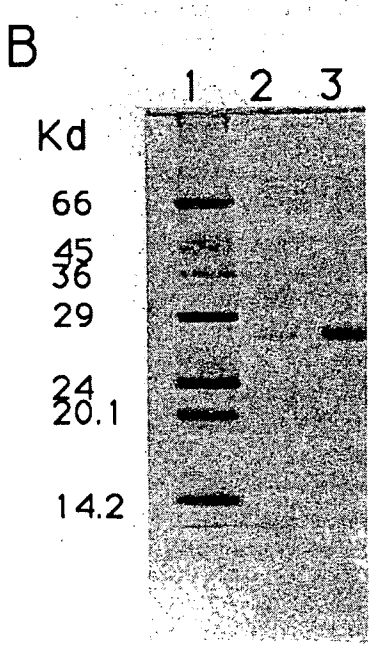


Fig. 2. Analysis of *E. coli* cell extracts by SDS-PAGE. Gels were stained with Coomassie blue. Origin of protein samples: molecular weight markers (both panels, lane 1); glutathione-Sepharose 4B purified lysates from IPTG-induced cells containing pGEX3X (B, lane 3) or pGPMT1 (both panels, lane 2). Protein was purified from cells grown in media supplemented with Cu (B, lane 2) or Zn (A, lane 2).

GST alone was detected in equivalent isolates from cells containing the pGEX3X vector alone (Fig. 2B). The bound metal content of GST-SmtA and GST alone was determined for protein purified from *E. coli* grown in media supplemented with Cu, Cd, Hg or Zn (Table I).

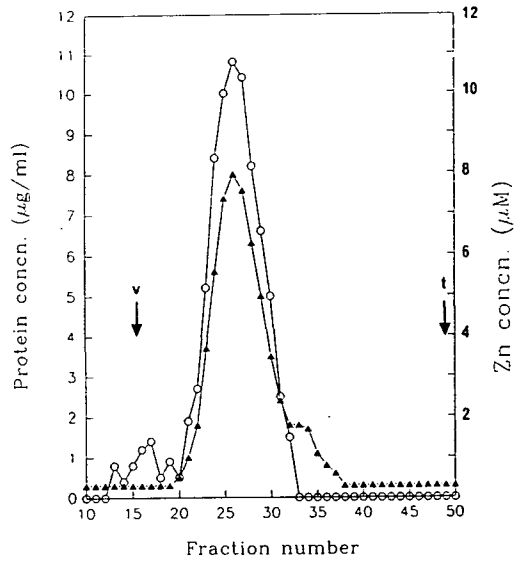


Fig. 3. Gel filtration chromatography on Sephadex G-50 of purified protein eluted from glutathione Sepharose 4B following incubation with factor Xa. Protein (○) was determined by dye binding assay and Zn (▲) was quantified by atomic absorption spectrophotometry. The void (v) and total (t) volumes of the column were determined by calibration with Blue dextran and Zn (II) respectively.

Relatively more metal was found to be associated with the fusion protein than with GST alone. However, variability in reactivities of different proteins (bovine serum albumin; GST; SmtA) in the protein assay may cause inaccuracies in the estimated stoichiometries. Furthermore, it is apparent that isolates from cells grown in media supplemented with Cu were impure (Fig. 2B). This could partly account for the lower estimates for the amount of Cu associated with the protein relative to the other three metal ions.

Data in Table I indicate that Cd, Cu, Hg and Zn all bind to the SmtA portion of the GST-SmtA fusion protein. To establish that metal ions associate with this part of the fusion protein, the SmtA domain was released from the fusion protein (purified from *E. coli* grown in Zn supplemented media) while associated with glutathione-Sepharose, using factor Xa. This releases SmtA plus an extension of three amino acids at the amino terminus (^{N}Gly-Ile-Pro^{c}) and an extension of seven amino acids at the carboxy terminus (^{N}Ser-Glu-Phe-Ile-Val-Thr-Asp^{C}). These additional amino acids arise from the GST coding sequence into which the *smtA* coding sequence is inserted in plasmid pGPMT1 (refer to Fig. 1). Eluate from glutathione-Sepharose was fractionated on Sephadex G-50 and fractions analysed for Zn and for protein (Fig. 3). A large coincident peak for Zn and protein was detected (fraction 26). Amino acid sequence analysis of an aliquot of this Zn-peak fraction gave the first 12 amino acids of SmtA, plus the anticipated N-terminal extension (Table II). A less abundant second amino acid was also detected at each cycle of Edman degradation. This sequence corresponded to SmtA se-

Table II

Amino acid sequence analysis of Zn peak fraction after gel filtration on Sephadex G-50 of cleaved SmtA

Cycle	1	2	3	4	5	6	7	8	9	10	11	12
Ala	15.8	0.0	−0.8	−3.8	−0.7	−0.4	0.1	3.9	*83.9*	0.0	15.6	0.0
Arg	102.7	0.0	0.0	0.0	−2.3	0.0	36.9	14.8	9.5	13.6	0.0	0.0
Asn	−0.2	0.3	2.9	−0.4	0.2	0.4	−0.7	0.4	−0.2	−1.0	1.5	−1.1
Asp	53.4	0.0	0.0	−0.3	−1.3	−0.4	−0.6	0.0	3.0	0.0	1.0	0.0
Cys	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	*−1.0*	−1.0	*−1.0*	−1.0	**−1.0**
Glu	2.4	−1.5	−0.6	33.4	0.0	0.0	−1.5	−0.5	−0.5	0.5	*54.5*	0.0
Gln	0.5	0.2	−0.1	−1.1	0.7	−0.8	−0.5	1.1	1.1	0.0	2.6	0.0
Gly	**478.3**	0.0	0.0	−1.4	0.0	0.0	0.0	23.1	−1.1	−0.5	0.7	10.7
His	−0.4	0.7	1.7	0.1	2.4	4.0	−0.9	0.0	8.3	0.0	5.3	0.6
Ile	−0.3	**528.6**	0.0	0.0	0.0	0.0	−1.5	24.8	14.8	0.0	0.0	0.0
Leu	−1.2	46.7	0.0	−1.4	*67.4*	0.0	−0.3	−6.3	**169.7**	92.2	0.0	0.0
Lys	21.2	0.0	0.0	−1.0	−1.9	−0.3	*51.4*	0.0	16.1	0.0	**159.5**	110.2
Met	4.9	−0.3	0.0	**433.6**	0.0	0.0	0.0	0.0	0.4	10.8	11.5	0.0
Phe	0.4	−0.1	−0.3	−0.1	0.4	0.0	−0.8	−0.1	0.2	0.2	0.6	0.2
Pro	0.3	7.6	**391.6**	0.0	0.0	0.0	−11.3	−3.8	2.5	11.3	−5.8	*34.9*
Ser	1.9	*23.1*	0.0	−2.1	0.0	**48.6**	0.0	4.2	0.0	0.4	−1.3	2.1
Thr	*76.2*	−0.2	*25.7*	*25.6*	**116.0**	0.0	**51.5**	**110.2**	22.7	2.1	0.0	0.0
Trp	0.7	−0.5	−0.4	−0.1	−0.1	0.1	0.3	0.1	0.2	−0.2	−0.2	0.2
Tyr	3.3	−0.1	−0.1	−0.3	0.2	0.3	−0.2	0.5	−0.4	−1.0	−0.7	0.2
Val	1.3	−0.8	−2.0	−1.3	5.5	*84.0*	0.0	14.8	0.0	**174.2**	105.9	0.0

The yield (pmol) of each amino acid is shown at each cycle with the anticipated sequence of SmtA plus N-terminal extension in bold and underlined. A secondary sequence corresponding to the anticipated sequence initiated at amino acid 5 is italicised and underlined.

quence initiated four amino acids beyond the factor Xa cleavage site. These data confirm that metal ions associate with the SmtA portion of the recombinant protein.

Comparison of the pH at which 50% of metal is dissociated is a criterion which has been used to distinguish MT from non-MT metal-binding proteins. From the graphs shown in Fig. 4 (panels A) such values can be estimated for the GST-SmtA fusion protein to be 4.10, 3.50 and 2.35 for Zn, Cd and Cu respectively. These values were obtained for fusion protein isolated from cells grown in media supplemented with the respective metal ions. A similar value was also obtained for Cu associated with GST-SmtA by exchange binding in vitro. Bound Hg could not be displaced under the conditions used in these experiments indicative of high affinity for this metal. From Fig. 4 the pH of half-dissociation of Zn from equine MT is estimated to be 4.50, which is in agreement with previously reported values [15,16]. This implies that SmtA may have a higher affinity for Zn than does equine MT and could have an important role in Zn metabolism/detoxification in Synechococcus cells. Metal displacement curves for Cu and Cd indicate that SmtA has a lower affinity for these metals than does equine MT.

Expression of the GST-SmtA fusion protein under the conditions reported here did not confer any detectable increase in Cd, Cu or Zn tolerance in *E. coli* containing plasmid pGPMT1 compared to cells containing the pGEX3X vector alone (data not shown). This does not preclude the possibility that metal-regulated expression of *smtA* confers enhanced tolerance to certain metals in Synechococcus cells. Table III shows the accumulation of these three metal ions in the same cells. There was no significant difference in the accumulation of Cd, Cu or Zn in cells grown in the presence of inhibitory concentrations of these metals. However, following growth in the presence of lower concentrations of these metals there was an increase in the accumulation of Zn in cells expressing GST-SmtA and also a slight, but statistically significant, increase in accumulation of Cu (Table III).

In conclusion, we have demonstrated that following

Table III

Accumulation of metal ions by intact *E. coli* cells containing either pGPMT1, or pGEX3X expressing GST-SmtA, or GST, respectively

	Expressed protein	Accumulated metal (nmol/8 × 10^8 cells)	
		[high]	[low]
Cd	GST	24.406 ± 3.825	5.789 ± 0.858
	GST-SmtA	23.531 ± 5.168	8.717 ± 2.410
Zn	GST	11.178 ± 0.885	1.998 ± 0.205
	GST-SmtA	12.172 ± 0.834	6.049 ± 1.038
Cu	GST	16.778 ± 7.371	6.193 ± 0.595
	GST-SmtA	21.762 ± 8.96	8.869 ± 1.164

Cells were grown in media supplemented with either inhibitory concentrations ([high]) of 0.6 mM $CdSO_4$, 1 mM $ZnSO_4$ or 2 mM $CuSO_4$ or lower concentrations ([low]) of 0.3 mM $CdSO_4$, 0.5 mM $ZnSO_4$ or 0.5 mM $CuSO_4$. Values are the mean of nine replicate determinations with standard deviation.

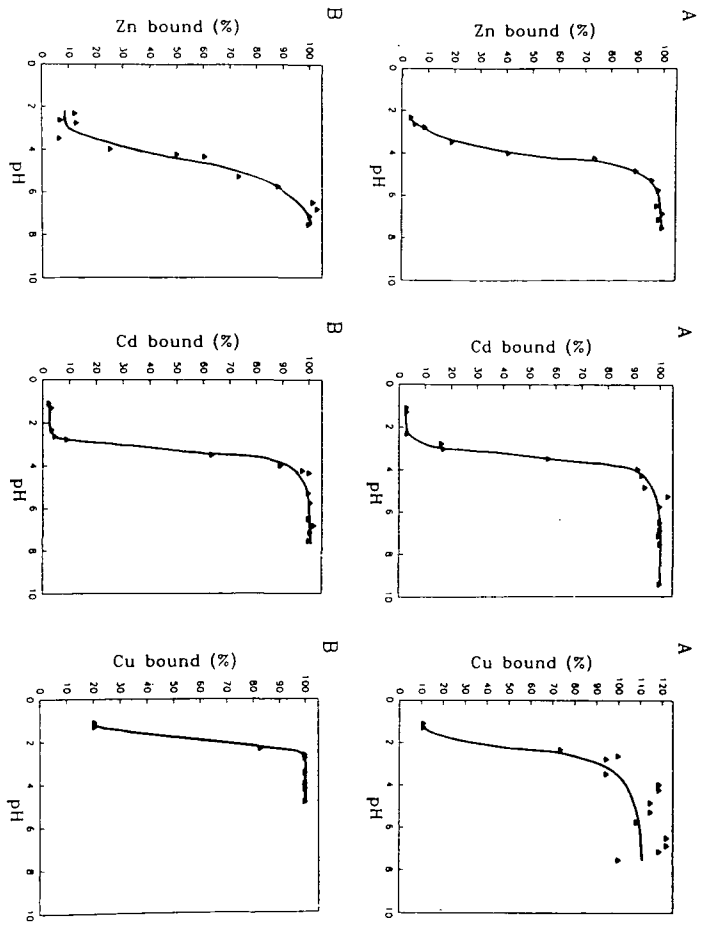


Fig. 4. Hydrogen ion competition of metal binding to GST-SmtA fusion protein (panels A). The data is expressed as a proportion of metal bound at the highest pH. Protein was purified from *E. coli* grown in media supplemented with either Zn, Cd or Cu. Aliquots of purified (via glutathione Sepharose 4B) protein were incubated for 1 h at the indicated pH. Free and bound Zn, Cd and Cu were resolved by gel filtration on columns of Sephadex G-25 (PD-10, Pharmacia) equilibrated with equivalent pH buffer. The analysis was also repeated with a commercial preparation of equine MT (panels B).

expression as a fusion protein in *E. coli*, the *smtA* gene product has high affinities for Cd, Cu, Hg and Zn supporting its designation as the first characterised prokaryotic MT gene. Continuing studies are elucidating the significance of this gene for metal-tolerance and -metabolism in cyanobacterial cells.

Acknowledgements: The authors thank Julia Bryden and John Gilroy for technical assistance. This work was supported by Research Grant GR/E 91257 from the Science and Engineering Research Council. N.J.R. is a Royal Society University Research Fellow.

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Prokaryotic metallothionein gene characterization and expression: chromosome crawling by ligation-mediated PCR

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SUMMARY

A strategy is described for the characterization of a novel gene employing the polymerase chain reaction, inosine-containing oligonucleotide primers, and ligation mediated, or anchored polymerase chain reaction (APCR). The same primers, designed from the known protein sequence, are used to amplify the coding region of the gene and subsequently to 'chromosome crawl' by APCR.

This strategy was applied to the characterization of a prokaryotic metallothionein gene, designated *smt*A, from the cyanobacterium *Synechococcus* PCC 6301 (= *Anacystis nidulans*). The abundance of *smt*A transcripts was examined in extracts from cells exposed to heat shock and elevated concentrations of cadmium, zinc and copper ions. There was no detectable change in *smt*A transcript abundance following exposure to heat shock, while exposure to all three metal ions led to an increase in abundance. A *smt*A homologue was also identified in *Synechococcus* PCC 7942 (= *Anacystis nidulans* R2).

1. INTRODUCTION

Metallothioneins (MTs) are a family of low molecular mass, cysteine-rich, metal-binding proteins and polypeptides, which have been isolated from a wide range of eukaryotes (see Kagi & Schaffer (1988) for a review). Functions which have been proposed for these ligands include the storage of certain essential trace metal ions and the detoxification of supra-optimal concentrations of the metals that they bind (Kagi & Schaffer 1988). Reports have suggested that similar ligands also occur in prokaryotes (cited in Silver & Misra (1988)), and the amino acid sequence of a cyanobacterial MT was reported by Olafson *et al.* (1988) (see Robinson (1989) for a review).

A number of different eukaryotic MT genes have been isolated and their nucleotide sequences determined (see Hamer (1986) for a review). These genes possess a complex array of transcriptional control elements that determine basal expression and responses to environmental factors. In mammalian cells MT genes are activated by exposure to: elevated concentrations of silver (Ag), bismuth (Bi), cadmium (Cd), cobalt (Co), copper (Cu), mercury (Hg), nickle (Ni) or zinc (Zn) ions; phorbol esters; iodoacetate; glucocorticoid hormones; interferon; ultraviolet (UV) irradiation; and inducers of the acute phase response (cited in Palmiter (1987)). Transcription of the *CUP*1 gene, which encodes MT in *Saccharomyces cerevisiae*, increases after exposure to elevated concentrations of Cu or Ag ions in the growth medium (Karin *et al.* 1984; Butt *et al.* 1984). Recently, progress has been made towards understanding the signal transduction mechanisms which elicit these responses (Culotta *et al.* 1989; Huibregtse *et al.* 1989).

Increased synthesis of cyanobacterial MT was detected after exposure to Cd and Zn, and inhibitor studies showed that, in common with eukaryotic systems, this induction was regulated at a transcriptional level (Olafson 1984; Olafson 1986). Confirmation of this observation requires the isolation of the corresponding prokaryotic MT gene.

As a first step in the characterization of a prokaryotic MT gene, the polymerase chain reaction (PCR) has been used to amplify part of the coding region of a MT gene from *Synechococcus* PCC 6301. PCR has rapidly become established as a powerful technique for both gene analysis and cloning (Saiki *et al.* 1988). Originally used with single primer species, it is now possible to obtain specific DNA amplification using redundant primers designed from known protein sequences (Lee *et al.* 1988; Gould *et al.* 1989). The base analogue inosine can be incorporated into primers which correspond to polypeptide regions that would otherwise require complex oligonucleotide mixtures (Fordham-Skelton *et al.* 1990).

Regions flanking known genes can be amplified by using inverse PCR (IPCR) to 'chromosome crawl' (Ochman *et al.* 1988; Triglia *et al.* 1988). IPCR depends upon the use of primers that associate with deoxyribonucleic acid (DNA) to give proximal 5′ ends, thereby facilitating outward DNA synthesis around circular-

ized fragments of genomic DNA. Recently, Shyamala & Ames (1989) have tested the feasibility of using ligation mediated, or anchored, PCR (APCR) as an alternative to IPCR for chromosome crawling. APCR depends upon the ligation of restricted genomic DNA fragments to a sequence of DNA containing a generic primer site (e.g. the M13 primer sites in pUC vectors) rather than the production of circular ligation products, as required for IPCR. Shyamala & Ames (1989) used APCR with single primer species to take two contiguous steps into the region flanking the known histidine transport operon of *Salmonella typhimurium*. In this paper a strategy combining and adapting several of these methods to amplify and clone a prokaryotic MT gene, designated *smt*A, by using inosine-containing oligonucleotide primers for both PCR and APCR has been described. The same primers used to amplify part of the coding region of *smt*A are subsequently applied to chromosome crawling by APCR. This strategy has general applications for the rapid characterization of novel prokaryotic genes when only limited amino acid sequence data are available.

2. MATERIALS AND METHODS

(*a*) *Materials and cyanobacterial culture*

Synechococcus PCC 6301 and PCC 7942 were cultured for 10 days under constant light (100 μmol photon m^{-2} s^{-1} photosynthetically active radiation (PAR)) at 32 °C in AC medium (Kratz & Myers 1955) modified according to Shehata & Whitton (1982). The trace element composition was the BG11 formula of Rippka *et al.* (1979), with added Ni (0.17 μM). The medium contained 0.77 μM Zn and 0.32 μM Cu. Cultures were grown to mid-log phase before exposure to toxic trace metals (0–10 μM) for 2 h, or elevated temperature (45 °C for 10 and 20 min; 55 °C for 10 and 20 min), followed by further growth up to 2 h under standard conditions. Restriction enzymes and T4 DNA ligase were supplied by NBL, Cramlington U.K., Taq polymerase was supplied by Stratagene, Cambridge U.K., or Perkin-Elmer/Cetus. [α-^{32}P] dCTP (14.8 TBq $mmol^{-1}$) and nylon (Hybond N) filters were obtained from Amersham International, Aylesbury, U.K. Phosphoramidite derivatives of all nucleotide bases were obtained from Applied Biosystems, Warrington U.K. Silica fines were a gift of Dr R. G. Alexander.

(*b*) *Genomic DNA isolation*

Genomic DNA was isolated by using part of a protocol described previously for the isolation of nucleic acids from plant cell cultures (Robinson *et al.* 1988), but excluding caesium chloride (CsCl) gradients.

(*c*) *Oligonucleotide synthesis*

Inosine-containing oligonucleotides were synthesized by using an Applied Biosystems 381A DNA synthesizer operated with a standard synthesis programme. After cleavage and deprotection the oligonucleotides were dried under vacuum, twice resuspended in water and vacuum dried. Oligonucleotides were stored at −20 °C either dry or as aqueous solutions and were used without further purification.

(*d*) *In vitro DNA amplification*

PCR was carried out essentially as described by Saiki *et al.* (1988) with minor modifications by Fordham-Skelton *et al.* (1990). Reactions were subjected to 28 cycles of the following series of temperatures and times: denaturation 92 °C for 1.5 min, annealing 55 °C for 1.5 min, extension 72 °C for 1.5 min (using a Hybaid Intelligent Heating Block). Amplified DNA samples were stored at −20 °C before further analysis. Control reactions lacking template DNA were also set up.

(*e*) *Analysis and cloning of PCR products*

Ten microlitres of reaction mixtures were analysed by electrophoresis on 2% agarose gels stained with ethidium bromide. Discrete PCR-amplified sequences were electroeluted from agarose gel slices and the DNA was recovered from solution by binding to silica fines. Fragments were digested with *Sal* I and *Eco*R I (restriction sites which were included near the 5′ ends of the PCR primers), ligated to *Sal* I and *Eco*R I cut pUC19, and transformed into *Escherichia coli* DH5α transformation competent cells, prepared by the method described by Alexander *et al.* (1984)

(*f*) *DNA sequence analysis*

Plasmid sequencing was performed by the dideoxy-sequencing method of Sanger *et al.* (1977) by using fluorescent dye-linked universal M13 primers and analysed by using an Applied Biosystems 370A DNA sequencer. Plasmids were sequenced in both directions using forward and reverse primers (except plasmids containing APCR fragments which were only sequenced in one direction due to the second reverse primer site introduced in APCR) as described in the supplier's protocol (Model 370A DNA Sequencing System, Users Manual Version 1.3A, Oct. 1988, pp. 3.22–3.25), using Sequenase enzyme with a Klenow (DNA polymerase I) chase.

(*g*) *Production of ^{32}P-labelled probes*

Cloned amplification products were used to prepare probes for Southern, and Northern blots by radiolabelling fragments isolated from agarose gels, with [α-^{32}P]dCTP according to the procedure of Feinberg & Vogelstein (1984).

(*h*) *Restriction and Southern analysis of genomic DNA*

Fifteen micrograms of genomic DNA were used for each restriction by using incubation conditions recommended by the manufacturers. Restriction fragments were first separated on a 0.8% agarose gel, and were then denatured, neutralized and the DNA transferred to a nylon filter as described by Sambrook *et al.* (1989). Standard prehybridization and hybridization conditions were used and the filters were washed to a final

stringency of 0.5 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate) at 65 °C for 15 min and autoradiographed at −80 °C by using pre-flashed X-ray film (Fuji RX) and intensifying screen.

(*i*) *Ligation mediated, or anchored,* PCR *(*APCR*)*

Genomic DNA (10 μg), restricted with *Hind* III, was ligated to 4 μg of pUC19 plasmid DNA, previously restricted with *Hind* III and *Pst* I. Aliquots (0.35 μg) of the ligation were used as template DNA for four separate APCR's using combinations of *smt*A gene primers, as specified in figure 1, and universal M13 forward and reverse sequencing primers, which hybridize to sites on pUC19, as described below. Reaction conditions were similar to those described previously for standard PCR by Fordham-Skelton *et al.* (1990), with the exception that 10 μg of *smt*A specific primer and 0.5 μg of generic primer were used in a 100 μl reaction, and the reactions were subjected to 32 cycles of the following series of temperatures and times 92 °C for 1.5 min; 55 °C for 2 min; 72 °C for 5 min.

(*j*) *RNA isolation and analysis*

Total RNA isolated from *Synechococcus* PCC 6301 by using standard techniques (Dzelkalns *et al.* 1988) was analysed on 15 g l^{-1} agarose gels and visualized with ethidium bromide to allow quantitation. Equivalent amounts of RNA (c.a. 20 μg) from different cell extracts were glyoxalated, resolved on 15 g l^{-1} agarose gels, and Northern blotted onto nylon membranes (Hybond-N, Amersham International, Aylesbury, U.K.) by standard techniques (Sambrook *et al.* 1989). Blots were hybridized to *smt*A probe, washed to 0.5 × SSC, 1 g l^{-1} SDS at 65 °C, and autoradiographed as before. All Northern blots were hybridized concurrently with the same batch of probe and subsequently exposed simultaneously to X-ray film.

3. RESULTS AND DISCUSSION

(*a*) *Amplification and cloning of part of the coding region of smt*A

Inosine-containing oligonucleotide primers were synthesized corresponding to the two peptide regions shown in figure 1. These regions were selected to minimize primer redundancy and maximize the proportion of the coding region that would be amplified. Inosines were incorporated at base positions with greater than twofold redundancy, and restriction endonuclease recognition sites were included at the 5′ ends, as shown, to facilitate cloning. The analysis of the PCR products obtained using these primers and *Synechococcus* PCC 6301 template DNA is shown in figure 2. Two amplification products of 144 b.p. and 116 b.p. were obtained. The nucleotide, and encoded amino acid, sequence of the insert in a plasmid containing the larger fragment, is shown in figure 3. This amino acid sequence corresponds to the known sequence (Olafson *et al.* 1988) of the class II MT in *Synechococcus* PCC 6301 inclusive of regions 1 and 2 shown in figure 1, with the exception of Cys_{32} being

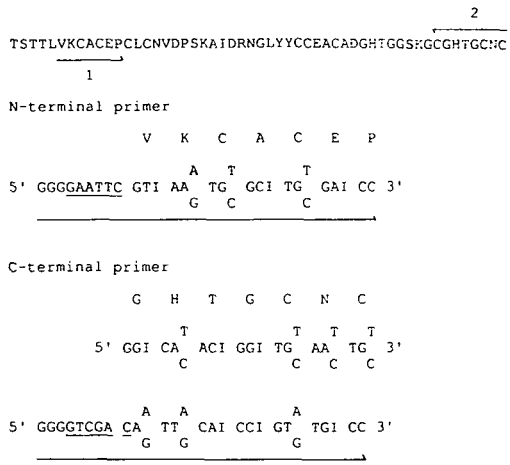


Figure 1. Amino acid sequence of *Synechococcus* MT and the deduced primer sequences used in DNA amplification reactions. The arrows show the relative location and orientation of the two primers. Restriction endonuclease recognition sites, added to the 5′ ends of the primers, are underlined.

Figure 2. Visualization of amplified fragments on an agarose gel. Amplification conditions (using *Synechococcus* PCC 6301 genomic DNA) were as described in the Materials and Methods.

substituted for Ser. It is noted that this Cys was one of only two amino acids, in the published MT amino acid sequence, which was not confirmed in two independent sequence analyses. The observed homology confirms that part of the MT gene has been amplified and hence designated *smt*A.

It is noted that sequence errors will arise due to the use of the base analogue inosine in the primer region or the low frequency of base misincorporation by *Taq* DNA polymerase, although three independent clones

```
GTG AAA TGC GCG TGT GAG CCC TGT CTC TGC AAC GTC GAT CCC AGC
Val Lys Cys Ala Cys Glu Pro Cys Leu Cys Asn Val Asp Pro Ser

AAA GCG ATC GAT CGC AAC GGT CTG TAC TAC TGC AGC GAA GCC TGT
Lys Ala Ile Asp Arg Asn Gly Leu Tyr Tyr Cys Ser Glu Ala Cys

GCC GAT GGC CAC ACC GGT GGT AGC AAA GGC TGC GGC CAC ACC GGC
Ala Asp Gly His Thr Gly Gly Ser Lys Gly Cys Gly His Thr Gly

TGC AAC TGN
Cys Asn Cys
```

Figure 3. Nucleotide, and encoded amino acid, sequence of the 144 b.p. fragment shown in figure 2. This corresponds to part of the known amino acid sequence of *Synechococcus* MT with the exception of the underlined Ser. A region of internal homology to the C-terminal primer site (underlined) corresponded to the 3′ end of the truncated 116 b.p. product shown in figure 2. Bases corresponding to the positions of inosines in the primers may be incorrectly assigned and are shown in bold.

(pJHNR11, pJHNR12, pAGNR11) were sequenced to detect such errors. Locations corresponding to I in the N-terminal primer contain G in the PCR product and locations corresponding to I in the C-terminal primer contain C in the PCR product (figure 3). This is because of preferential I/C base pairing (Ohtsuka *et al.* 1985). These six bases are shown in bold type-face (figure 3) as they may not correspond to the nucleotides present in the template DNA.

The nucleotide sequence of the 116 b.p. fragment (figure 2) showed that it is a product of a primer mismatch to an internal region of homology to the C-terminal primer-binding site within *smt*A, shown on figure 3, to produce a truncated product.

The sequence obtained for part of the *smt*A gene and shown in figure 3 was missing 15 bases of *smt*A coding region at its 5′ end. Therefore, APCR was employed to isolate and sequence this region and also associated translational signal sequences.

(*b*) *Chromosome crawling by* APCR

A genomic Southern blot of restricted DNA from *Synechococcus* PCC 6301, probed with labelled *smt*A insert from a clone containing the 144 b.p. PCR product, shows a single major hybridizing band for each digest (figure 4). *Hind* III was selected for APCR because of the relatively small size (2.8 kb) of the *smt*A *Hind* III fragment. pUC19 was digested with *Pst* I, the linear form recovered from an agarose gel and then digested with *Hind* III in order to obtain plasmid DNA with only one *Hind* III compatible end which could subsequently ligate to *Hind* III digested genomic DNA. *Hind* III digested genomic DNA (10 μg) was ligated to 4 μg of digested pUC19. Four separate APCR's were set up using combinations of the two *smt*A specific primers, and two generic M13 universal primers which hybridize to the vector. A negative control reaction was also set up containing the two *smt*A specific primers but without template DNA. Diverse *Hind* III fragments will ligate to pUC19 and both universal M13 primers will hybridize to all of these ligation products. However, only fragments containing the *smt*A gene will amplify exponentially when the ligation mix is used as a template for PCR because of the specificity of the

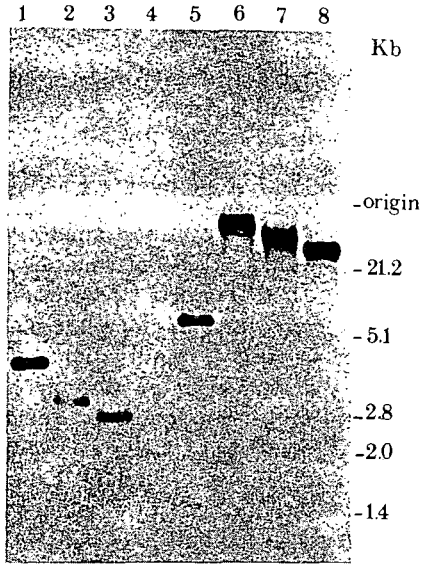


Figure 4. *Synechococcus* PCC 6301 genomic Southern blot hybridized with ^{32}P-labelled *smt*A probe prepared from the insert in plasmid pJHNR11, which contains the 144 b.p. fragment shown in figure 2. DNA was digested to completion with the following restriction endonucleases: lane 1, *Sal* I & *Nco* I; lane 2, *Sal* I & *Bgl* II; lane 3, *Hind* III; lane 4, *Pst* I; lane 5, *Sal* I; lane 6, *Bam*H I; lane 7, *Kpn* I; lane 8, *Eco*R I.

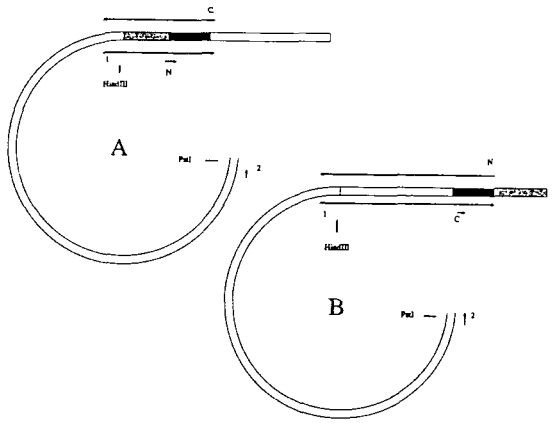


Figure 5. Diagrammatic representation of ligation mediated, or anchored, PCR. Total genomic DNA, digested with *Hind* III, was ligated to pUC19 digested with *Hind* III and *Pst* I (represented as partial circle). The universal M13 reverse primer (1) will prime unidirectional single strand synthesis of all ligated fragments but only *Hind* III fragments containing the *smt*A gene will be exponentially amplified (long arrows). Fragments containing *smt*A will be ligated in both orientations A and B. In reactions containing the *smt*A N-terminal primer (N) there will be exponential amplification of fragments ligated in orientation B of part of the *smt*A coding region (black box) and 3′ flanking sequence (open box) to the first 3′ *Hind* III site. In reactions containing the C-terminal primer (C) there will be exponential amplification of fragments ligated in orientation A of the *smt*A coding region and 5′ flanking sequence (shaded box) to the first 5′ *Hind* III site. There will be no amplification of *smt*A fragments in reactions containing the M13 forward primer (2). Primers that will not give exponential amplification of *smt*A fragments in a particular ligation are shown as short arrows.

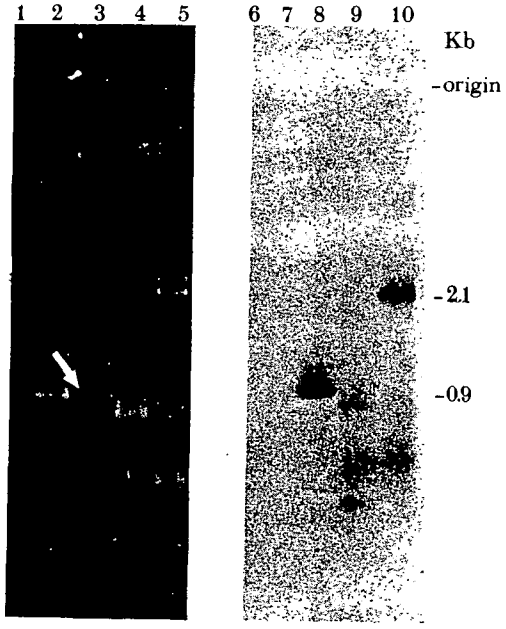


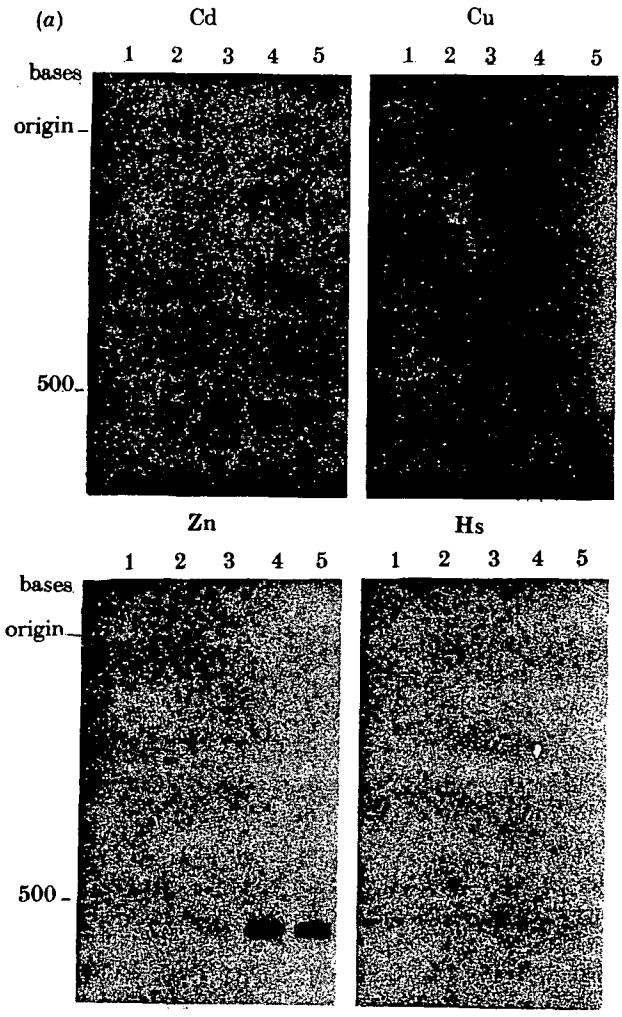
Figure 6. Visualization of APCR products on an agarose gel. The primers used were; lane 1, *smt*A N- and C-terminal primers, no template control; lane 2, M13 forward & *smt*A C-terminal; lane 3, M13 reverse & *smt*A C-terminal; lane 4, M13 forward & *smt*A N-terminal; lane 5, M13 reverse & *smt*A N-terminal. Lanes 6 to 10 are a Southern blot of lanes 1–5 probed with ^{32}P-labelled *smt*A probe. Two specific products are revealed in lanes 8 and 10. The 5′ amplification product which was subsequently cloned and sequenced is shown with an arrow in lane 3.

```
TCAAAGGAGTTGCTGTCATG ACC TCA ACA ACC TTG GTC AAA TGC GCG
                     Thr Ser Thr Thr Leu Val Lys Cys Ala

TGT GAG CCC
Cys Glu Pro
```

Figure 7. Nucleotide sequence of part of the APCR fragment produced from reactions containing the *smt*A C-terminal primer giving the correct sequence (corrected base in bold) of the complement to the N-terminal primer binding site, the 15 b.p. of *smt*A coding sequence missing from figure 3, and an additional 20 b.p. of 5′ region including AAGGAG and ATG sequences.

*smt*A primer in each reaction. Furthermore, only reactions containing the M13 reverse primer will lead to amplification of ligated fragments as only this primer is adjacent to the *Hind* III ligation site in pUC19, while the M13 forward primer is adjacent to the incompatible *Pst* I site (figure 5). APCR with the *smt*A C-terminal primer (figure 1) will amplify the upstream coding sequence of *smt*A and its 5′ flanking region to the first upstream *Hind* III site, while the *smt*A N-terminal primer will amplify the downstream *smt*A coding sequence and its 3′ flanking region to the first downstream *Hind* III site (figure 5). Both sets of amplification products will therefore contain the diagnostic coding sequence, shown in figure 3, at one end. Figure 6 shows that a variety of non-specific amplification products were produced; however specific *smt*A products were revealed on a Southern blot probed with the insert of pJHNR11, and the 5′ fragment which was subsequently cloned is denoted



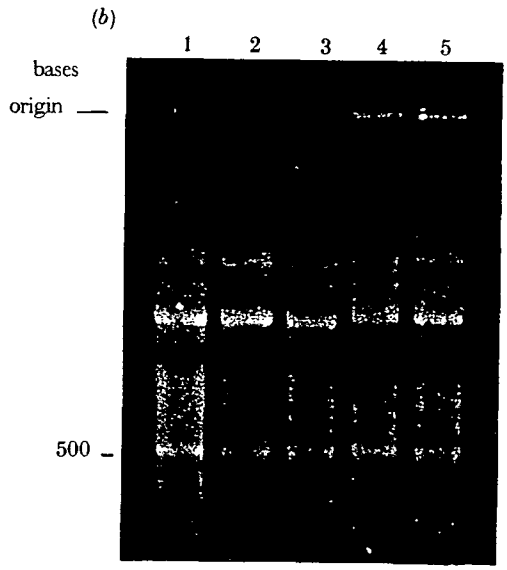


Figure 8. (*a*) Northern blots of RNA from *Synechococcus* PCC 6301 grown in the presence of different concentrations (lane 1 = 0 μM; 2 = 1 μM; 3 = 2.5 μM; 4 = 5 μM; 5 = 10 μM) of added metals (Zn; Cu; Cd) or elevated temperature (Hs) (lane 1 = 32 °C; lane 2 = 45 °C for 10 min; lane 3 = 45 °C for 20 min; lane 4 = 55 °C for 10 min; lane 5 = 55 °C for 20 min), and probed with *smt*A. (*b*) Equivalent amounts of RNA were loaded onto each track as shown by the visualization of rRNA bands for isolates from cells exposed to Cu.

with an arrow in the first panel of figure 6. The sum of the estimated sizes of the two specific *smt*A fragments, 0.9 + 2.1 = 3.0 kb, is equivalent to the size (2.8 kb) of the *Hind* III fragment indicated from genomic Southern blots (figure 4), plus an additional 0.2 kb (comprising 144 b.p. of *smt*A coding region, since this has been amplified twice, and $2 \times 25 = 50$ b.p. of amplified pUC19 sequences). The 0.9 kb APCR fragment was purified from agarose gels incubated with T4 DNA polymerase and blunt end ligated to pUC19. Part of the sequence of the inserts in plasmids (pJHNR21, pJHNR22, pJHNR23, pJHNR24) containing this fragment, is shown in figure 7. It is noted that this APCR strategy introduces a second M13 primer site (either forward or reverse depending upon the vector used in the initial ligation) and therefore only the alternative M13 site can be used for subsequent sequence analysis, unless the duplicate site is removed.

The sequence shown in figure 7 corresponds to the N-terminal primer site, 15 bases of coding region upstream from the N-terminal primer site which were missing from the previous *smt*A clones, and 20 bases of 5′ region flanking the coding sequence, which include the AAGGAG and ATG sequences for ribosome-binding and start of translation. The five codons absent from figure 3 encode the five amino acids at the N-terminus of *Synechococcus* MT as determined by Olafson *et al.* (1988) and this confirms the utility of APCR for chromosome crawling. One of the bases in the N-terminal primer region was incorrectly assigned as G in figure 3 but shown to be C in figure 7. This base corresponds to I in the N-terminal primer and will have arisen because of preferential I/C pairing as described above. Therefore, using APCR the correct sequence of the N-terminal primer region has been obtained.

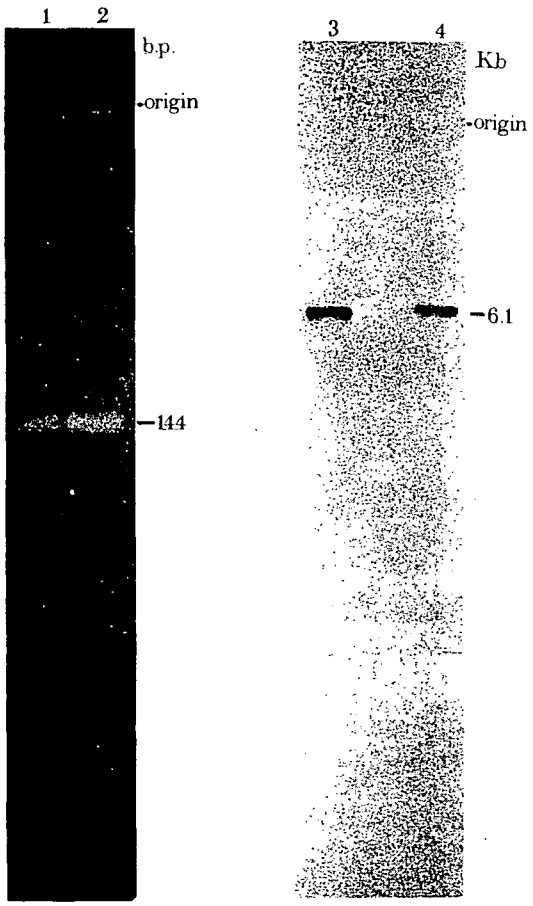


Figure 9. Identification of a *smt*A homologue in *Synechococcus* PCC 7942. Lanes 1 & 2 show visualization of amplified *smt*A fragments on an agarose gel by using template DNA from: lane 1, *Synechococcus* PCC 6301; lane 2, *Synechococcus* PCC 7942. Lanes 3 & 4 are a genomic Southern blot of *Sal* I digested genomic DNA from: lane 3, *Synechococcus* PCC 7942; lane 4, *Synechococcus* PCC 6301, probed with *smt*A.

(*c*) *Expression of smt*A *in Synechococcus PCC 6301 and identification of a homologue in Synechococcus PCC 7942*

The abundance of *smt*A transcripts in *Synechococcus* PCC 6301 increases following exposure to increased concentrations of Cd, Zn and Cu ions in the growth medium (figure 8*a*). Visualization of the rRNA bands showed that similar amounts of RNA were loaded on each track (figure 8*b*). In response to elevated concentrations of Cd, *smt*A transcripts are maximally abundant after exposure to 5 μM, and slightly less abundant following exposure to 10 μM. By contrast, *smt*A transcripts are more abundant following exposure to 10 μM compared to 5 μM Zn. There is an increase in *smt*A transcript abundance following exposure to elevated concentrations of Cu, although Cu appears to be a less potent inducer than either Zn or Cd. The transcripts did not increase in abundance following exposure to conditions which induce synthesis of heat-shock proteins (figure 8 and Borbely *et al.* (1985)).

Using the *smt*A primers (figure 1) a fragment (*ca.* 144 b.p.) was amplified from genomic DNA from *Synechococcus* PCC 7942 (= *Anacystis nidulans* R2) (figure 9). Furthermore, a *smt*A homologue was also identified on a genomic Southern blot of *Sal* I digested DNA from *Synechococcus* PCC 7942 (figure 9). Therefore *smt*A genes have been detected in both of these two strains which have been referred to as *Anacystis nidulans*.

In conclusion, it has been possible to characterize the first prokaryotic MT gene, *smt*A, without screening genomic DNA libraries, thus demonstrating the feasibility of the strategy described. This has allowed subsequent examination of the effect of elevated concentrations of certain trace metals on *smt*A transcript abundance. Exposure to elevated concentrations of Zn, Cd and Cu ions lead to an increase in *smt*A transcript abundance. It remains to be established whether these changes are due to transcriptional activation of the prokaryotic MT gene, analogous to eukaryotic MT genes (see Palmiter (1987) for a review), or changes in transcript stability. The possible role of these genes in mechanisms of metal tolerance, and in the metabolism of essential trace metals such as Zn, in cyanobacteria requires investigation.

N.J.R. is a Royal Society University Research Fellow. J.W.H. is supported by a N.E.R.C. research studentship and A.G. by a Nehru Memorial Trust scholarship. This work was partly financed by S.E.R.C. research grant GR/E 91257. The authors thank J. Bryden and J. Gilroy for technical

assistance, and gratefully acknowledge the work of R. W. Olafson *et al.*, on which this research was based.

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(*Received 3 September 1990; accepted 18 September 1990*)

Amplification and rearrangement of a prokaryotic metallothionein locus *smt* in *Synechococcus* PCC 6301 selected for tolerance to cadmium

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SUMMARY

Metal-tolerant cyanobacteria have been isolated from metal-polluted aquatic environments and also selected in culture, but no genes which confer metal tolerance have been described. To investigate the possibility that amplification of a prokaryotic metallothionein gene (*smtA*), or rearrangement of the *smt* locus, could be involved in the development of Cd tolerance in *Synechococcus* PCC 6301, Cd-tolerant lines were selected by stepwise adaptation of a *Synechococcus* culture. An increase in *smtA* gene copy number and the appearance of unique additional *smtA* restriction fragments (both larger and smaller) were detected in these tolerant lines (tolerant to 0.8 μM Cd, 1.3 μM Cd and 1.7 μM Cd). Stepwise adaptation was repeated by using a culture of *Synechococcus* PCC 6301 inoculated from a single plated colony to obtain four new lines (tolerant to 1.4 μM Cd, 1.8 μM Cd, 2.6 μM Cd and 3.2 μM Cd). Amplification of the *smtA* gene and development of unique *smtA* restriction fragments (larger and smaller) were once again detected in these tolerant lines. Amplification and rearrangement of the *smt* locus were only detected in the seven Cd-tolerant lines, with no evidence of amplification or rearrangement in the non-tolerant lines from which they were derived. As a control, another gene, *psaE*, was also monitored in these cell lines. There was no evidence of amplification or rearrangement of *psaE* in the non-tolerant or any of the Cd-tolerant lines.

1. INTRODUCTION

Anthropogenic mobilization of toxic trace metals into the biosphere, and the consequent adaptation of certain organisms to supra-optimal concentrations of these metals, has been extensively documented (see citations in Antonovics *et al.* (1971); Bradshaw (1984)). At moist sites combining high concentrations of metal with high pH, cyanobacteria are often the dominant microorganisms. Some cyanobacterial isolates from metal-polluted sites tolerate considerably higher concentrations of metal in subsequent culture than do cyanobacterial strains isolated from environments not enriched with metal (Shehata & Whitton 1981). Cyanobacteria have also been selected in the laboratory for increased tolerance to a number of different metals by using stepwise adaptation. However, the mechanisms of metal tolerance in metal-adapted cyanobacteria have not been fully described, and no genes which confer metal tolerance have been identified in cyanobacteria.

Several studies have shown that diverse mechanisms of tolerance to different metals operate in cyanobacteria (for examples see Fernandez-Piñas *et al.* 1991; Jardim & Pearson 1984; Verma & Singh 1991). It has been proposed that metal tolerance in cyanobacteria referred to as *Anacystis nidulans* and *Synechococcus* TX-20 (*Anacystis nidulans*, *Synechococcus* PCC 6301, *Anacystis nidulans* TX-20 and *Synechococcus* PCC 7942 are all suggested to belong to the same species) could involve intracellular binding of Cd/Zn to ligands similar to eukaryotic metallothioneins (MTs) (Maclean *et al.* 1972; Olafson *et al.* 1980).

MTs (class I and class II) have been isolated and characterized from such a wide range of eukaryotes that they are often considered to be ubiquitous (for reviews see Hamer 1986; Kägi & Schäffer 1988). In eukaryotes, MTs are known to be involved in cellular responses to elevated concentrations of certain metal ions. MTs bind specific metal ions, and rapid induction of MT in response to elevated concentrations of these metals is thought to confer tolerance. Additionally, animal MT genes respond to a variety of endogenous factors, suggesting an undefined role in cellular regulation (Kägi & Schäffer 1988; Zeng *et al.* 1991).

Olafson (1984, 1986) and Olafson *et al.* (1988) purified and chemically characterized an MT-like cyanobacterial protein, reporting its amino acid sequence. This protein is the first (characterized) prokaryotic MT (see citations in Kägi & Schäffer (1988)). Based upon the known amino acid sequence, degenerate inosine-containing oligonucleotides were

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designed and used to amplify part of the corresponding MT gene in the polymerase chain reaction (PCR) (Robinson *et al.* 1990). Subsequently, the gene has been isolated from a size-fractionated genomic DNA library. The nucleotide sequence was determined and the gene designated *smtA* (J. W. Huckle, unpublished data).

Exposure of a *Synechococcus* culture to highly elevated concentrations of Cd resulted in a prolonged lag. Subsequent resumption of growth coincided with an increase in cellular MT (Olafson 1984; Olafson 1986). On transfer of these cells to fresh medium in the absence of Cd, MT reduced to near-basal levels. However, no lag was observed upon re-transfer of these cells into Cd-containing medium, whereas non-tolerant cells grew only after a lag. This apparent acquisition of Cd tolerance was thought unlikely to be related to a chromosomal mutation because it would require a mutation frequency considered to be unreasonably high (Olafson 1984, 1986). It was proposed that metal tolerance may result from the amplification of an extrachromosomal MT gene (Olafson *et al.* 1980; Olafson 1986). This strain of *Synechococcus* has two plasmids of *ca.* 8.0 kilobase pairs (kb) and 48.5 kb (Laudenbach *et al.* 1983), which could potentially harbour the MT gene, although no plasmid-encoded functions have previously been identified in cyanobacteria (Ciferri *et al.* 1989). Analysis of DNA isolated from R2-PIM8, a derivative of *Synechococcus* PCC 7942 cured of the small plasmid (R2-SPc) (van der Plas *et al.* 1990), confirms that this strain contains the *smtA* gene (J. S. Turner, personal communication). Therefore, *smtA* must be either chromosomal or located on the 48.5 kb plasmid. However, the sizes of *Sal*I, *Hin*dIII and *Bam*HI restriction fragments containing the *smtA* gene in DNA isolated from *Synechococcus* PCC 6301 (Robinson *et al.* 1990) do not correspond to the known sizes of *Sal*I, *Hin*dIII and *Bam*HI restriction fragments of DNA isolated from the 48.5 kb plasmid (Laudenbach *et al.* 1983). It is therefore assumed that *smtA* is chromosomal. In eukaryotes, however, amplification of MT genes (initially chromosomal) has been observed in metal-tolerant cell lines (Beach & Palmiter 1981; Crawford *et al.* 1985).

To investigate the possible involvement of amplification of the prokaryotic MT gene, *smtA*, or rearrangement of the *smt* locus in Cd tolerance, we report here: (i) the selection of two different sets of Cd-tolerant lines of *Synechococcus* PCC 6301; and (ii) the analysis of *smtA*, and also another gene *psaE*, in a series of Southern blots of restricted DNA isolated from both non-tolerant *Synechococcus* PCC 6301 and these selected lines.

2. MATERIALS AND METHODS

(*a*) *Materials and cyanobacterial culture*

Synechococcus PCC 6301 and *Synechocystis* PCC 6803 were cultured as described previously (Robinson *et al.* 1990). Absorbance at 540 nm was used as an indirect estimate of cell density. Restriction enzymes were supplied by Northumbria Biologicals Ltd, Cramlington, U.K.; Taq polymerase was supplied by Stratagene, Cambridge, U.K. or Perkin-Elmer/Cetus. [α-^{32}P]dCTP (14.8 TBq mmol^{-1}) and nylon (Hybond N and Hybond N+) filters were from Amersham International, Aylesbury, U.K.

(*b*) *Stepwise adaptation*

Cd-tolerant lines of *Synechococcus* PCC 6301 were developed by repeated subculturing in liquid medium containing a range of Cd concentrations. Cells which grew in the highest concentration of Cd were used as inocula for further subculture. The most tolerant lines obtained after each step of selection were also maintained in media supplemented with the respective Cd concentrations. Two different sets of Cd-tolerant lines of *Synechococcus* PCC 6301 were selected: (i) a culture (A0) that had been maintained for a prolonged period in liquid medium was adapted to 0.8 μM Cd (A0.8), 1.3 μM Cd (A1.3) and 1.7 μM Cd (A1.7); and (ii) a culture (C0) inoculated from a single plated colony (to minimize initial genetic variability) was adapted to 1.4 μM Cd (C1.4), 1.8 μM Cd (C1.8), 2.6 μM Cd (C2.6) and 3.2 μM Cd (C3.2). The inoculum and harvesting densities were 2×10^5 cells ml^{-1} and 2×10^3 cells ml^{-1}, respectively, throughout selection and subsequent maintenance of lines C0, C1.4, C1.8, C2.6 and C3.2.

(*c*) *Isolation and quantification of DNA*

Genomic DNA was isolated as described previously (Robinson *et al.* 1990). DNA concentration was determined by the Fluorometric Diaminobenzoic acid (DABA) assay of Thomas & Farquhar (1978). To estimate the number of copies of the *smtA* gene in DNA (10 μg) isolated from *Synechococcus* PCC 6301, standard amounts (multiples of 8.81 ng) of pJHNR11 (plasmid pUC19 containing 144 base pairs (b.p.) of the *smtA* coding region) DNA were also analysed. It was assumed that the genome size of *Synechococcus* PCC 6301 is 3.212×10^6 b.p. (Herdman *et al.* 1979). Therefore, 10 μg of genomic DNA would contain 2.988×10^9 copies of the genome, and an equivalent number of copies of *smtA* will be contained in 8.81 ng of pJHNR11 DNA.

(*d*) *Amplification and cloning of a fragment of the psaE gene from Synechocystis PCC 6803*

The *psaE* gene (a photosystem I gene) was used as a control probe for subsequent Southern analyses. Oligonucleotides suitable for *in vitro* amplification of *psaE* from *Synechocystis* PCC 6803 were synthesized based upon the nucleotide sequence reported by Chitnis *et al.* (1989); N-terminal primer, 5′CCA TGG CCT TAA ATC GTG GTG ACA AA 3′; C-terminal primer, 5′ AAG CTT TGC CGC CGC TTG CAC CAA TTC C 3′ (underlined sequence represents restriction endonuclease recognition sites included in the primers). PCR was done as described previously (Robinson *et al.* 1990). Reaction products were analysed by electrophoresis on a 2% agarose gel, and a 215 b.p. fragment of *psaE* was recovered, cloned and sequenced. Plasmid sequencing using M13 forward and reverse primers was done by the dideoxy-sequencing method of Sanger *et al.* (1977), as described previously (Robinson *et al.* 1990). The nucleotide sequence of the cloned (in pJHNR61) fragment corresponded to the known nucleotide sequence (data not shown). The cloned fragment of *psaE* was then used to prepare radiolabelled probes suitable for the detection of a homologous gene in *Synechococcus* PCC 6301.

(*e*) *Production of* [^{32}*P*]*-labelled probes*

PCR products and also cloned fragments of *smtA* and *psaE*

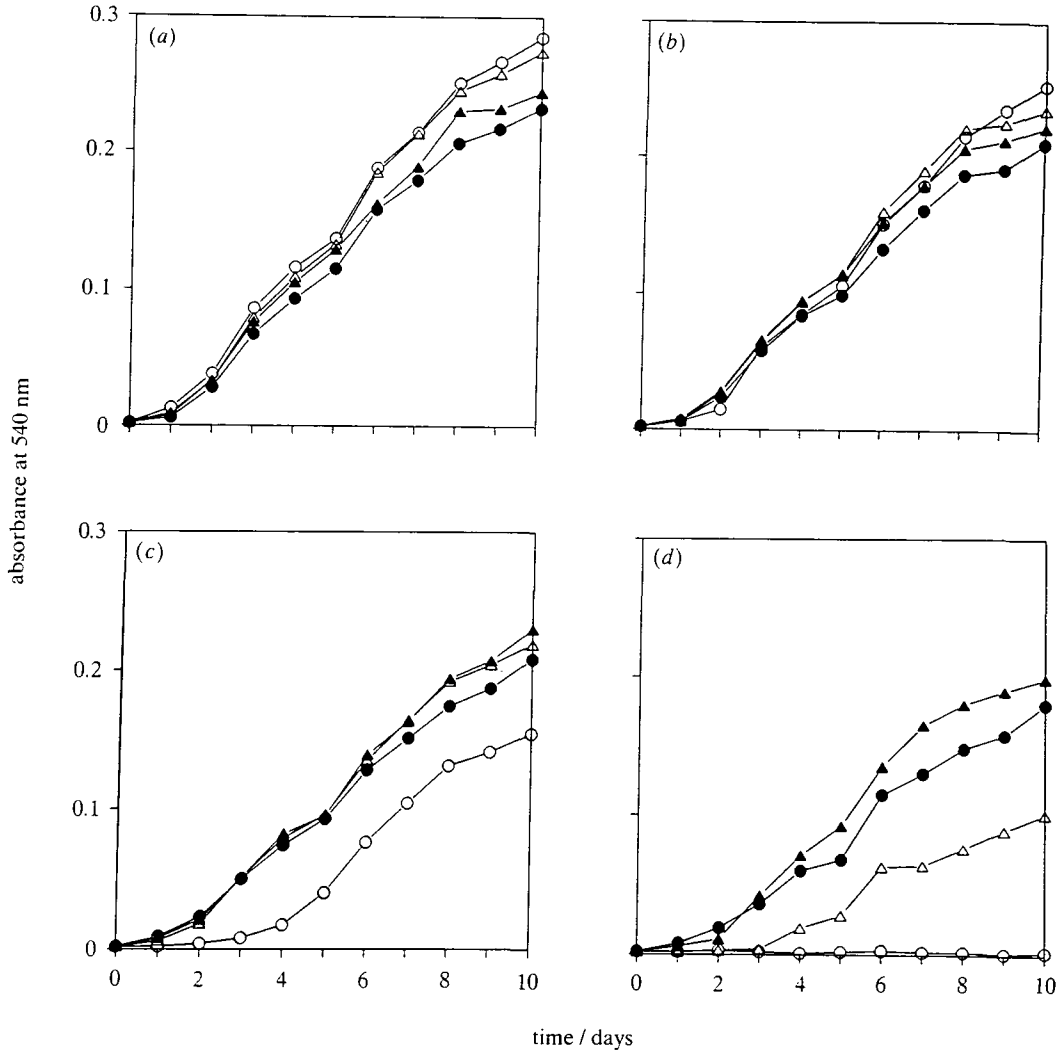

Figure 1. Growth of non-tolerant A0 (○) and Cd-tolerant lines (A0.8 (△), A1.3 (▲), and A1.7 (●)) in different concentrations of Cd. Growth in (*a*) 0 μM Cd, (*b*) 0.8 μM Cd, (*c*) 1.3 μM Cd, and (*d*) 1.7 μM Cd.

(in pJHNR11 and pJHNR61, respectively) released from vector by restriction (*Sal*I and *Eco*RI; *Eco*RI and *Bam*HI, respectively) were electroeluted (Sambrook *et al.* 1989) from agarose gel slices and DNA recovered by binding to silica fines (Robinson *et al.* 1990). Recovered DNA was radiolabelled with [α-^{32}P]dCTP, according to the procedure of Feinberg & Vogelstein (1983).

(*f*) *Restriction and analysis of DNA*

Genomic DNA (10 μg) was digested with *Sal*I, using incubation conditions recommended by the manufacturers. Digested genomic DNA and portions (equivalent to one, two, three, four, five, six and eight gene copies) of digested (*Eco*RI) plasmid pJHNR11 were separated by agarose gel electrophoresis, transferred to Nylon (Hybond N) filters (Sambrook *et al.* 1989) and hybridized with a [^{32}P]-labelled 144 b.p. fragment of the *smtA* gene. For lines C0, C1.4, C1.8, C2.6 and C3.2, restriction fragments were separated on a 0.7% agarose gel and transferred to Nylon (Hybond N+) membrane by alkali transfer (manufacturer's protocol). Standard prehybridization and hybridization conditions were used and the filters washed to a final stringency of 1.0 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) at 65 °C for 15 min and exposed at −80 °C to X-ray film (Fuji RX).

3. RESULTS

(*a*) *Growth of non-tolerant A0 and Cd-tolerant lines A0.8, A1.3 and A1.7 in Cd-containing media*

Growth of A0 and the three tolerant lines was monitored in four different concentrations of Cd (0 μM, 0.8 μM, 1.3 μM, 1.7 μM) by measuring absorbance at 540 nm (figure 1). Growth of A0 was totally inhibited, and line A0.8 was partly inhibited, in media containing 1.7 μM Cd.

(*b*) *Analysis of genomic DNA isolated from non-tolerant A0 and Cd-tolerant lines A0.8, A1.3 and A1.7*

A *ca.* four-fold increase in [^{32}P]-labelled *smtA* hybridization, relative to *Sal*I digested DNA from A0, was observed in *Sal*I digested genomic DNA from line A1.7 (figure 2*a*). However, ethidium bromide staining of the agarose gel before Southern blotting showed that equivalent amounts of DNA from each of the four lines was present (figure 2*c*). The filter was subsequently probed with a [^{32}P]-labelled fragment of the *psaE* gene (figure 2*b*). Relative to DNA from A0, there was no increase in *psaE* hybridization in line A1.7; indeed,

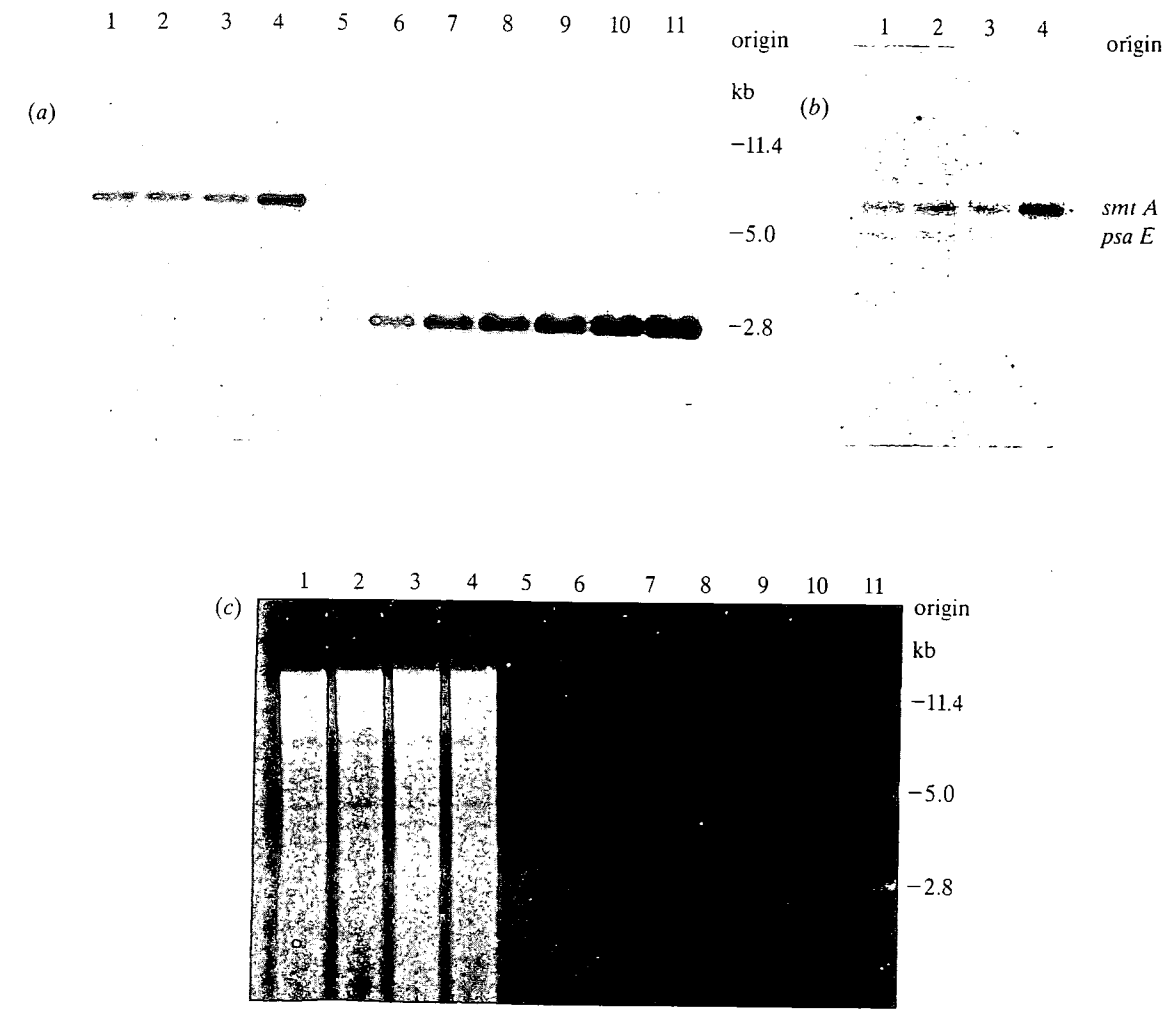

Figure 2. Analysis of genomic DNA isolated from A0 and Cd-tolerant lines. (*a*) Hybridization to *smtA*. (*b*) Hybridization to *psaE*. (*c*) Visualization of ethidium bromide-stained DNA. *Sal*I restricted genomic DNA was isolated from: lane 1, A0; lane 2, A0.8; lane 3, A1.3; and lane 4, A1.7. Lanes 5–11 contain standard amounts of plasmid pJHNR11 DNA, equivalent to one, two, three, four, five six and eight gene copies, respectively. Two bands in (*b*): upper band corresponds to *smtA*; lower band corresponds to *psaE*.

slightly less hybridization was observed to DNA from this most tolerant line.

DNA isolated from A0 and the tolerant lines was further analysed after two, four, seven and 12 subcultures (figure 3). Increased hybridization of *smtA*, relative to A0, was repeatedly detected in the tolerant lines. Additionally, unique *smtA* restriction fragments, both larger and smaller than that detected in the line A0, were observed in DNA isolated from lines A1.3 and A1.7. After seven subcultures (figure 3), a similar restriction pattern to A0 occurred in DNA isolated from the tolerant lines. However, prolonged exposure revealed larger and smaller *smtA* restriction fragments in the tolerant lines, although these fragments were relatively less abundant (data not shown). No evidence of rearrangement was observed in DNA from A0 when probed with *smtA*, or in any of the lines when probed with the control gene *psaE*. All Southern blots were done with gene copy number reconstructions (data only shown in figure 2).

To confirm whether or not the appearance of unique restriction fragments was reproducible (not merely caused by anomalous, possibly incomplete, restriction), DNA isolated from tolerant line A0.8 after two subcultures in 0.8 μM Cd was independently restricted three times with *Sal*I. A similar banding pattern of larger and smaller *Sal*I *smtA* restriction fragments was obtained in all three restrictions (data not shown).

(*c*) *Reselection of Synechococcus PCC 6301 (cell line C0) for Cd tolerance and analysis of genomic DNA isolated from non-tolerant C0 and Cd-tolerant lines C1.4, C1.8, C2.6 and C3.2*

A culture inoculated from a single plated colony was reselected, independent of the first adaptation protocol, for Cd tolerance. Rapid development of Cd tolerance was achieved. Coincident with adaptation to increasing Cd concentrations, an increase in lag before growth was observed. This lag decreased upon subsequent maintenance of Cd-tolerant lines in media supplemented with the respective Cd concentration (data not shown).

DNA isolated from C0 and tolerant lines C1.4, C1.8, C2.6 and C3.2 was analysed by Southern hybridization to a [^{32}P]-labelled fragment of the *smtA* gene, after one, two, three and four subcultures in media supplemented with the respective Cd concentration (figure 4). After

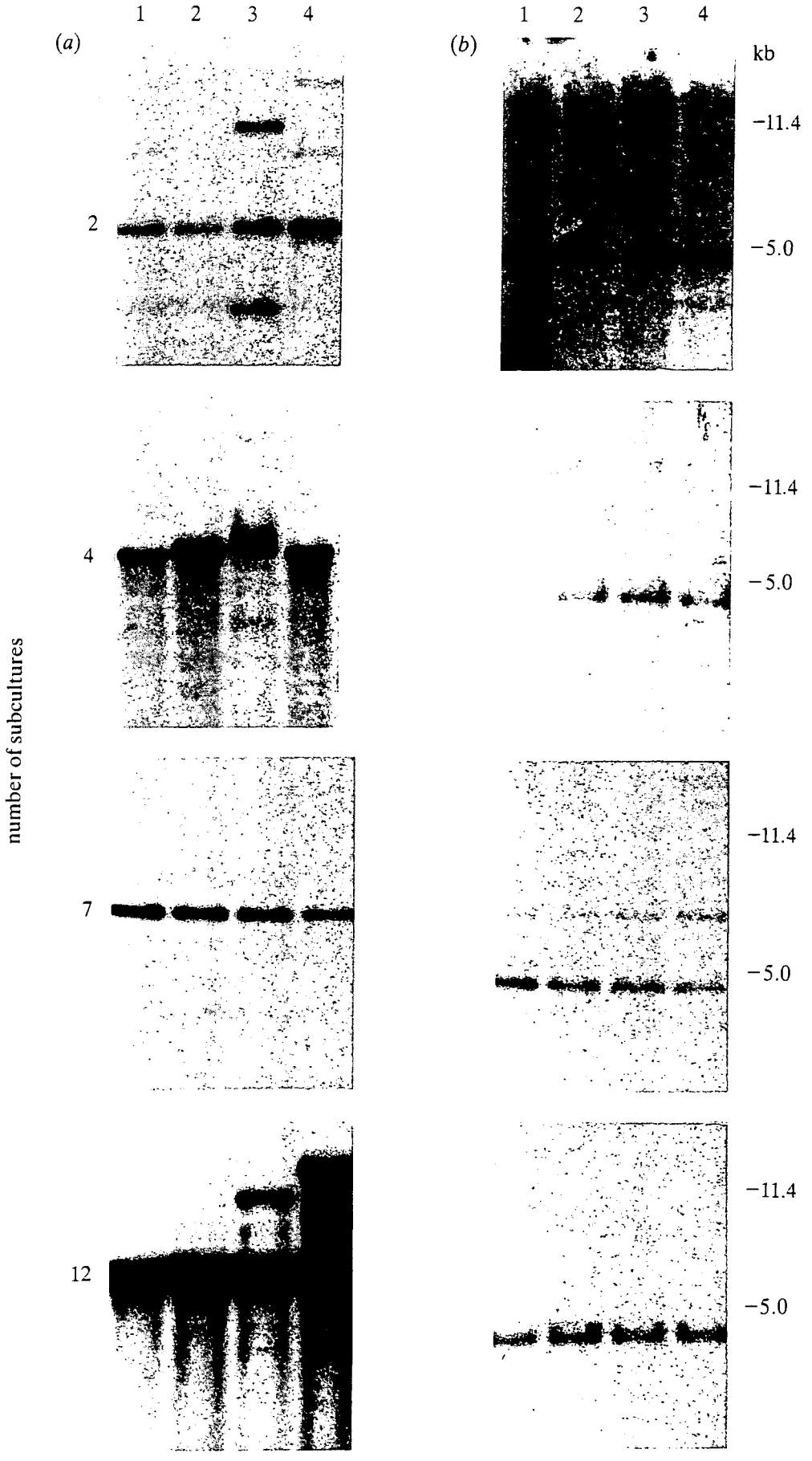

Figure 3. Analysis of genomic DNA isolated from A0 and Cd-tolerant lines (A0.8, A1.3 and A1.7) after two, four, seven and twelve subcultures. (*a*) Hybridization to *smtA*. (*b*) Hybridization to *psaE*. Equivalent amounts of DNA isolated from: lane 1, A0; and tolerant lines, lane 2, A0.8; lane 3, A1.3; and lane 4, A1.7; was restricted with *Sal*I.

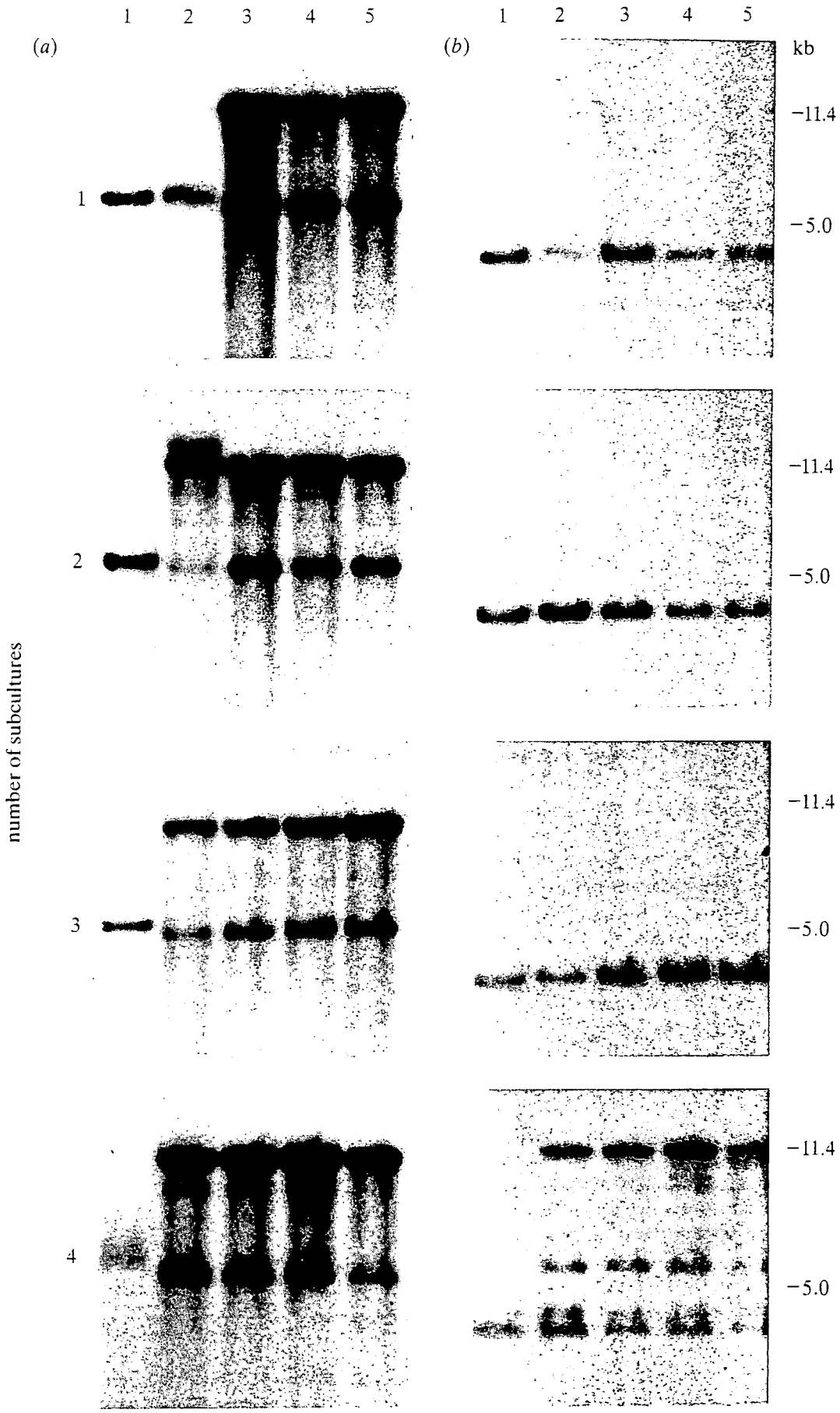

Figure 4. Analysis of genomic DNA isolated from C0 and Cd-tolerant lines (C1.4, C1.8, C2.6 and C3.2) after one, two, three and four subcultures. (*a*) Hybridization to *smtA*. (*b*) Hybridization to *psaE*. DNA isolated from: lane 1, C0; and tolerant lines, lane 2, C1.4; lane 3, C1.8; lane 4, C2.6; and lane 5, C3.2; was restricted with *Sal*I.

the first subculture, unique larger and slightly smaller *smtA* restriction fragments were obtained in the DNA isolated from lines C1.8, C2.6 and C3.2. No *Sal*I *smtA* fragments equivalent to that observed in C0 (5.8 kb) were detected in these three Cd-tolerant lines. The hybridization intensities of the two bands, relative to C0, in these lines (C1.8, C2.6 and C3.2) also suggests an increase in *smtA* gene copy number per unit DNA. No unique *smtA* restriction fragments were observed in the DNA isolated from line C1.4 at this time. After two, three and four subcultures, unique larger and smaller *smtA* restriction fragments were obtained in DNA isolated from all of the tolerant lines. Furthermore, no *Sal*I *smtA* fragment equivalent to C0 (5.8 kb) was present in any of these tolerant lines. As a control, the blots were subsequently hybridized with a [^{32}P]-labelled fragment of the *psaE* gene. No evidence of rearrangement was observed for *psaE* in any of the lines. All Southern blots were done with gene copy number reconstructions (data not shown). Further analysis of genomic DNA from C0 and tolerant lines after subsequent (after the fourth) subculture has generated similar results (data not shown).

(*d*) *Effect of Cd on digestion of genomic DNA with SalI restriction endonuclease*

There was concern that minute quantities of Cd which might be present in DNA isolated from Cd-exposed, Cd-tolerant lines could affect restriction of genomic DNA with *Sal*I restriction endonuclease causing artefacts that might have been misinterpreted as gene rearrangements. Genomic DNA (10 μg) isolated from C0 was restricted with *Sal*I in the presence of a range of Cd concentrations from 10^{-10} μM to 10^{-1} μM. Furthermore, to investigate any potential short-term (2 h) *in vivo* effects (e.g. modification of restriction endonuclease recognition sites), a culture of C0 grown to mid-log phase was divided into two portions. To one portion, 3.2 μM Cd was added and incubated for 2 h, whereas the second portion was incubated for 2 h without the addition of Cd. Subsequent Southern analysis of *Sal*I-digested DNA showed no differences in [^{32}P]-labelled *smtA* hybridization between the DNA from C0 cells restricted in either the presence or absence of added Cd, or grown in either the absence or presence of Cd for 2 h (data not shown).

4. DISCUSSION

A comparison of [^{32}P]-labelled *smtA* hybridization with *Sal*I-digested DNA from *Synechococcus* PCC 6301 line A0 with standard amounts of plasmid DNA containing the *smtA* gene is consistent with *smtA* occurring at a low copy number (probably one) on the cyanobacterial chromosome (figure 2).

Cell lines selected by stepwise adaptation to increasing concentrations of Cd were phenotypically distinct, displaying enhanced Cd tolerance when compared with A0 (figure 1). In Cd-tolerant lines obtained from the two independent sets of stepwise adaptation, amplification (increase in gene copy number per unit DNA) and apparent unique *smtA* restriction fragments were observed (figures 2, 3 and 4). There was concern that these apparent unique fragments could possibly be caused by some effect of Cd on restriction. The addition of Cd *in vitro* did not directly affect *Sal*I restriction endonuclease activity, and no rapid indirect effect of Cd *in vivo* (e.g. modification of restriction endonuclease recognition sites) was apparent after exposure of cells to Cd for 2 h. Moreover, DNA isolated from line C1.4 after one subculture (figure 4*a*, panel 1), did not show unique *smtA* restriction fragments despite being cultured in the presence of Cd, although unique restriction fragments were subsequently detected in DNA isolated from the same line after two, three and four subcultures. The unique *smtA* restriction fragments in DNA isolated from Cd-tolerant lines (A0.8, A1.3, A1.7, C1.4, C1.8, C2.6 and C3.2) are therefore ascribed to rearrangement of the *smt* locus. These smaller and larger restriction fragments may be attributed either to deletion in the flanking regions of *smtA* or integration of *smtA* into another region of the chromosome. There was no evidence of rearrangement or amplification of *smtA* in the non-tolerant lines A0/C0.

By contrast to *smtA*, hybridization of DNA from lines A0/C0 and Cd-tolerant lines to another gene, *psaE*, showed no evidence of unique restriction fragments, either during selection or the subsequent maintenance of these lines. It is noted that *psaE* hybridization to genomic DNA isolated from all lines, including A0/C0, identifies two restriction fragments, the larger fragment being more apparent upon prolonged exposure to X-ray-sensitive film (e.g. figure 3*b*, panel 2). Additionally, some blots show residual *smtA* hybridization because the blots were first hybridized with *smtA* and then washed before *psaE* hybridization (figure 3*b*, panel 7; figure 4*b*, panel 4). It is concluded that, unlike *smt*, there is no rearrangement of *psaE* in the Cd-tolerant lines. Continuing studies have identified specific changes in the nucleotide sequences flanking the *smtA* gene which give rise to the unique restriction fragments observed in the Cd-tolerant lines, thus confirming rearrangement of DNA at the *smt* locus.

Whereas *smtA* hybridization increased in some Cd-tolerant lines, there was no unequivocal evidence of amplification of the *psaE* gene (e.g. figure 2*b*). In some blots it was noted that there was some variability in *psaE* hybridization. Upon visualization of the corresponding ethidium bromide-stained genomic DNA (data not shown), these apparent small changes in *psaE* hybridization were found to correlate with slight variation in the amounts of DNA loaded on the agarose gel. The magnitude of variation in *psaE* hybridization was less than that observed for increases in *smtA* hybridization to DNA isolated from Cd-tolerant lines (figure 3*b*, panel 4; figure 4*b*, panels 1 and 3), and the variation in *smtA* hybridization did not correlate with variations in DNA loading (see figure 2, for example).

Olafson (1986) observed rapid development of Cd tolerance in a *Synechococcus* culture and a coincident increase in MT levels in Cd-tolerant cells. He proposed

that such rapid development of Cd tolerance may be associated with the amplification of an extrachromosomal MT gene. The prokaryotic MT gene, *smtA*, is now thought to be located on the chromosome. However, data presented here confirm amplification of *smtA* and rearrangement of the *smt* locus in Cd-tolerant lines, raising additional questions concerning the swiftness of adaptation. In the first set of stepwise adaptation, the development of Cd-tolerant lines could result from selection of particular variants from the genetic diversity occurring within the culture after prolonged maintenance in liquid medium. However, rapid adaptation was also observed upon stepwise adaptation of a culture generated from a single plated colony to minimize initial genetic variability. The molecular basis for such an apparent rapid amplification and rearrangement of a chromosomally located MT gene remains to be investigated. Greater frequencies of homologous recombination in prokaryotes than higher eukaryotes may be important.

These studies have established that, in *Synechococcus* PCC 6301, development of tolerance to Cd (in lines A0.8, A1.3, A1.7, C1.4, C1.8, C2.6 and C3.2) is associated with the amplification of *smtA* and rearrangement of the *smt* locus. Amplification of *smtA* may be analogous to the observed amplification of MT genes (initially chromosomal) in Cd-tolerant cultured eukaryotic cell lines (Beach & Palmiter 1981). For example, chinese hamster ovary (CHO) cell lines tolerant to successively higher Cd concentrations had threefold to 60-fold coordinate amplification of MT-I and MT-II genes, and accumulated increased levels of both MT-I and MT-II mRNA and polypeptides (Crawford *et al.* 1985).

It is proposed that increased internal metal ion sequestration by the *smtA* protein facilitates increased Cd tolerance in these stepwise-adapted *Synechococcus* PCC 6301 cell lines. It will be of interest to investigate whether similar phenomena of amplification and rearrangement of homologous genes occur in metal-tolerant cyanobacteria selected in metal-polluted natural environments.

A.G. is supported by a Jawaharlal Nehru Memorial Trust Scholarship. This work was supported in part by a research grant No. GR3/7883 from the U.K. Natural Environment Research Council. The authors thank Dr A. H. Shirsat and J. S. Turner for technical advice and assistance, and acknowledge the work of R. W. Olafson and co-authors. N.J.R. is a Royal Society University Research Fellow.

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Received 25 February 1992; accepted 23 March 1992

METALLOTHIONEIN GENES FROM *SYNECHOCOCCUS* PCC6301 AND PCC7942

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INTRODUCTION

In eukaryotes, metallothioneins (MTs) are involved in cellular responses to elevated concentrations of certain trace metal ions including those in the Cu and Zn triads (refer to 1). Genes encoding equivalent proteins have not previously been isolated from prokaryotes, although reports have indicated the presence of MT-like metal-ligands in several species (cited in 2). There is only one published amino acid sequence of such a protein, purified from *Synechococcus* sp. (3). Based upon this sequence PCR primers were synthesised. The resulting PCR products were used as probes to identify increases in the abundance of the corresponding transcripts following exposure to specific metal ions (4) and to isolate the corresponding MT locus, designated *smt*. The structure, function and regulation of the *smt* locus is reviewed.

STRUCTURE OF THE METALLOTHIONEIN LOCUS

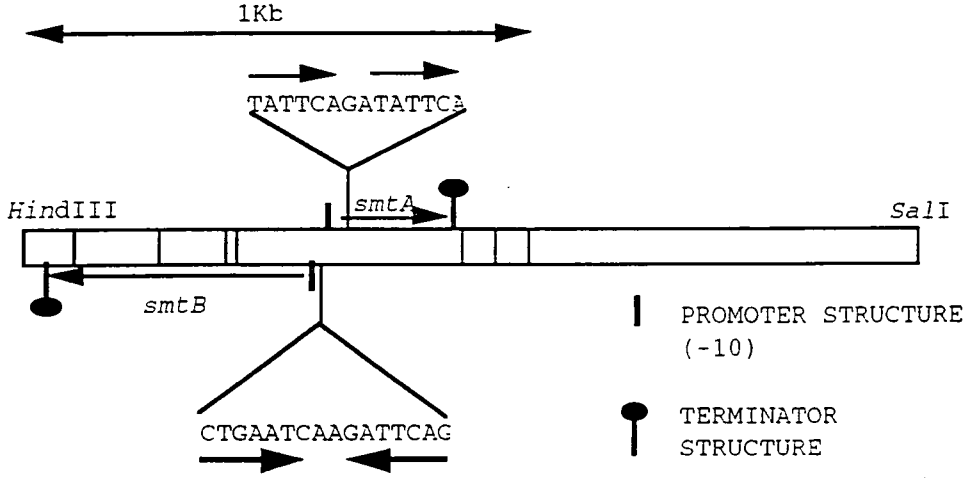

Figure 1 Representation of the structure of the *smt* locus, showing a 1.8 kb *Hin*dIII / *Sal*I fragment containing the MT gene, *smtA*, and divergent *smtB*. Sequence features within the *smt* operator-promoter region include a 7-2-7 hyphenated inverted repeat and a 6-2-6 hyphenated direct repeat located adjacent to, and 3' of, the *smtA* transcription start site. Vertical lines represent HIP1 sequences.

The MT locus from *Synechococcus* PCC7942 includes *smtA*, which encodes a class II MT, and a divergent gene *smtB* (Figure 1). The predicted sequence of the SmtA protein is similar to that purified from a *Synechoccocus* sp. (2) but with two additional amino acids at the C-terminus, N-His-Gly-C, and the substitution of Ser for Cys-32. The divergent ATG (encoding UAC translational start sites) sequences of both genes are separated by 100 bp. The sites of transcription initiation of both genes have been mapped within this region. Identified -10 concensus promoter motifs flank a 7-2-7 hyphenated inverted repeat (5'CTGAATC-AA-GATTCAG 3'). A 6-2-6 hyphenated direct repeat (5'TATTCA-GA-TATTCA 3') also lies within the region encoding the 3' untranslated portion of the *smtA* transcript.

METAL-BINDING PROPERTIES

To examine the metal-binding properties of its product the *smtA* gene was expressed in *E. coli* to generate SmtA as a carboxyterminal extension of glutathione-S-transferase (5). The protein was purified from *E. coli*, by glutathione affinity chromatography, and its amino acid sequence determined to confirm its identity. The pH of half dissociation of Zn, Cd and Cu ions from the expressed protein was determined to be 4.10, 3.50 and 2.35 respectively, indicating a high affinity for these metals (in particular for Zn in comparison to mammalian MT). Zn was displaced by Hg *in vitro*, using Zn-associated protein prepared from *E. coli* cells exposed to elevated concentrations of Zn for *in vitro* metal exchange. Associated Hg could not be displaced at low pH indicative of a high affinity for this metal.

CHARACTERISATION OF METALLOTHIONEIN DEFICIENT MUTANTS

Mutants deficient in functional *smt* have been generated via homologous-recombination-mediated insertional inactivation (6). A chloramphenicol acetyl transferase gene was inserted into the *smt* locus with the concomitant deletion of 373 bp including the *smt* operator-promoter region. Northern blots confirmed that the resultant *smt* cells were deficient in expression of *smtA* transcripts. Cultures of *smt* cells were hypersensitive to Zn and also showed reduced tolerance to Cd. No significant reduction in Cu tolerance was detected. *smt*-mediated restoration of Zn tolerance has subsequently been used as a selectable marker for transformation of these mutants.

METAL INDUCTION

smtA transcripts increase in abundance following exposure to elevated concentrations of certain trace metal ions including all of the Zn triad (Zn, Cd and Hg) and also Cu. Heat shock does not elicit an equivalent response indicating metal specificity, rather than a general "stress"

response. Metal-induction is repressed by a transcriptional inhibitor (rifampicin) (6). There is no detectable effect of metal ions on *smtA* transcript stability with equivalent rates of transcript decay in rifampicin treated cells regardless of metal exposure.

Sequences upstream of *smtA* fused to a promoterless *lacZ* gene confer metal-dependent *beta*-galactosidase activity. At maximum permissive concentrations Zn is the most potent elicitor followed by Cu and Cd. At maximum permissive concentrations, Hg did not confer significant increases in *beta*-galactosidase activity although induction was observed in response to 2 h exposures to higher concentrations of Hg. Induction curves, showing steady state expression of *beta*-galactosidase at different concentrations of metal ions, indicate that the *smtA* promoter is ultrasensitive with respect to Zn and Cd.

The deduced SmtB polypeptide has structural similarity to ArsR (a metal-dependent repressor) and CadC proteins involved in resistance to arsenate/ arsenite/ antimonite and to Cd respectively. SmtB also contains a predicted helix-turn-helix DNA-binding motif. In *smt* mutants (also deficient in functional *smtB*) there is some residual induction of *beta*-galactosidase activity from the *smt* operator-promoter in response to Zn (possibly due to a general stimulation of rates of transcription by elevated Zn). Most significantly there is a substantial (>20 fold) increase in basal expression in non-metal supplemented media which declines (although not to normal basal levels, possibly due to the absence of SmtA) upon reintroduction of a plasmid borne copy of *smtB* (6). It is therefore proposed that SmtB is a repressor of *smt* transcription.
Electrophoretic mobility shift assays (EMSA) have identified several protein complexes which associate with the *smt* operator-promoter region. Protein-binding sites include the 7-2-7 hyphenated inverted repeat and (a) site(s) located in a short region (39 bp) between the 7-2-7 hyphenated inverted repeat and the Shine/Dalgarno sequence of *smtA*. This region includes a 6-2-6 hyphenated direct repeat. Complexes which form with the latter region are absent in *smt* mutants, but are restored in *smt* mutants complemented with a plasmid borne copy of *smtB*. These data indicate that SmtB binds to a *cis*-element within this 39 bp region (6).

AMPLIFICATION AND REARRANGEMENT IN CADMIUM TOLERANT CELLS

Amplification and rearrangement of the MT locus has been observed in cells selected for tolerance to Cd (7). The rearranged locus has been cloned and sequenced from a Cd-tolerant cell line of *Synechococcus* PCC6301, C3.2. A 352 bp region of *smtB*, encoding the C-terminal portion of SmtB, is deleted in these cells. The functional deletion of *smtB* in cells selected for Cd-tolerance is consistent with the proposal that SmtB is a transcriptional repressor of *smtA* as derepressed expression of *smtA* may be beneficial for continuously metal challenged cells.

An octameric palindrome (5'GCGATCGC 3') traverses the borders of the excised element (6). Database analyses

reveal that this is a highly iterated palindrome (HIP1) in the genomes of all cyanobacteria represented within the database. It occurs once every 664 bp in *Synechococcus* genera, and is present both within genes and in intragenic regions. HIP1 is therefore proposed to have a fundamental role in genome plasticity and hence adaptation to environmental change in these organisms.

ACKNOWLEDGEMENTS

This work is supported by research grant GR3/7883 from the Natural Environment Research Council. J.S.T and J.W.H. are supported by research studentships from the Natural Environment Research Council and A.G. is supported by a Jawaharlal Nehru Memorial Trust Scholarship. The authors thank J. Gilroy and J. Bryden for technical assistance and acknowledge the work of R.W. Olafson and co-workers. N.J.R. is a Royal Society University Research Fellow.

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