Algal Metallothioneins: Synthesis and regulation of two putative metallothioneins within the blue-green cyanobacteria anacystis nidulans

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Algal Metallothioneins: Synthesis and regulation of two putative metallothioneins within the blue-green cyanobacteria Anacystis nidulans.

by

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ABSTRACT

Metallothioneins are low molecular-weight, cysteine-rich, trace metal-binding proteins. Whereas class III metallothioneins (MTs) have been isolated in eukaryotic algae, *Anacystis nidulans (Synechococcus TX20)* remains the only prokaryote in which a class II MT has been isolated and characterized. Cadmium binding ligands produced in response to cadmium were separated using gel permeation HPLC, DEAE and a variety of physical methods. Results indicated that, in addition to the class II MT previously isolated, putative class III $(gammaEC)_nG$ polypeptides may have been induced by the cadmium ions. An attempt was made to isolate and characterize the first prokaryotic metallothionein gene. Two synthetic oligonucleotides analogous to the *Synechococcus* TX20 MT protein were used as extension primers in an attempt to amplify the MT gene locus.

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ABBREVIATIONS

Amp = Ampicillin

A_{414} = Absorbance at 414 nm

BSA = Bovine serum albumin

bp = base pair

CdBP = Cadmium-binding peptide

dNTP = deoxyribonucleoside triphosphate

EDTA = Ethylenediaminetetra-acetic acid

GSH = Glutathione

hMT = Human metallothionein

HPLC = High pressure liquid chromatography

Kb = Kilobase

MT = Metallothionein

MRE = Metal regulatory element

NTA = Nitrilotriacetic acid

PCR = Polymerase chain reaction

RNAse = Ribonuclease

SDS = Sodium dodecyl sulphate

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Chapter I

INTRODUCTION

1.0.1 Introduction to metallothioneins

The isolation and characterization of metallothioneins (MTs) has now been documented for many species of vertebrate, invertebrate, and fungi. Most recently, an MT gene has been isolated from a higher plant but its translational product has not yet been identified (Evans *et al.,* pers. com.). MTs are thought to play an essential role in the intracellular regulation of the trace elements zinc and copper. MTs are cysteine-rich, low molecular weight proteins of extremely high metal content and are currently of considerable scientific interest through their involvement in the detoxification of heavy metals.

1.0.2 Biochemistry of metallothioneins

The term metallothionein was initially used to designate the cadmium and zinc containing sulphur-rich protein first extracted from equine renal cortex (Kagi and Vallee, 1960). This protein had been characterised as having a low molecular weight, high metal content, an amino acid composition high in cysteine but with no aromatic residues or histidine, a unique amino acid sequence with characteristic distributions of cysteinyl residues (such as Cys-X-Cys, where X is any other amino acid), spectroscopic features characteristic of metal thiolates (mercaptides) and metal thiolate clusters. With the isolation of MTs from other evolutionary diverse species, such as crab, locust and fission yeast (Olafson *et al.,* 1979. Martoja *et al.,* 1983. Murasugi

et al., 1981), there are now three internationally recognized subclasses of metallothionein:

Class I: Polypeptides with locations of cysteine closely resembling those of equine renal MT, such as those isolated from *Neurospora crassa* and *Agaricus bisporus* (Lerch and Beltramini, 1983; Munger and Lerch, 1985).

Class II: Polypeptides with locations of cysteine only distantly related to those in equine renal MT, such as those isolated from *Saccharomyces cerevisiae* and *Synechococcus* TX-20 (Winge *et al.*, 1985; Olafson *et al.*, 1988).

Class III: Nontranslationary-synthesized metal-thiolate polypeptides isolated from higher plants (Grill *et al.*, 1987; Jackson *et al.*, 1987; Rauser, 1987.), the fission yeast *Schizosaccharomyces pombe* (Kondo *et al.*, 1985) and eukaryotic algae (Gekeler *et al.*, 1988; Shaw *et al.*, 1988; Hart and Bertram, 1980).

Despite the wide range of metallothioneins discovered, they remain the only polypeptides isolated that contain cadmium. The class II MT isolated by Olafson (1988) from *Synechococcus* TX20 complexes cadmium and zinc. It has a high thiol content (19%) for a molecule, although this is low in comparison with other MT sequences, and cysteine sequences similar to those observed in eukaryotic MTs with characteristic clusters of Cys-X-Cys and the commonly found Cys-X-X-Cys or Cys-Cys sequences. Synthesis of prokaryotic MT is induced by the presence of zinc or cadmium within the media. The cyanobacterial molecule under discussion may have some covergent evolutionary relationship with eukaryotic MTs, and some similarities in secondary structure with regard to the metal thiolate region, However, it is believed to be the first prokaryotic MT isolated. Data presented here also indicates that *Anacystis nidulans* contains a putative class III MT or $(gammaEC)_nG$. If this is confirmed, it will be the first evidence for such metal-binding polypeptides within a prokaryote.

1.0.3 The role of metallothioneins in trace metal homoeostasis

Several intracellular/extracellular mechanisms of defense have evolved in response to the presence of trace metals within the environment. The role of Class I MTs in animals is thought to be primarily that of a regulator in the control of zinc homoeostasis (Karin, 1985: Olafson *et al.*, 1988). Zinc ions are important constituents of the active site of many enzymes involved in transcription, protein synthesis and degradation, replication and energy metabolism. As the major zinc-binding proteins within the cell, MTs can potentially regulate the supply of trace metal and thus directly effect the outcome of many important biological processes. Increased dosage of cadmium and zinc in animals leads to the transcriptional activation of MT genes and the subsequent accumulation of MT within the liver and kidneys (Durnam and Palmiter, 1981). The transcription rate of the MT genes increase with the concentration of free zinc ions within mammalian cells, leading to the synthesis of extra MT to bind the excess metal. In addition, the level of zinc regulates the turn over of MT. When zinc levels are low intracellularly, MTs are rapidly degraded. The complexity of MT synthesis and transcriptional control suggest that MTs occupy a central role in cellular metabolism. Such a role is unlikely to have developed in response to fluctuations in environmental levels of toxic trace metals and natural selection for intracellular detoxifying-metal binding proteins.

The role of the class III MT, $(gammaEC)_nG$, is less clear although there is direct evidence that these polypeptides are involved in the detoxification of excess cadmium and copper in plant cells (Jackson *et al.*, 1987). Their role in zinc homoeostasis is doubtful as $(gammaEC)_nG$ are only weakly associated with zinc (Reese and Wagner, 1987). In *Euglena gracilis*, studies found evidence that intracellular cadmium bound to $(gammaEC)_nG$, whilst the majority of the zinc was to be found in a low molecular weight pool (Gingrich *et al.*, 1984; Shaw

et al., 1989). Exposure to zinc did not induce synthesis of zinc-(gammaEC)$_n$G in *Euglena gracilis* (Weber *et al.*, 1987; Shaw *et al.*, 1989).

1.1 Cadmium

1.1.1 The uses of cadmium.

Cadmium is a major environmental and occupational pollutant. Many commercial products contain cadmium. These include; cooking utensils; sleeve bearings for cars, aeroplanes and marine engines; jewellery production; chemicals (as in the halides) used in photography, coloured pigments used in the glass industry; storage batteries; paints; plastics; phosphors in television tubes; household appliances and transistors (Schroeder, 1965).

1.1.2 Cadmium exposure and toxicity

Humans are exposed to cadmium through the inhalation of cigarette smoke and emission from the combustion of fuels and plastic waste. Industrial workers are exposed to cadmium within metal smelters, paint pigment, battery, ceramic, alloy and welding industries (Nriagu, 1980). Trace amounts of cadmium have been detected in almost all types of food; shellfish, wheat, soybean and rice accumulate high amounts of cadmium (Jayasekara *et al.*, 1986; Casterline and Bennett, 1982). The tissue distribution of cadmium has been studied in various parts of the world. Renal cadmium (mean level) amongst Japanese men was 6,030 $\mu g\ g^{-1}$in ash with a range of 1,350-19,500 $\mu g\ g^{-1}$, compared to the average Englishman with 1050 $\mu g\ g^{-1}$ (Schroeder, 1960; Schroeder and Balassa, 1961). Exposure to cadmium causes anaemia, osteomalacia, hypertension,

hepatic, renal and cardiovascular disorders (Flich *et al.*, 1971). The inhalation of CdO fumes leads to emphysema, chronic bronchitis and bronchial carcinoma (Morgan,1971; Gunn *et al.*, 1963). Such chronic effects are a result of the long half-life of cadmium in most tissues, ranging from 80 days in blood and 10 years in the liver to 25 years in the kidney. Within the USA cadmium is recognized as a "priority pollutant "and drinking water limits are set at 10 ng ml^{-1}. European levels for the Rhine and Neckar Rivers in 1979 were reported to have reached 3.7 and 6 $\mu g\ l^{-1}$, respectively (Förstner and Wittman, 1979).

1.1.3 Cadmium, metal antagonism

The chemistry of cadmium is essentially homologous to that of zinc. Cadmium shows a strong affinity for ligands such as phosphates, cysteinyl and histidyl side chains of proteins, purines, pteridines, and porphyrins. In a similar way to lead and mercury, cadmium can act at a large number of biochemical sites. Premsagar (1969) noted the effect of cadmium on the conformation of polyriboadenylic acid and the physical properties of DNA, whilst other workers have noted the inhibition of enzymes with functional sulphydryl groups and the disruption of oxidative phosphorylation pathways. In higher organisms the biological action of metals both toxic and essential are often conditioned by metal ion antagonism. Cadmium can substitute for the zinc atom at the active site of bovine pancreatic carboxypeptidase A (Vallee and Ulmer,1972). The cadmium-enzyme displays a significantly greater activity than the native zinc-enzyme during the hydrolysis of other substrates, but in contrast to zinc-carboxypeptidase it will not hydrolyse peptide substrates. Thus cadmium by altering the catalytic efficiency and specificity of the enzyme is both "activating " and "inhibiting " carboxypeptidase depending on the

substrate. Several other unnatural Cd-protein complexes are known (Table 1). The number of enzymes altered suggests that cadmium and zinc are likely to be isomorphic and compete readily for the same binding sites, i.e 3-SH groups, when cadmium is present intracellularly. It is therefore a serious environmental toxin. In cyanobacteria, the documented damage induced by cadmium includes mitochondrial membrane inactivation, chromosome aberrations, destruction of photosynthetic pigments and photosynthesis inhibition, release of cellular potassium and the inhibition of nitrogen fixation (Kunisawa and Cohen-Bazire, 1970; Singh and Yadava, 1984 and 1986).

Table 1: Cadmium-zinc metal antagonism (Vallee and Ulmer, 1972.)

Cadmium enhanced	Cadmium inhibited
Rat liver acid phosphatase	Canine liver acid phosphatase
Chicken adenosine triphosphatase	Rat liver adenosine triphosphatase
Rat alkaline phosphatase	Calf Duodenum alkaline phosphatase *E. coli* alkaline phosphatase
Bovine pancreatic carboxypeptidase A	Calf duodenum and *E.coli* carboxypeptidase A
Rat cholinesterase	Calf duodenum and *E. coli* cholinesterase
Pigeon brain cytochrome oxidase	Rat liver mitochondrial cytochrome oxidase
Mouse malic dehydrogenase	
Rat phosphorylase	
Pigeon succinic dehydrogenase	Rat liver succinic dehydrogenase

1.1.4 Cadmium contamination of the environment

The presence of toxic trace metal contaminants in industrial and agricultural wastes is of growing environmental and toxicological concern. Cadmium is commonly associated with zinc in carbonate and sulfide ores and as a byproduct in the refining of other metals (copper and lead). Global cadmium production in 1979-1980 was 1.5 x 10^5 t. In Europe cadmium emissions into the environment during the same year reached 2,700 t. The UK total alone was almost 100 t. The largest source of emission were the zinc-cadmium mining operations (Tables 2a and 2b). The close association of cadmium in nature with zinc results in the recovery of 6kg of cadmium for every ton of zinc metal mined and smelted.

Many industrial and domestic effluents are contaminated with toxic trace metals in solution. If not removed prior to discharge these metals (uranium, cadmium, lead and copper) can pose a serious health hazard (Scott *et al.*, 1973). Running water spreads pollutants either as solutes or in the suspended sediment load, and they may be deposited on the floodplain. Although the quality of effluent water from mines and processing plants in the UK is now controlled, the floodplain soils in the old lead mining areas of Britain are still contaminated by lead, zinc and cadmium residues (Davies, 1983), with levels of cadmium up to 1 mg g^{-1} recorded (Colbourn and Thornton, 1978). In 1988 the village of Shipham, built on the site of old zinc mines, was the subject of a special report (Morgan, 1988). Cadmium concentrations within the soils were found to have reached 300 $\mu g\ g^{-1}$with a median value of 90 $\mu g\ g^{-1}$. An order of magnitude higher than those associated with the "itai, itai" disease in the Japanese community of Jinzu. Itai, itai, the Japanese for pain, is a syndrome of long term cadmium and other toxic trace metal exposure (Flick *et al.*, 1971). The unfavourable effects that cadmium containing effluents have on sewage should

Table 2a: European sources of cadmium emission into the atmosphere in 1979

Source	tons/yr
Combustion of fuels	312
Zinc-cadmium metal production	1550
Copper-nickel metal production	595
Ferroalloy manufacture	58
Phosphate fertilizers	84
Others	74
European total	2,700

Table 2b: The emission by European countries of cadmium into the atmosphere in 1979 (Nriagu and Davidson, 1979)

Source	tons/yr
USSR	816
UK	99
Poland	207
Italy	124
G.F.R.	324
France	170
Belgium	171
Austria	137
Others	648
European total	2,700

also not be overlooked, i.e. inhibition of nitrification and interference with biological oxidation by microorganisms. Sewage wastes, both solid and liquid, are increasingly applied to agricultural land. The solid waste, or sludge, is commonly applied annually to land at 25 t dry matter ha^{-1}. As a result any constituent trace metal will accumulate within the soil. Cadmium-containing sludges of between 60-1500 $\mu g\ g^{-1}$ dry matter have been recorded (Berrow and Webber,1972). Plants grown on these soils have demonstrated marked increases in intracellular cadmium concentrations and may pose a serious health hazard when ingested in quantity.

1.1.5 Decontamination of cadmium in solution

Decontamination can be achieved by physiochemical processes such as ion precipitation and exchange. Cadmium will also form moderately stable complexes with a variety of organic compounds and synthetic chelating agents such as nitrilotriacetic acid (NTA) form relatively stable cadmium-NTA chelates. However, the use of microorganism's biomass to absorb trace metal ions intracellularly appears to offer an alternative solution to the problem. A number of eukaryotic algae are predominant in sewage/effluent treatments and several algal species have been recorded to contain high intracellular levels of trace metals (Table 3). Other species have been used as biological indicators to monitor toxic trace metal pollution in aquatic environments. The efficiency of algae to remove trace metals will be determined primarily by.

(1) Algal growth rate.

(2) The environmental concentration of the metal.

(3) The ability of the species to absorb and concentrate the metal.

(4) Percentage recovery of the metal required and achieved.

Table 3: Toxic trace metal accumulation within algae.

(5) Cost and effectiveness of the operation.

Species	mg kg^{-1}	Author
Lemanea	10,000	Harding and Whitton, 1981
Cladophora	500	Förstner and Wittman,1979
Anabaena	10,000	Laube *et al.*, 1980
Coelastrum	2,000	Soeder *et al.*, 1978

1.2 Prokaryotic gene induction and its implications for MT induction

In comparison to mammalian MTs, in which transcription has been activated and inhibited by a variety of stress factors besides that of toxic trace metals, the transcription of the *Synechococcus* MT gene has been induced only by zinc and cadmium ions. Although copper induction of the fungal MTs has been recorded within *Saccharomyces cerevisiae*, *Agaricus bisporus* and *N. crassa*, it has not been reported within *Synechococcus*. Induction of the mammalian MT genes is controlled by short DNA sequences present in multiple copies upstream of the transcriptional start. The arrangement and sequence of several of these metal regulatory elements (MREs) is known. MREs have also been isolated within *Saccharomyces cerevisiae*. To date, although the amino acid structure and some physical characteristics of the *Synechococcus* TX20 MT gene product are known, the presence of upstream regulatory elements and the precise base pair sequence of the MT locus has not yet been determined. Two theories concerning the induction of eukaryotic MT by cadmium have been

proposed. The first is that cadmium directly interacts with a DNA binding factor increasing its affinity for the regulatory site. The second is that cadmium acts by stimulating or inhibiting a protein-protein interaction, i.e activating the formation of a complex between the DNA-binding molecule and a "co-activator "protein thereby altering the conformation and hence its affinity for the MRE (Saquin and Hamer, 1987). Regulation of *Synechococcus* MT genes is also thought to be controlled at a transcription level (Olafson, 1986).

In a potentially analogous system the mer operon of *E. coli* confers resistance to the effects of mercury salts. Mercury resistance is controlled through the coordinated action of the plasmid bo rne MerT, MerP and MerA genes within the operon (MerT and MerP encode membrane and periplasmic proteins involved in mercury uptake, whilst MerA encoded for mercuric ion reductase that detoxifies mercury salts through reduction to the non toxic Hg(0)). Resistance is acquired via amplification of the coordinate transcription rate of the MerTPA genes (Lund and Brown, 1987). This positive transcriptional control of the **mer** operon is affected by a metalloregulatory DNA binding protein (merR), that activates transcription from the promoter in the presence of mercury and represses transcription in its absence. In addition, the merR protein has also been found to regulate its own synthesis in the absence and presence of mercury (Shewchuk *et al.*, 1989). Two regions within the transcriptional control area of the MerR operon are thought to be potential binding sites for merR. A 16bp dyad centered on position -79/-80 with respect to the start point of mer mRNA and a 18bp inverted repeat embedded between the "-35 " and "-10 " recognition elements of the **mer** promoter. The merR protein has two domains, a carboxyl-terminal mercury binding site and an amino-terminal DNA binding domain. Specifically two regions within the N-terminal have the conformational ability to form the helix-turn-helix DNA binding motif common to many prokaryotic repressor/activator molecules. A

configuration of four cysteine residues within the C-terminal domain are thought to form the mercury binding site and contribute to transcriptional activation. The arrangement of cysteine and histidine residues within this region has been suggested by some authors to bear some similarities to the zinc-binding domain of the "zinc finger " proteins (O'Halloran and Walsh, 1987).

Several gene sequences, such as the mouse Krox-20, Drosophila "hunchback" , Xenopus transcriptional factor III_A and *S. cerevisiae* SW15 sequence (Wilkinson *et al.*, 1988; Tautz *et al.*, 1987; Miller *et al.*, 1985: Stillman *et al.*, 1988), have been shown to contain tandem repeats of base pairs, these repeats on translation are thought to form structural domains around zinc bound ions - the zinc fingers. The sequences seem to occur quite commonly and are thought to play a role in developmental and metabolic control through nucleic acid recognition. The zinc ion is thought to play an important role in maintaining the tertiary structure required for sequence-specific recognition (Nagai *et al.*, 1988). The zinc fingers direct binding of the transcriptional factor to its recognition sequence within the promoter region. These zinc-binding proteins are very different from MT in that pairs of metal-binding cysteine residues are seperated by 2 to 4 residues. The question regarding the ability of other trace metals such as copper and cadmium to substitute for the zinc ion has not been resolved (Miller *et al.*, 1985). In comparison with the merA protein of *E. coli*, it is conceivable that metal-binding sites such as these are either a target of intracellular cadmium, reducing the ability of a transcription factor or repressor to bind to an operon, or, by modifying the conformation of the metal-binding domain, through metal antagonism allow binding of an inducer protein to the MREs and transcription through the MT operon.

In comparison to Class II *Synechococcus* TX20 MT, Class III MT is a metal thiolate polypeptide which has identified as a secondary metabolite (Robinson *et al.*, 1988). These polypeptides are rapidly synthesised from an

intracellular pool of glutathione. The synthesis of these peptides, in response to the presence of cadmium, is rapid and has been detected within 5 minutes of cadmium exposure. It is, therefore, too fast a response to be a transcriptionally controlled process and it is also insensitive to cycloheximide.

1.3 Justification in using Anacystis nidulans

To date *Anacystis nidulans* remains the only prokaryotic organism identified as containing a recognized MT sequence. The cyanobacteria represent a diverse group of prokaryotic organisms, enough is known about the metabolism, biochemistry and physiology of *A. nidulans* for the species to be regarded as a "model "cyanobacteria (Golden *et al.*, 1989; Wilmotte and Stam, 1984). The study of gene expression is well developed within this organism and gene transfer techniques have been developed (Buzby, 1985; Golden, 1987; Kuhlemeire and Van Arkel, 1987). The investigation into the physiological role of MTs, their regulation, transcription and translation within an organism would be greatly enhanced by the use of a prokaryotic organism. Furthermore a MRE and MT gene which function in a cyanobacteria may allow the production of cyanobacteria which accumulate large amounts of cadmium from solution, possibly via the introduction of multiple copies of these genes. There are potential applications for such an organism in waste management.

1.4 Aims and objectives of the project

The overall aim was to further characterise the molecular and biochemical responses of a cyanobacteria to cadmium and thereby identify genetic resources which may be applied to the bioaccumulation of toxic trace metals. Specific

objectives were as follows:

(1) Characterize cadmium-ligands produced in *A. nidulans* in response to different cadmium concentrations.

  (1a) By separating different MW species using gel permeation HPLC.

  (1b) Further characterize ligands by a variety of physical methods

(2) Select Cd-resistant *A. nidulans* using stepwise selection.

(3) Examine Cd-ligands produced in Cd-resistant *A. nidulans.*

(4) Attempt to amplify the first prokaryotic MT gene from *A. nidulans* using the polymerase chain reaction.

  (4a) To design two synthetic oligonucleotide probes analogous to the *Synechococcus* TX20 MT gene N- and C-terminals.

  (4b) To use the two oligonucleotides as extension primers in an attempt to amplify the MT gene locus through the polymerase chain reaction.

(5) Attempt to isolate and characterise the first prokaryotic MT gene and its regulatory region.

  (5a) If the PCR amplification was successful to isolate the amplified gene and clone it into a suitable vector for nucleotide sequencing.

  (5b) To make radioactively-labelled probes from the oligonucleotides and probe the *A. nidulans* genome.

  (5c) To isolate the regions flanking the gene.

Chapter II

METHODS AND MATERIALS

2.0.1 Anacystis pedigree

The literature concerning cyanobacterial genetic research is confused by the different taxonomic names appended to the same strain by different authors. The strain of *Anacystis* under examination in this thesis was obtained originally from the CCAP (1405/1) and has been nominated as *Anacystis nidulans* **33A** under the University of Durham stock collection. Strains named *Anacystis nidulans* (TX20, UTEX 625 and UTEX 1550), *Synechococcus leopoliensis* CCAP 1405/1, *Synechococcus* PCC 6301 (ATCC 27144), and *Synechococcus* TX20 (Table 4). All originated from the immotile, apparently unicellular, rod-shaped blue-green algae isolated by Kratz and Myers (1955) and subsequently brought into pure culture by M. Allen (Pringsheim *et al.*, 1968). This organism was identified by Drouet (unpublished) as *Anacystis nidulans*, under which name it has been used for numerous physiological, biochemical and genetical investigations. *A. nidulans* sometimes produces short filaments of cells, particularly when grown in stationary cultures and such observations may have confused previous workers, whilst bringing into question its classification as a unicellular organism.

Table 4: Alternative generic designations of *Anacystis nidulans*.

Organism	PCC	ATCC	UTEX	CCAP
Synechococcus	6301	27144	TX20	
Anacystis nidulans			TX20	
Anacystis nidulans			625,1550	
Synechococcus leopoliensis				1405/1

ATCC= American type Culture Collection; UTEX= Culture Collection of Algae at the University of Texas (formerly IU); CCAP= Cambridge Culture Collection of Algae and Protazoa; PCC= Pasteur Culture Collection of Cyanoacteria.

2.0.2 Algal growth media and buffers

The strains were maintained on ACM (modified AC media) buffered with 2.5 mM HEPES (pH 7.6) and grown in constant light at 32^0C (Table 5).

AC micronutrients: Stock solution (1 ml), made up to 1 litre with distilled water. 2.5 mM HEPES (0.6 g in 500 ml distilled water adjusted to pH 7.6 with 1 M NaOH) added and adjusted to pH 7.6 before adding the rest of the stock solutions. Care was taken to add the phosphate last to reduce the risk of precipitation. Due to the Oxygen requirement of the organism during growth, only 25 ml in 100 ml flasks and 400 ml in 1 litre flasks was used. The media was autoclaved for 20 min and allowed to stand for a 2 hours. Stock culture (10 ml) was inoculated into 400 ml (0.5 ml of stock culture to 25 ml of medium). Cultures were incubated at 32^0C in continuous light (37^0C would be ideal). Stock cultures were renewed every 7-10 days.

Table 5: ACM modified medium and BG11 trace metal stock solution

macronutrients		quantity
$K_2HPO_4.3H_2O$.		Use 0.1 ml of 131.03 gl^{-1} stock
$CaCl_2.2H_2O$.		Use 1 ml of 19.86 gl^{-1} stock
$MgSO_4.7H_2O$.		Use 5 ml of 50.00 gl^{-1} stock
NaCl.		Use 0.5 ml of 46.00 gl^{-1} stock
KNO_3.		Use 5 ml of 100.00 gl^{-1} stock
Fe.EDTA.		Use 1 ml of 1.21 $FeCl_3$. and 0.50 EDTA gl^{-1}
BG11 Trace metal		Use 1 ml of stock solution
BG11 Trace metals		
micronutrient	MW	quantity
H_3BO_3	61.83	2.86 gl^{-1}
$MnCl_2.4H_2O$.	197.72	1.81 gl^{-1}
$ZnSO_4.4H_2O$.	287.55	0.22 gl^{-1}
$NaMoO_4.2H_2O$.	241.95	0.39 gl^{-1}
$CuSO_4.5H_2O$.	249.86	0.079 gl^{-1}
$Co(NO_3)_2.6H_2O$.	291.05	0.049 gl^{-1}
$NiSO_4.7H_2O$.	280.76	0.048 gl^{-1}

2.0.3 Bacterial media and buffers.

1. **2XL Media (for 1 litre).**
   20 g of Trypticase
   10 g of Yeast extract
   1 g of NaCl (pH 7 with NaOH)
   autoclave
   10 ml of 20% (w/v) Glucose

2. **10x. TBE (for 1 litre).**
   108 g of Tris-Base
   55 g of Boric acid
   40 ml of 0.5M EDTA (pH 8)
   (14.6g in 100 ml)

3. **20x SSC (for 1 litre).**
   175.3 g of NaCl
   88.2 g of Citric acid
   pH 7 with 10N NaOH

4. **TE Buffer.**
   10 mM of Tris.HCl
   1 mM of EDTA (pH 8)

5. **Restriction Buffer (medium)**
   50 mM of NaCl
   10 mM of Tris.HCl (pH 7.5)
   10 mM of $MgCl_2$
   1 mM of Dithiothreitol

6. **Loading Buffer.**
   10 mM of Tris.HCl (pH 7)
   10 mM of KCl
   1.5 mM of $MgCl_2$
   50 mM of β-mercap.

2.0.4 Isolation of genomic DNA from A. nidulans

Several methods of DNA extraction were attempted (Tomioka *et al.*, 1981.

Marmur, 1961; Dzelzkalns and Bogorad, 1986; Dzelzkalns *et al.*, 1984). Early efforts at restriction by endonucleases were hampered by the purity of the resulting isolated DNA. Finally the method of Robinson *et al.*, (1988), a protocol previously used to extract DNA from plant cells, yielded nucleic acids of sufficient purity for restriction digest, Southern blotting and the Polymerase Chain Reaction (PCR). Approximately 50 ml of cells were harvested in mid-late log phase of growth, washed once with ACM media and three times in sterile ice-cold extraction buffer containing 100mM Tris-HCl (pH 8.0), 20 mM EDTA and 1.4 M NaCl, then placed in a sterile mortar and frozen in liquid nitrogen. Frozen cells were ground to a fine powder, then suspended in boiling extraction buffer containing 50 mM 2-mercaptoethanol. An equal volume of 1:1 chloroform/buffered phenol (80% (v/v) phenol, 20% buffer containing 10 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA) was added and the phases mixed for 10 min, then separated by centrifugation at 5000 x g. The upper aqueous phase was collected and extracted twice more with 1:1 chlorophorm/buffered phenol then three times with an equal volume of 24:1 (v/v) chloroform/isoamyl alcohol. The nucleic acids were precipitated from the final aqueous phase at -20^{0}C by addition of 1M ammonium acetate and 2.5 volumes of ice-cold 95% ethanol. Nucleic acids were collected by centrifugation at 5000 x g for 20 min and the pellet washed with 70% (v/v) ethanol and air dried prior to resuspension in 30 μl of TE buffer (20 mM Tris-HCl (pH 8.0) and 1 mM EDTA). The purity of the resulting DNA was determined by measuring the absorbance at 260 and 280nm, and determining the ratio between the two. A ratio of between 1.8 and 2.0 indicated that the DNA was suitable for further manipulation. If the ratio was below this the DNA was assumed to be contaminated with phenol or protein.

2.0.5 Digestion of plasmid DNA with restriction endonuclease

Genomic and plasmid DNA from the previous isolation method were restricted according to the protocol of Maniatis *et al.*, (1982). TE Buffer (50μl, pH 8) containing 20μg ml^{-1} DNase-free pancreatic RNase was added and the tube vortexed briefly. To 10 μl of this solution, 1.2 μl of restriction buffer and 1 unit of restriction enzyme was added. This was then incubated at 37^0C for 2 hr.

2.0.6 Gel electrophoresis of restricted DNA

Restricted DNA was analysed by agarose gel electrophoresis using the methods of Maniatis *et al.*, (1982). Gels were prepared using 1.5% (w/v) agarose dissolved in TBE, plus 10 μl ethridium bromide and DNA was separated by electrophoresis in tanks with volumes of 400 ml (minigel) and 2.2 l (full sized gels) containing TBE plus 100 μl of Ethidium bromide. A voltage of 30-100V was applied, and the gel run for 3-12 hrs respectively. Gels were photographed under uv-light using an aperture of 1.8 and 1 s exposure.

2.0.7 Detection of cadmium binding peptides

Cell cultures of *Anacystis* 33A were grown up ACM media, approximately 10 x 400 ml of culture was required for analysis. Cadmium was added at the required dosage, at a set time and the cultures allowed to continue to grow. The cells were then harvested and spun down and collected finally in a 50 ml Falcon tube. Washed twice in ice-cold buffer (10 mM Tris-HCl (pH 7.0), 10 mM KCl, 1.5 mM $MgCl_2$) and then resuspended in a equal volume of buffer containing 50 mM β-mercaptoethanol in a 1.5 ml Eppendorf (36 μl β-mercap in 10 ml of buffer). The cell suspension was sonicated for 10 x 30 s on ice with a 1 min gap between each sonication to break open the cells. The 1 min rest

between sonication was found to be necessary to allow the dissapation of heat from the sample. Each cell extract was then centrifuged at 10,000 x g for 10 min. The supernatant collected and respun to remove any further debris. Four x 100 μl samples were then injected onto a SW 3000 (7.5 x 250 mm, Anachem.) gel permeation HPLC chromatography column. The samples were overlaid for each 100 μl run with 15 ml of buffer. Sixty fractions were collected in total at 30 s intervals. Fractions were analysed by passing the fractions through an Atomic Absorption Spectrophotometer set to detect the absorption 228 nm for the presence of cadmium.

2.0.8 Determination of acid-labile sulfide

In a tightly stoppered tube 0.5 ml of zinc acetate (2.6% in water), 0.1 ml of Sodium hydroxide (6% in water) and 0.7 ml of the sample under analysis were added. The tube was sealed and vortexed vigorously for 1 min. The tube was then opened and 0.25 ml of diamine reagent was rapidly added (N.N-Dimethyl-p-phenylenediamine monohydrochloride (0.1% (w/v) in 5N HCl). The solution was gently swirled then opened and 0.1 ml of ferric chloride (11.5 mM in 0.6N HCl) was quickly added with the test tube being rapidly resealed and the mixture vortexed for 1 min. The mixture was then allowed to stand at room temperature for 30 min, before adding 0.85 ml of distilled water. The resulting precipitate was removed by centrifugation at 10,000 x g for 10 min. The solution and standards made at 5, 10, 20 and 40 mM of sulfide (Standard Sodium sulfide 0.2M in water corresponding to 0.015, 0.1, 0.2 and 0.4 ml of diluted solution, respectively) were read against a blank reagent at 670 nm.

2.0.9 Synthesis and purification of oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems DNA synthesiser model 381A. The method of synthesis utilises phosphoramitide chemistry with the oligonucleotides bound to a solid phase support during the reaction cycles (Marreucci and Caruthers, 1981; Beaucage and Caruthers, 1987). The sequences were based on previously published work by Olafson (1988) which contained the amino acid sequence of a recently isolated cyanobacterial metallothionein. Before cleavage of the oligonucleotide from the support the 5'dimethoxy trityl group (DMP) is removed by treating with tri-fluoro acetic acid (TCA). The oligonucleotide is subsequently released from the synthesis column by concentrated NaOH treatment. This cleaves the oligonucleotide from the support and removes the β-cyanoethyl groups protecting the phosphates of the internucleotide linkages ("trityl off synthesis"). Subsequent incubation of the eluted oligonucleotides in concentrated NH_4OH (55^0C , 8-15 hrs) deprotects the exocyclic amines of the bases. Oligonucleotides are dried down under vacuum, twice resuspended to remove any ammonia (which inhibits any 5' end-labelling) and resuspended finally in water and stored until use at -20^0C. .

2.0.10 Structure of two synthetic oligonucleotide extension primers

As mentioned the two synthetic oligos were based on previously published data by Olafson: The N-terminus probe: 5'-GTI AAY TGX GCI TGX GAI CC-3'. where A, C, G and T represent deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine respectively. X is deoxythymidine 50% of the time and deoxycytidine 50% of the time. Y is deoxyadenosine 50% of the time and deoxyguanosine 50% of the time. I is deoxyinosine, a synthetic oligonucleotide which will not prevent the formation of the DNA helix no matter which naturally occuring nucleotide is paired opposite. Deoxyinosine

was used within the probe structure to reduce the level of four-base redundancy within the twentymer (Mullis and Faloona, 1987). It was decided to place the 5 $\mapsto$ 3 probe six amino acids in from the N-terminus to reduce the level of redundancy that would have to be accommodated within the probe structure and to include a the sequence Cys-X-Cys, characteristic of MTs. In the case of the triplet GAI, it has been used to reduce further redundancy within the probe by replacing a A/G mix. This population of sequences should have a sixteen fold redundancy and hybridize to all possible DNA sequences encoding the polypeptide sequence Val-Lys-Cys-Ala-Cys-Glu-Pro. The C-terminus probe: 5'-CAY-TTY-CAI-CCI-GTY-TGI-CC-3'. This population of sequences should have a sixteen fold redundancy and hybridise to sequences encoding for Gly-His-Thr-Gly-Cys-Asn-Cys. It is notable that both probes were constructed to correspond with regions of Cys-X-Cys sequences, where X can be any amino acid, associated with previously isolated metallothioneins (Figure 1).

2.0.11 The polymerase chain reaction (PCR)

Genomic DNA was obtained from *A. nidulans* as described previously. Approximately 1μg of genomic DNA was placed in 100 μl of 1x PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$./ 0.1% gelatin,/200 μM of each dNTP and 1μM of each extension primer) following the method of Salki (1985) and Mullis (1987). Five units of Taq (*Thermus aquaticus*) DNA Polymerase (purchased from Perkin Elmer Cetus or Stratagene) diluted 5:1 was added and the reaction mixture was overlaid with 100 μl of mineral oil. The polymerase amplification reaction took place in previously siliconized Eppendorf tubes (Maniatis 1982) sealed with silica gel. The reaction tube was then subjected to several cycles of: a) Denaturation (2 min at 95^0C , first cycle 5 min), to dissociate the double-stranded DNA template. The two strands will remain free in solution until the temperature is reduced sufficiently to

allow annealing; b) Annealing of extension primers (3 min at 47^0C). The extension primers will anneal to only one of the strands of DNA. Since the primers anneal to opposite strands , they can be viewed as having their 3' ends facing each other. The primers are present in excess over the DNA template, therefore the formation of the primer-template complex will be favoured over the reassociation of the two DNA strands, at the primers' annealing sites, when the temperature is lowered. The annealing temperature was further raised to 55^0C , 60^0C and 65^0C in latter re-runs to assertain the degree of specificity between the extension primers and the potential MT gene regions; c) Synthesis and extension (amplification step, 3 min at 72^0C). Through this process the extension primers will become incorporated into the amplification product and form the templates of subsequent PCR reactions. The typical set of three steps (i.e., denaturation, annealing, extension) are referred to as a cycle. Thirty cycles were run per reaction mixture. During the final cycle tubes were placed at the annealing temperature for 3 min, followed by 6 min at 72^0C. PCR products were examined either on a 2% agarose mini-gel (run at 80v) or on an alkaline 3.75% agarose gel (NuSieve GTG) run overnight at 30V. A successful PCR reaction will result in the exponential increase of the DNA sequence between the 5' ends of the extension primers. The method relies on the the ability of the synthetically produced oligonucleotides to function as extension primers for the Taq polymerase. It was intended that in this case the degree of redundancy within the oligonucleotides would not inhibit this function in the early cycles. In latter cycles of amplification these primers will anneal primarily to the more abundant amplified products rather than to the original genomic sequences.

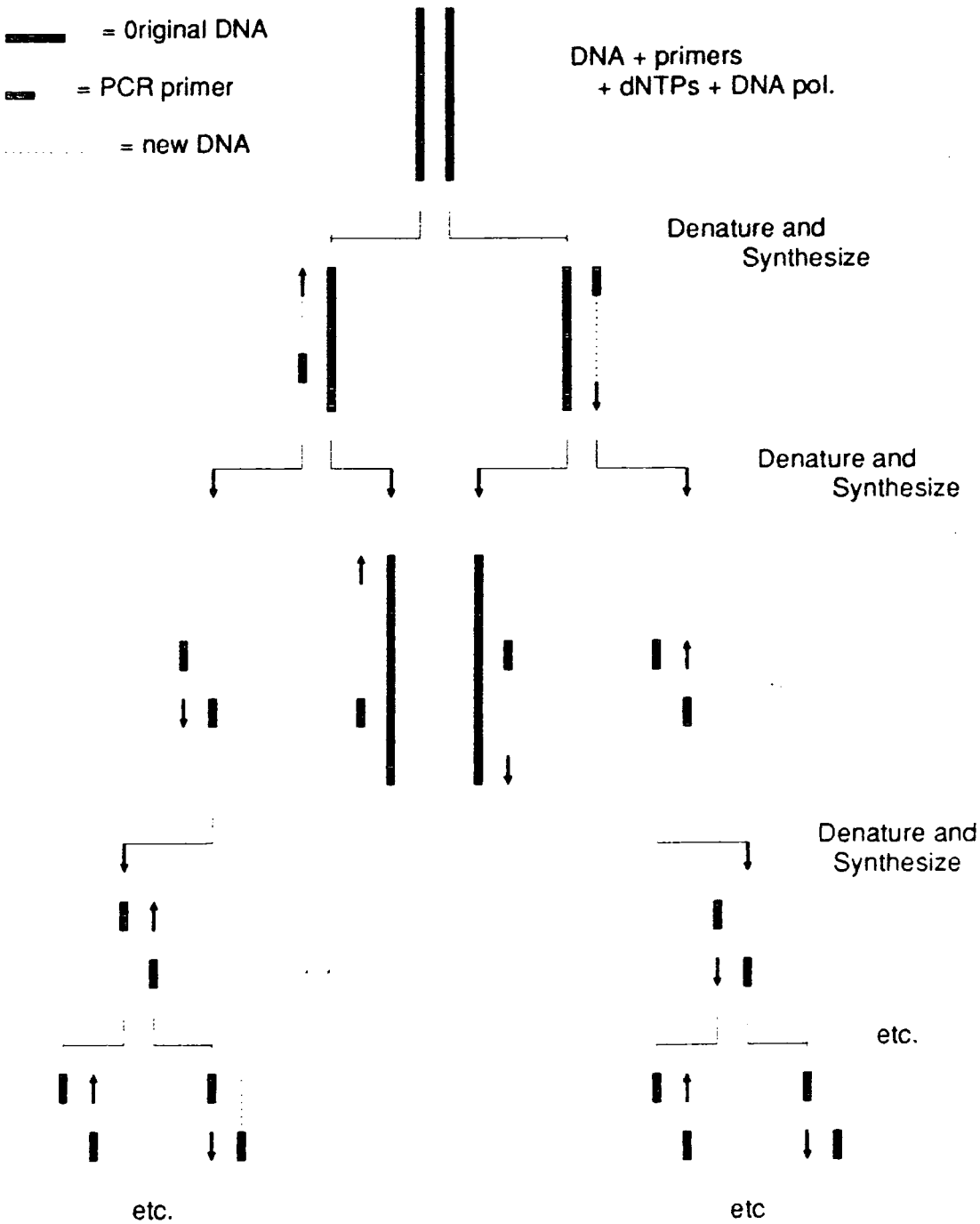

= 0riginal DNA
= PCR primer
= new DNA
DNA + primers
+ dNTPs + DNA pol.
Denature and
Synthesize
Denature and
Synthesize
Denature and
Synthesize
etc.
etc.
etc

Figure 1: Amino acid sequence of the recently isolated *Synechococcus* TX20 MT protein, the base pair sequence of two synthetic oligonucleotides and their proposed annealing sites upon the MT gene locus. A, C, G, T and I represent deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine and deoxyinosine repectively within the probe sequence. X is deoxythymidine 50% of the time and deoxycytidine 50% of the time. Y is deoxyadenosine 50% of the time and deoxyguanosine 50% of the time.

Figure 1: Design of the MT oligonucleotide probes.

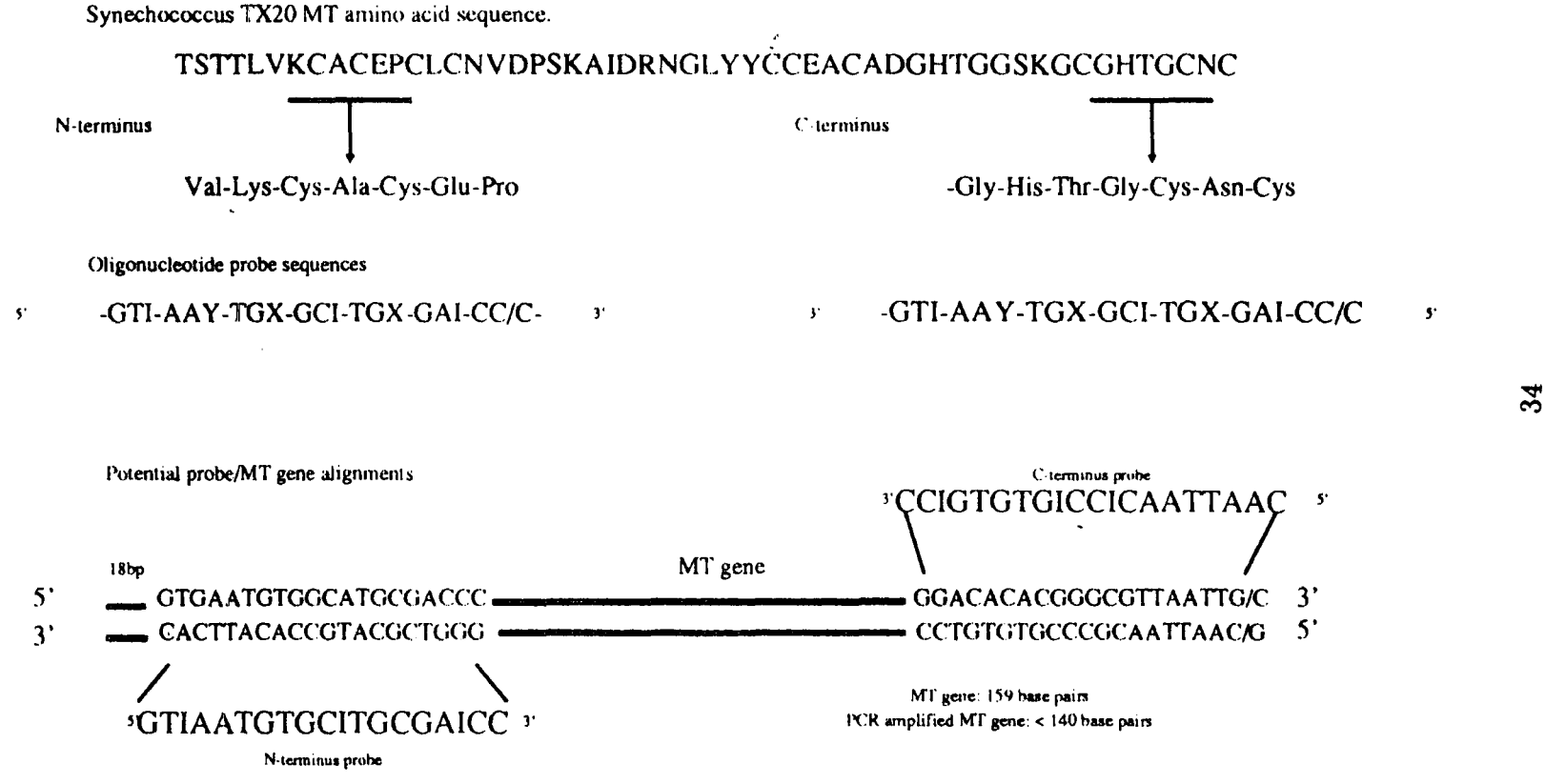


Figure 2: Schematic diagram of the polymerase chain reaction. Only the first two cycles are shown completely. Beginning with the third cycle, the diagram doesn't show the fate of the original DNA and the extension products made from it. Note that the longer primer extensions from the original template can increase additively with each cycle. In contrast, the short discrete, primer-terminated copies, which first appear in the third cycle, proceed to double with each subsequent cycle and rapidly become the predominant form of the amplification product.

2.0.12 PCR fragment isolation and recovery from agarose gels

The band of DNA of comparible size to the PCR amplified MT gene was excised from the agarose gel, frozen in liquid nitrogen for 15 min, removed and spun for 10 min at 10,000 g through sterile glass wool before the fragment had a chance to melt. The resulting solution was then extracted with 100 μl (equivalent volume) of TE buffered chloroform. The aqueous solution was removed to a separate tube, nucleic acids were precipitated by the addition of 3 M Sodium acetate, 0.1 M Magnesium acetate (pH 5.2) to a tenth of the residual volume plus two volumes of 100% ethanol. The solution was then returned to the liquid nitrogen for 30 min. On removal the Eppendorf was centrifuged at 10,000 x g for 10 min. The DNA pellet was then washed once with 70% ethanol and dried briefly in a desicator, before being resuspended in 10 μl of 10 mM Tris-HCL (pH 7.6) and 1 mM EDTA solution.

2.0.13 Ligation of the potential MT gene amplified sequence

After the PCR reaction and recovery of the PCR reaction products, the DNA was treated with T4 DNA polymerase to create linear strands of DNA with blunt ends or directly ligated into a suitable vector. For polymerisation, 1 μl of the recovered DNA (approximately 100 μg) was added to 2 μl of 10x polymerase reaction buffer, 1 μl of each deoxynucleotide triphosphate (0.5 mM, 4 μl total) and 1 μl of the large fragment DNA polymerase (Klenow fragment, T4 polymerase, 0.5 units μl^{-1}, NBL). The reaction mixture was then incubated at room temperature (22^0C for 15 min). Once completed 25 μl of TE satuarated Phenol and 25 μl of chloroform/isoamyl alcohol (24:1) was added and the solution vortexed. The aqueous solution was removed after centrifugation to briefly seperate the layers, and transfered to a new tube where 3 M sodium acetate was added to a final concentration of 0.3 M. The solution was precipitated by the

addition of two volumes of ice-cold 95% ethanol before freezing in liquid nitrogen for 15 min. On removal the solution was centrifuged at 4^0C for 10 min at 10,000 x g, the supernatant was discarded and the pellet washed with 0.5 ml of 80% ethanol. Care being taken not to disturb the pellet, before centrifuging at 10,000 x g for 1 min, discarding the remaining supernatant and vacuum drying the pellet before resuspending in 6 μl of TE buffer. To ligate the resulting blunt or ragged-ended fragments into a vector (pUC18), approximately 1 μg^{-1} of the vector was combined with 100μg of amplified DNA, 3 μl of Ligase Kinase buffer, 3 μl of 10 mM ATP and 1 μl of T4 DNA Ligase for blunt ended fragments). The reaction mixture was then incubated at 22^0C for 12 h and phenol/chloroform extracted as above. Due to the low annealing frequency normally experienced between blunt ended fragments and the blunt ended linear plasmid site during ligation, the level of Ligase had to be increased to compensate for the poor efficiency of this reaction: The K_m for the activity of T4 ligase on blunt ended fragments is nearly 100x higher than its K_m on DNA with cohesive ends. During blunt ended ligation a fraction of the plasmid blunt ends will reanneal. Also due to the high concentration of fragments to be cloned many recombinant plasmids will contain more than one insert.

2.0.14 Transformation into pUC18

DH5α-*E. coli* was transformed with the pUC18-PCR amplified fragment. The competent cells were grown up for 12 h on 2XL media (2 g Bacto Tryptone, 1 g Bacto Yeast extract and 0.1 g of NaCl, adjusted to pH 7.0, autoclaved and 1 ml of 20% sucrose added per 100 ml) at 30^0C. Upto 40 ng of DNA (dissolved in 100 μl of ligation buffer or TE) was added to each 200 μl of cells. The cells were then cold-shocked on ice for 30 min before being placed in a 37^0C water bath for a further 5 min. The mixture of cells and DNA was then diluted to a volume of 4 ml with 2XL broth (prewarmed to 37^0C and shaken gently at 37^0C for a 1-2

hours before being plated onto 2XL/ampicillin/X-Gal plates with 1.5 g Bacto agar/100 ml of media. Colonies were allowed to grow for 12 h at 37^0C.

2.0.15 Plasmid miniprep of pUC18 transformants

Colonies were picked and grown up for 12 h in 2XL media (+ 20 μl ampicillin) at 37^0C with constant agitation. Approximately 1.5 ml of the bacterial solution was pipetted into an Eppendorf and centrifuged for 1 min, before being resuspended in lysis buffer (8% sucrose (v/v), 0.5% Triton-X (v/v), 50 mM EDTA (pH 8.0) and 10 mM Tris-HCl (pH 8.0)). To this buffer 25 μl of freshly prepared lysozyme (10mg/ml, Sigma) in TE buffer was added, before vortexing for 3 min. The tube was then incubated in a boiling water bath for 90 s before being centrifuged for 10 min. After centrifugation the bacterial pellet was removed and discarded. To the supernatant 0.7 ml of a saturated Sodium Iodide solution was added (90.8g NaI and 1.5g $NaSO_3$ (0.5 g added afterwards to saturate the solution and act as an antioxidant) in 100 ml of water. The stock solution was then filter sterilized and stored in the dark at 4^0C. Both agarose and DNA are soluble in high concentrations of NaI; and in NaI, glass binds DNA. Previously prepared glass fines were used (Vogelstein and Gillespie., 1979). The glass fines were shaken to resuspend and 5 μl of the slurry was added. The DNA was bound onto the glass by incubating the mixture at room temperature for 30 min, before spinning for 15 s at 10,000 x g. The supernatant was removed and the fines were resuspended in 1 ml of 70% ethanol (70% ethanol/30% TE buffer). The solution was recentrifuged for 15 s, the ethanol removed and resuspended in 30 μl of TE buffer and incubated at 37^0C to release the DNA.

2.0.16 Preparation of a hybridization probe

One 1 μg of the template (synthetic oligonucleotides) was used with 20 μl of

water. Heated to 100^0C in a boiling water bath for 5 min and chilled in ice-cold water. Ten microlitres of BSA (50 mg/ml) and 20 μl of 5x random primer buffer (0.25 M Tris-HCl (pH 8.1), 10 mM dithiothreitol, 25 mM $MgCl_2$ and 0.2 M KCl) were added, followed by 2 μl of a 2 mM solution of each unlabelled dNTP is added, plus 250 pmoles (100 μCi) [α-^{32}P] dNTP (sp.act >400 Ci/mM) and 4 μl of Klenow fragment (Sigma). The reaction mixture was made up to 100 μl with water. Mixed and incubated for 2 h. Then 2 μl of 0.5 M EDTA was added and the solution was passed through a column of G-50 Sephadex to separate unlabelled dNTPs from labelled DNA. Approximately 30% of the [α-^{32}P] dNTP should have been incorporated into the DNA.

2.0.17 Lysis and Incorporation of bacterial colonies onto nitrocellulose

Bacterial colonies were grown for 12 h and smeared onto nitrocellulose filters placed on fresh plates of 2XL agar. They were then allowed to grow up for 48 h before the filter was removed and laid on top of a 10% SDS solution. Care was taken at all times not to allow the top side of the filter to become saturated. After 10 min the filter was removed and floated on top of 0.5 M NaOH and 1.5 NaCl for a further 10 min. This was subsequently repeated with 1 M Trisma (pH 8.0) and 1.5 M NaCl solution. The filter was then removed placed carefully between a sheet of 3MM Whatmans Filter paper and baked in a glass oven for 1 hr and 2 hrs in a 80^0C vacuum oven. Controls added to the filter paper included pUC18, pUC18 containing *E.coli* colonies and re-amplified MT.

2.0.18 Hybridization of bacterial colony and "Southern blotting "

"Southern blotts " of electrophoresis gels were produced by the method of Maniatis (1982). Southern blots and *in situ* colonies were first floated on the surface of 6x SSC (20x SSC: 173 g of NaCl and 88.2 g of sodium citrate in 1 l (pH

7.0), sterilized by autoclaving) until the filter wetted from beneath. The filter was immersed in the solution for 2 min. The filters were placed in a heat sealable plastic bag and 10 ml of prehybridisation fluid (6x SSC, 0.5% SDS, 5x Denhardt's solution and 100 $\mu g\ ml^{-1}$ denatured, salmon sperm DNA) preheated to 68^0C was added. Any air was removed from the bag before sealing and incubating for 4 h submerged in a 68^0C agitated water bath. The bag was removed and as much as possible of the prehybridization fluid squeezed out. Using a Pasteur pipette hybridization solution (6x SSC, 10 mM EDTA, ^{32}P-labelled denatured probe DNA, 5x Denhardt's solution (50x: 5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g BSA in 500 ml water), 0.5% SDS and 100 ug/ml denatured salmon sperm DNA) was added, using just enough to keep the filter wet (50 $\mu l\ cm^3$ of filter). Any air was removed and the bag resealed and incubated at 68^0C for 16 h. The filter was latter removed and placed immediately into a 2x SSC and 0.5% SDS solution at room temperature for 5 min. The filter was then transferred to a 2x SSC and 0.1% SDS solution and incubated at 68^0C for 2 hours in a gently agitated water bath. The filter was then dried at room temperature on a sheet of Whatman 3MM paper, wrapped in Saran Wrap and applied to X-ray film to obtain an autoradiographic image (Maniatis, 1982).

Chapter III

RESULTS

3.0.1 Cd-binding ligands in *A. nidulans*

Growth of the cyanobacteria was recorded by measuring the absorbance of the photosynthetic pigments (OD at 670 nm) as an indication of cell density. Figure 3 presents the data on the growth kinetics of a cadmium-sensitive culture in the presence of cadmium. The addition of 5 μM cadmium on day 7 inhibited the growth of the cells within 48 h. Although no indication of cell death was found, the results suggest that the cells had ceased to divide. At 1.5 μM cadmium the culture continued to divide for a further 72 h before a decline in absorbance was observed. The selection of a cadmium-tolerant strain was slow but relatively simple and was achieved through the stepwise selection of isolates through staggered increases in cadmium concentration. Large spontaneous increases in resistance were not observed and culture flasks often required several re-inoculations before a tolerant culture was established. No significant difference in this study was detected between cadmium-tolerant and sensitive cultures with regard to growth in the absence of cadmium.

It was observed, however, that prolonged exposure to inhibitory levels of cadmium gave rise to differences in morphology and pigmentation. Clumping of cells, a normal occurrence during late log phase of growth, was seen to occur much earlier within the growth cycle of cadmium exposed cells. Examination under the light microscope revealed the presence of extra mucilage upon

the cyanobacteria's outer surface. After passage of the extracts through the gel permeation HPLC (SW3000) cyanobacterial pigments separated out amongst fractions 18-30 in cultures exposed to 5 μM cadmium (Figure 4). In cultures exposed to 1 μM cadmium for 20 days, a blue pigment was found to be associated with fractions 25-40. This pigment absorbed strongly in the region of 660-680 nm and may have interfered with sulfide assays. The distribution of cystolic cadmium following the chromatography of extracts from cadmium-sensitive and tolerant cells (Expt A and B) exposed to 5 μM cadmium is shown in Figures 4 and 5. Analysis of the fractions by atomic absorption spectrophotometry revealed that the cadmium was distributed between three distinct peaks. Approximately 50-80% of the cadmium, respectively, was associated with a low molecular weight complex resolved from the peaks of void-associated and 2-mercaptoethanol bound cadmium (Tables 6 and 7). The peak fractions centred between 42-43 accounted for approximately 15% of the recovered cadmium within both cultures. The retention time for this material was similar to that reported previously for cadmium-(gammaEC)$_n$G complexes (Robinson *et al.*, 1988) and is designated putative (gammaEC)$_n$G. A cadmium-tolerant culture was grown for 20 days in media containing a sublethal concentration of cadmium. When compared by HPLC analysis to a cadmium-sensitive culture grown under the same conditions, the profiles of the cystolic cadmium were notably different (Figure 6). The profile of the cadmium-tolerant culture was comparable to those previously obtained in expts A and B. Fifty-five percent of the cadmium was associated with the low molecular weight fraction tentatively thought to be (gammaEC)$_n$G. This contrasted with the HPLC profile of the cadmium-sensitive culture . No discernable putative (gammaEC)$_n$G peak was recorded, but 56% of the cadmium was associated with a higher molecular weight fraction resolved between fractions 23-28. A cadmium-sensitive culture grown for 9 d in 1 μM

cadmium with a further 5 μM added on day 7 (Expt. C) contained 44% of the cadmium associated within fractions 23-28. The putative $(gammaEC)_nG$ fractions accounting for only 4% of the cystolic cadmium (Figure 7). To determine if the cadmium binding fractions were constitutively present within the cultures. A cadmium-tolerant strain was grown in the absence of cadmium for 9 d (Figure 8). An equivalent amount of cadmium (200 μg in 100 μl of buffer) to that found to be present within the homogenate of the cells of expt B, was added prior to HPLC analysis. Void and 2-mercaptoethanol-bound cadmium accounted for 70% of the recovered metal, whilst no cadmium was associated within fractions 23-28. Only 14.4% of the cadmium was associated with the putative $(gammaEC)_nG$ fractions, its presence suggesting that there may have been some constitutive cadmium binding ligands present within the cells. However, when compared to the percentage cadmium bound to the putative $(gammaEC)_nG$ in the cadmium-sensitive and tolerant profiles, the results indicate that these cadmium binding ligands are induced by cadmium within 48 h of exposure.

Table 6: Expts A-F; Cadmium exposure of Cd-sensitive and tolerant cultures.

Expt.no.	Cds	Cdt	Cd_{Add}	Cd_{uM}	Cd_{Extra}	Cd_{mM}	Days
A	yes		7	5			9
B		yes	7	5			9
C	yes		1	1	7	5	9
D	yes						9
E	yes		1	1			20
F		yes	1	1			20

Cds= cadmium-sensitive culture; Cdt= cadmium-tolerant culture; Cd_{Add}= The day on which cultures were exposed to cadmium; Cd_{uM}= the amount of cadmium added (μM); Cd_{Extra}= date of further cadmium addition to the culture.

Table 7: Comparison of cystolic metal distribution for cadmium-tolerant and sensitive cultures.

Expt.no	MT-Cd	$(gammaEC)_nG$-Cd	Void Cd	Free Cd
A	2%	49%	10%	39%
B	0	80%	0.6%	16%
C	44%	4%	23.6%	17.3%
D	2%	14.4%	10.7%	59%
E	56%	1%	20.3%	1.3%
F	4%	55%	14%	16%

Figure 3: Growth kinetics of an cadmium-sensitive *A. nidulans* strain in the presence and absence (⊟-) of cadmium. Growth was inhibited within 24 hrs and 72 hrs at 5 μM (⊠-) and 1.5 μM cadmium (◇-). Cadmium was added to the ACM media on day 7 of growth.

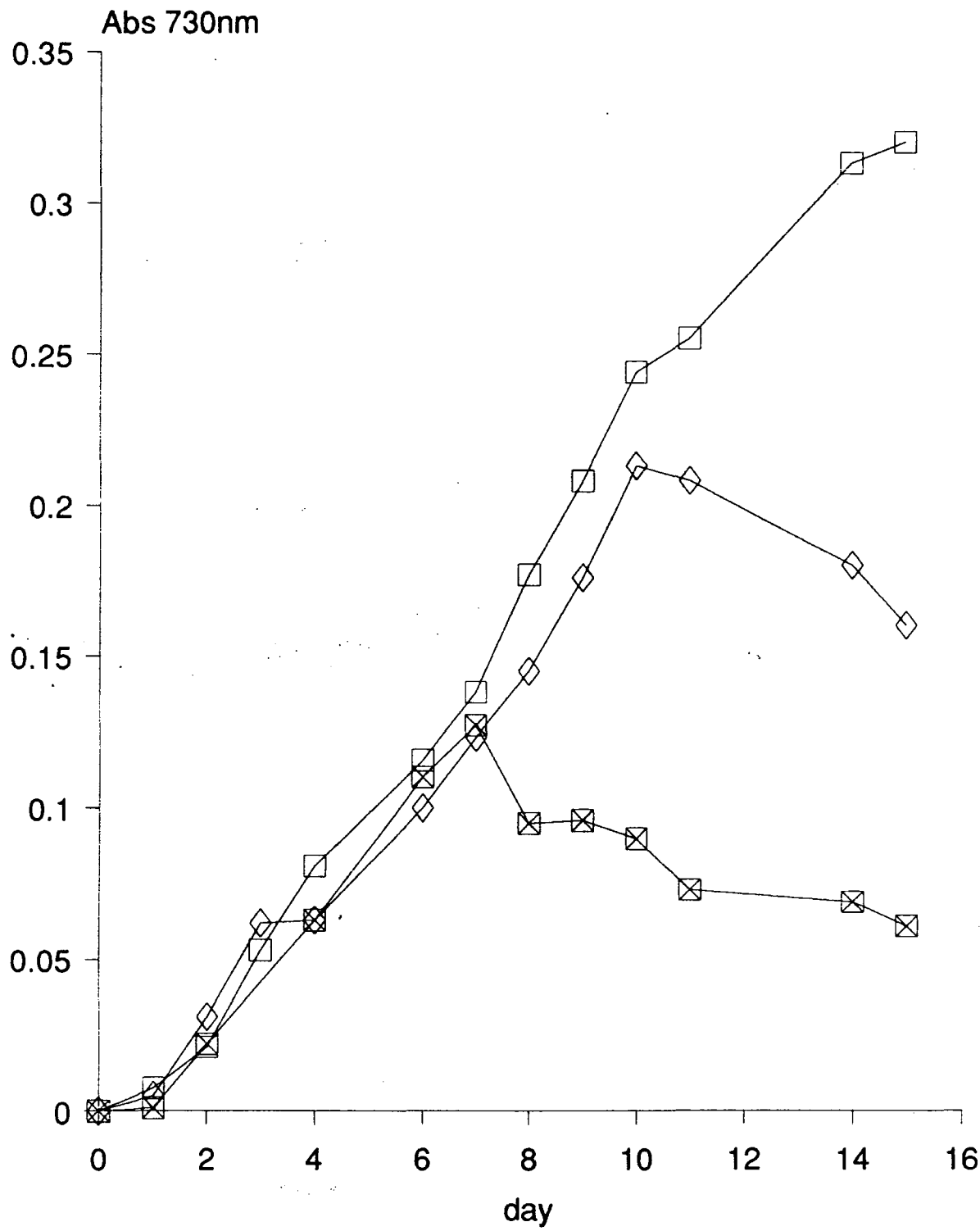

Abs 730nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
12
14
16
day

Figure 4: Distribution of cystolic cadmium in a cadmium-sensitive culture of *A. nidulans*. Cells were exposed to 5 μM cadmium for 48 h on day 7 of growth. Extracts of cells were centrifuged and the soluble portion of the extract was analysed by passage through a SW 3000 HPLC column. (⊟) concentration of cadmium; (◇) photosynethetic pigments; A= void volume associated cadmium; B= β -mercaptoethanol associated cadmium.

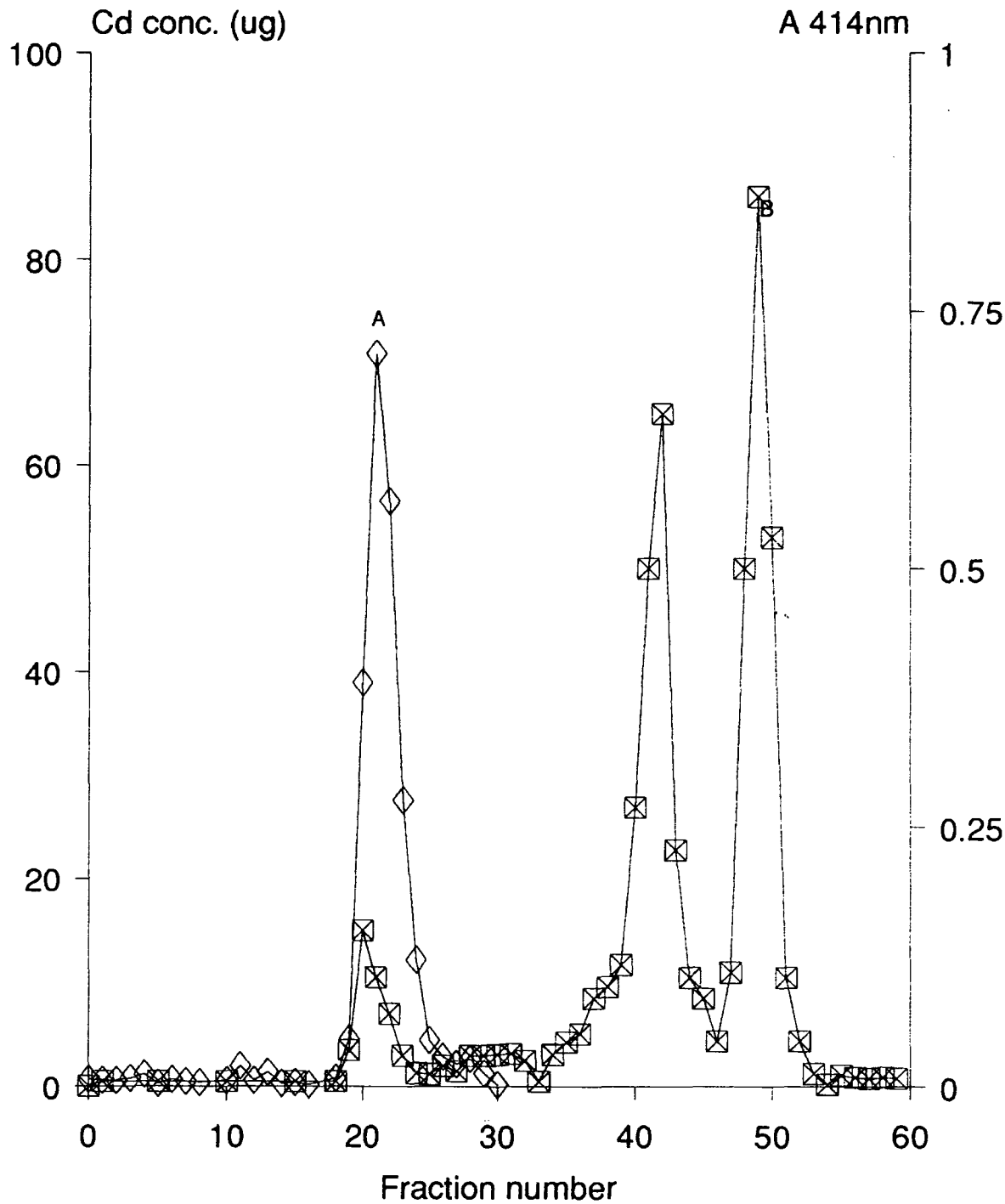

Cd conc. (ug)
A 414nm
100
80
60
40
20
0
1
0.75
0.5
0.25
0
A
B
0
10
20
30
40
50
60
Fraction number

Figure 5: Distribution of cystolic cadmium in a cadmium-tolerant culture of *A. nidulans*. Cells were exposed to 5 μM cadmium for 48 hrs on day 7 of growth. Extracts of all cells were analysed by passage through a SW 3000 HPLC column. (⊟-) concentration of cadmium;

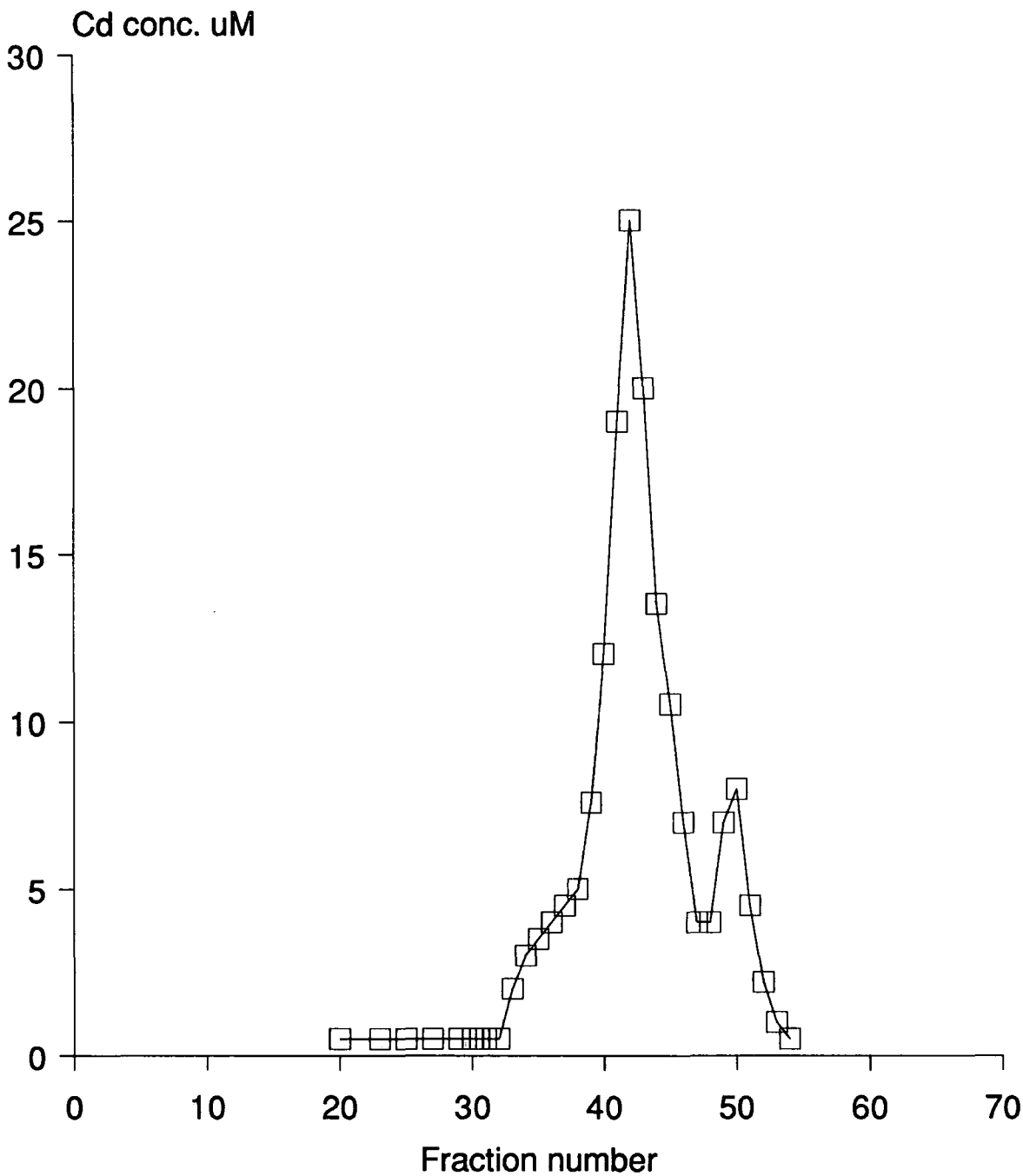

Cd conc. uM
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
Fraction number

Figure 6: Distribution of cystolic cadmium in cadmium sensitive (-◆-) and tolerant (-⊟-) cultures maintained in 1 μM cadmium for 20 d. Extracts of cells were centrifuged and the soluble portion of the extract was analysed by passage through a SW 3000 HPLC column.

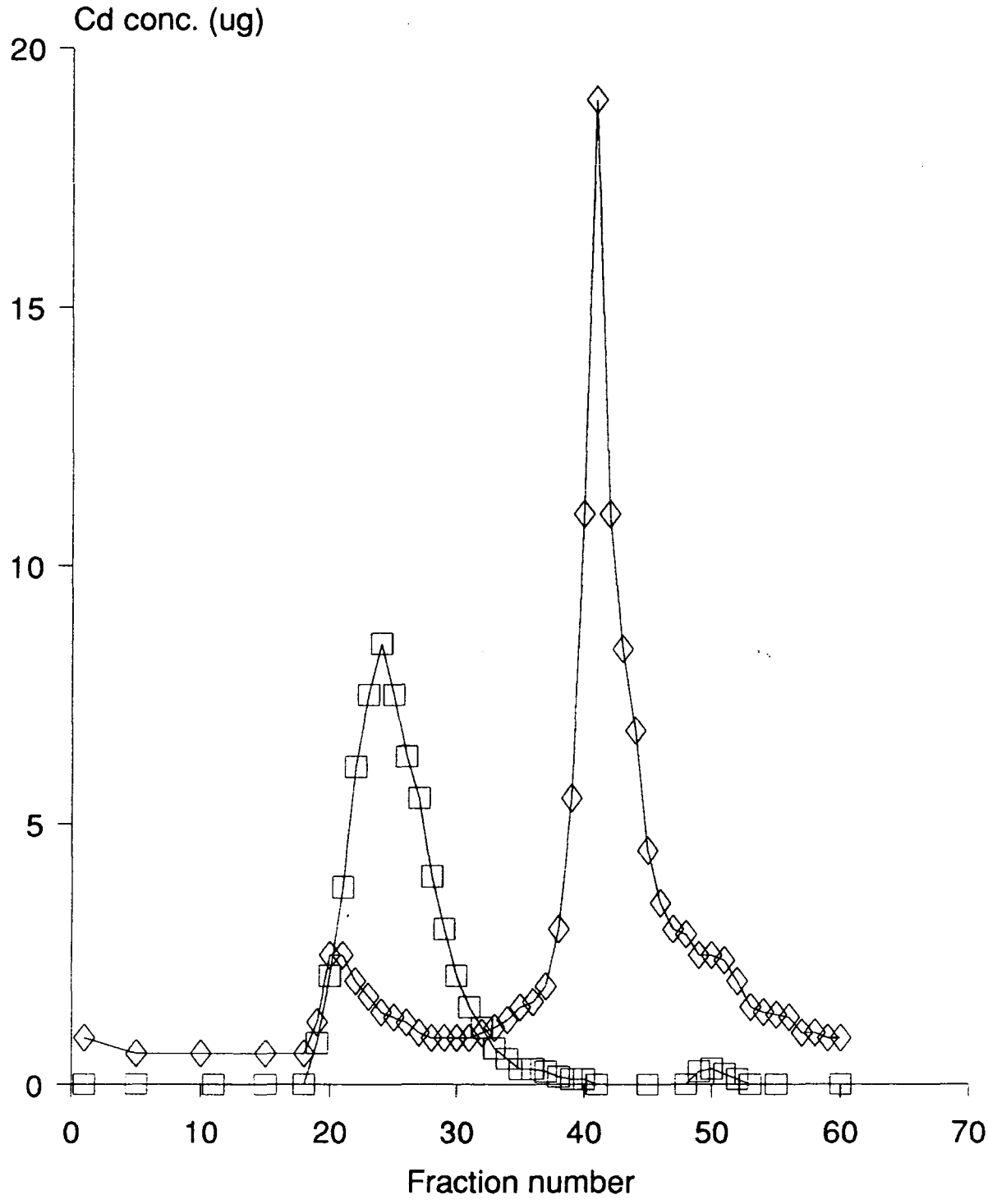

Cd conc. (ug)
20
15
10
5
0
0
10
20
30
40
50
60
70
Fraction number

Figure 7: HPLC chromatography of cadmium-binding ligands in a cadmium-tolerant culture of *A. nidulans* exposed to 1 μM cadmium for 7 days before a further 5μM cadmium was added for 48 hours. Extracts of the cells were centrifuged and the soluble fraction analysed by passage through a SW 3000 HPLC column. (⊟) cadmium concentration; (⊠) sulfide concentration.

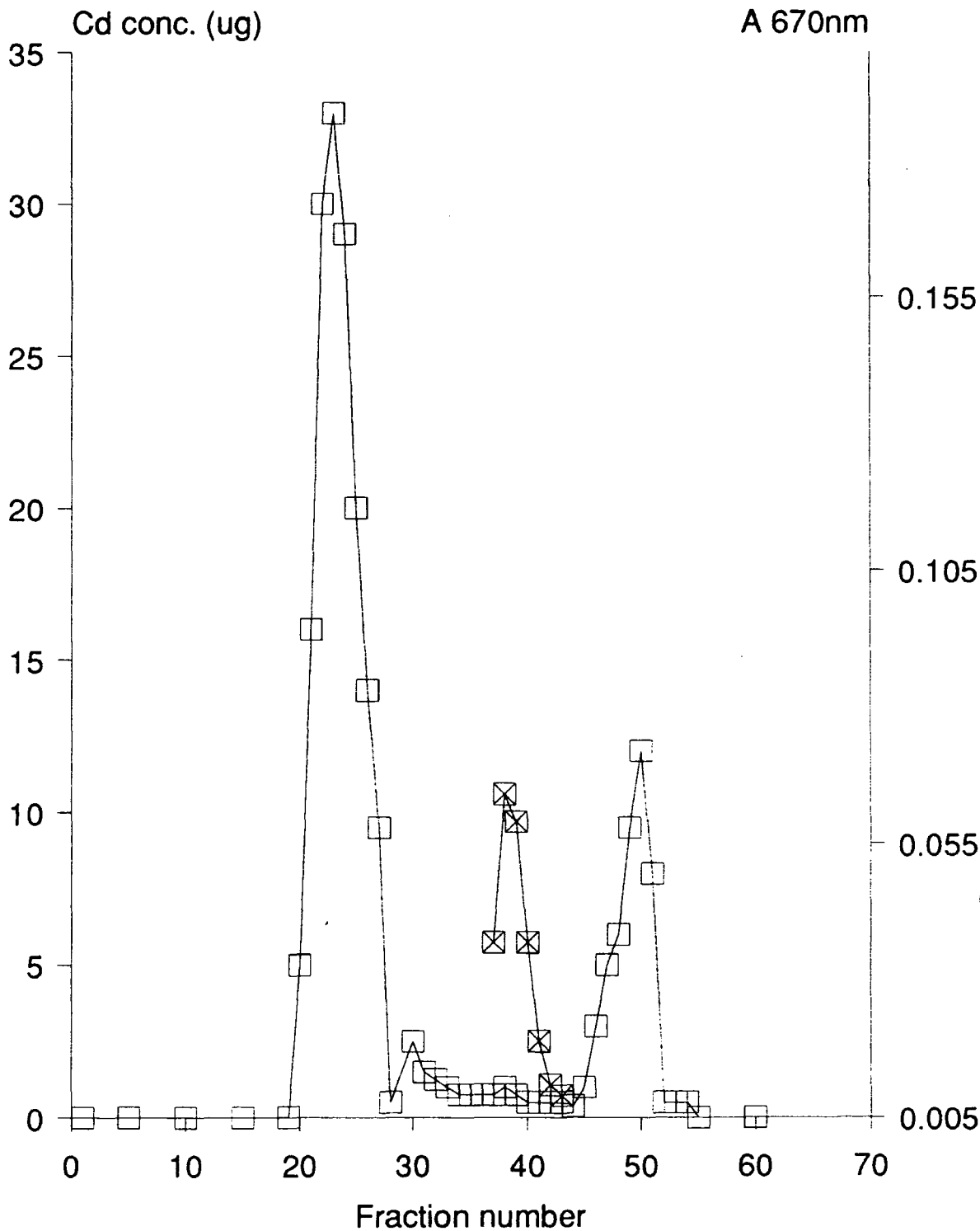
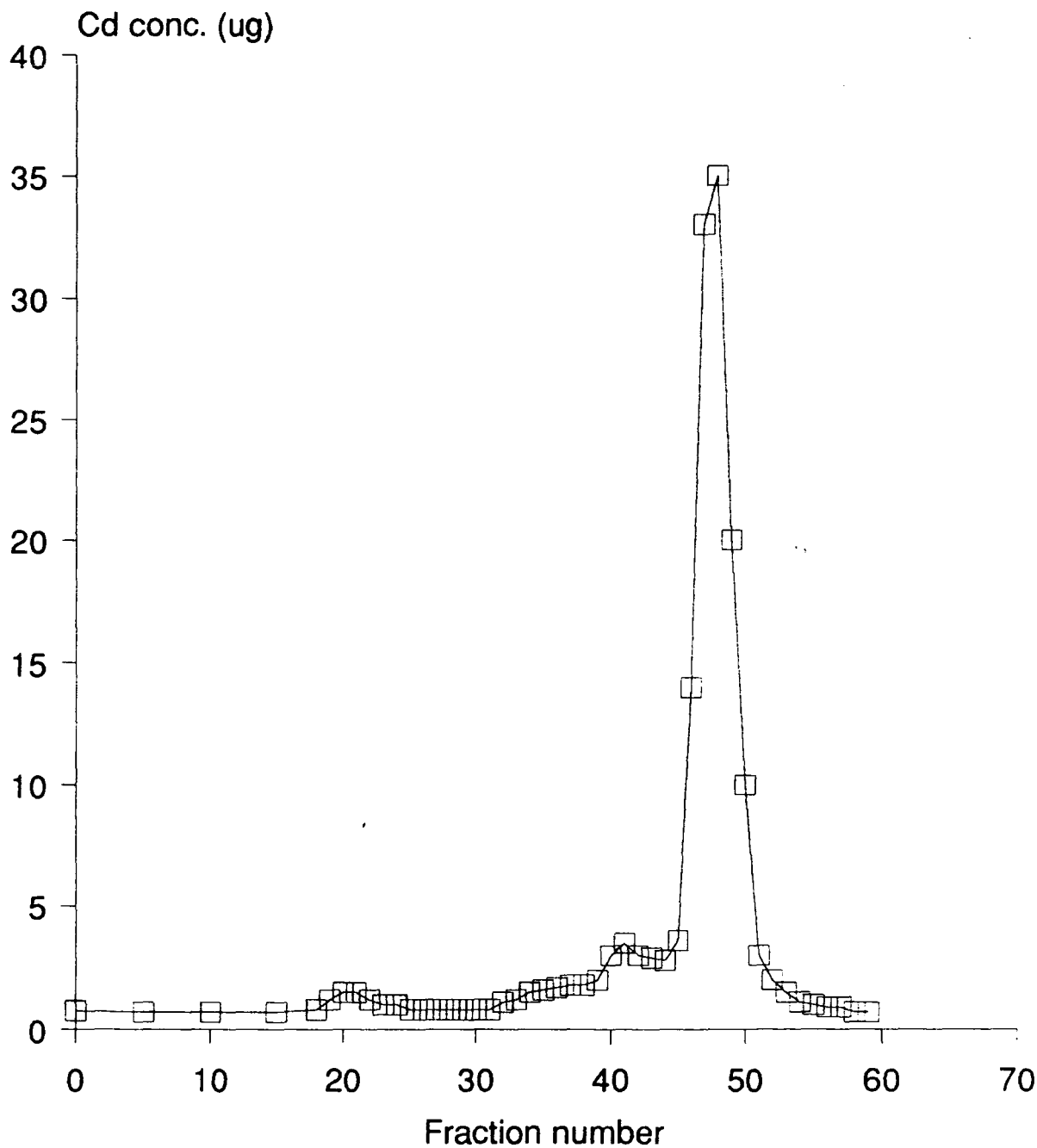

Cd conc. (ug)
A 670nm
35
30
25
20
15
10
5
0
0.155
0.105
0.055
0.005
0
10
20
30
40
50
60
70
Fraction number

Figure 8: HPLC profile of a cadmium-sensitive *A. nidulans* culture grown for 9 d in the absence of cadmium. Extracts of the cells were centrifuged and 200 μg of cadmium was added to the soluble portion of the extract. The extract was then analysed by passage through a SW 3000 column to determine if constitutively produced cadmium-binding ligands were present within the sample. (⊟) cadmium concentration.


Cd conc. (ug)
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
Fraction number

3.0.2 Characterization of the two cadmium-binding ligands

The putative (gammaEC)$_n$G fractions were collected and subjected to analysis for the presence of free sulfide. Free sulfide has been documented as occuring within the CdBPs of *E. gracilis* and *S. pombe* (Weber *et al.*, 1987; Murasugi *et al.*, 1983; Hayashi *et al.*, 1986). Both complexes are known to be composed of aggregates of (gammaEC)$_n$G and it was decided to investigate whether S^{2-} is associated with Cd-binding ligands in *A. nidulans*. The assay revealed that a concentration of free sulfide was centered around fraction 41 (Table 8). The fractions were partially occluded by the presence of a blue pigment that absorbed in the region of 670 nm, the same wavelength used in the sulfide assay. To correct for absorbance due to the pigment, the absorbance of the fractions were read prior to the sulfide assay and the result subtracted from the final reading. Analysis of the high molecular weight associated cadmium shown in Figure 4 had indicated that it was resolved from the photosynthetic pigments and eluted in advance of the pigments. However, in comparison, expt E reveals that although 20% of cystolic cadmium is associated with the void fraction, 56% of the cadmium is resolved behind the photosynthetic pigments (Figure 9). The fractions were subjected to the Bradford assay to detect the presence of protein. The results indicated that although the majority of proteins were resolved with the void volume of the column, it could not be discounted that the cadmium ions were bound to lower molecular weight proteins travelling behind the main protein body (Figure 10).

One of the key features of metallothioneins is the mercaptide bond. The cadmium-thiol bond forms a chromaphore that can be detected at an OD of 255 nm. In the presence of excess H^+ ions the cadmium is displaced and lost from the molecule. The displacement of the cadmium ion results in the chromophore

bond being lost and a reduction in the OD at 255 nm. Thus pH titrations can be used to assess the strength of metal-binding. Fractions 23-28 were collected and pooled and the optical density of the solution read between the range of 200-300 nm. The pH of the sample, initially pH 7 within the column, was reduced by the addition of hydrochloric acid. Aliquots of the putative MT were subjected to progressively lower pH. Increasing acidity was seen to reduce the OD, until at pH 1.5 the cadmium is completely displaced from the putative MT (Figure 11). When the absorbance values of the cadmium-thiol chromophore (A_{255}) is plotted against pH (Figure 12) the characteristic cadmium displacement curve of a MT was produced. The pH of half displacement (i.e. the pH at which half the bound metal had been displaced by protons) was approximately pH 3.5.
In an attempt at further isolation, the material was passed through an ion exchange chromatographic column of DEAE-cellulose. Separation is obtained by utilising the different affinities of solute molecules for the ion exchanger within the column. These affinities can be controlled by altering ionic strength and pH conditions within the column matrix. The first peak eluted marks the start of the linear salt gradient followed by a small broad peak of putative MT (Figure 13a). For comparison the work of Olafson (1979) on the isolation of the *Synechococcus* MT through a similar DEAE column are reproduced below (Fig 13b). The putative MT isolated within *A. nidulans* possesses a similar affinity for the DEAE-sephadex matrix as the *Synechococcus* (strain RRIMP NI) MT-1 protein.

In conclusion these results demonstrate the presence of two putative MTs within *A. nidulans*. Although potentially present within the cell at a low constitutive level both peptides are induced by the presence of cadmium. In adapted cultures, or cells exposed to 5 μM cadmium synthesis of the low molecular weight ligand was observed which had a retention time equivalent to Class III MT, or cadmium-(gammaEC)$_n$G. This ligand was induced within two

days of exposure and was associated with free sulfide. The second putative MT had a greater molecular weight and was only induced within a cadmium-sensitive culture exposed to sublethal levels of cadmium. It was shown to possess a mercaptide chromophore characteristic of MT, a pH of half displacement value around pH 3.5 and a similar affinity on DEAE-Sephadex to another MT isolated within the *Synechococcus* species.

Table 8: Sulfide assay

Fraction no.	A	B	A-B
35	0.071	0.064	0.008
36	0.086	0.048	0.038
37	0.078	0.037	0.041
38	0.057	0.035	0.022
39	0.060	0.029	0.041
40	0.077	0.019	0.058
41	0.082	0.030	0.052
42	0.038	0.018	0.020
43	0.041	0.013	0.028
44	0.026	0.005	0.021
45	0.031		

A= Sulfide assay, B= Absorbance of the fraction at 675 nm prior to sulfide analysis

Table 9: Changes in the uv absorption spectrum of Anacystis MT with increasing pH

Wavelengh (nm)	pH 6	pH 4.5	pH 3.5	pH 2	pH 1.5
220	2.530	2.368	2.350	1.988	1.878
225	2.206	2.060	1.991	1.544	1.527
230	1.908	1.793	1.697	1.187	1.179
235	1.571	1.495	1.388	0.832	0.828
240	1.321	1.266	1.104	0.639	0.628
245	1.192	1.143	1.050	0.567	0.550
250	1.126	1.081	0.992	0.550	0.530
255	1.134	1.087	1.004	0.577	0.562
260	1.058	1.017	0.936	0.554	0.542
265	1.023	0.986	0.909	0.614	0.607
270	0.988	0.955	0.881	0.549	0.537
275	0.945	0.916	0.844	0.535	0.524
280	0.894	0.868	0.797	0.505	0.495
285	0.831	0.808	0.737	0.460	0.453
290	0.754	0.736	0.664	0.397	0.391
295	0.676	0.662	0.590	0.329	0.325
300	0.612	0.601	0.530	0.274	0.270

Figure 9: HPLC analysis of cadmium-binding ligands within a cadmium-sensitive culture of *A. nidulans*. The main cadmium resolved peak (⊟) is passing through the column behind the void associated photosynthetic pigments (⊖). The cultures were exposed to 1μM cadmium for 20 days prior to extraction and HPLC analysis.

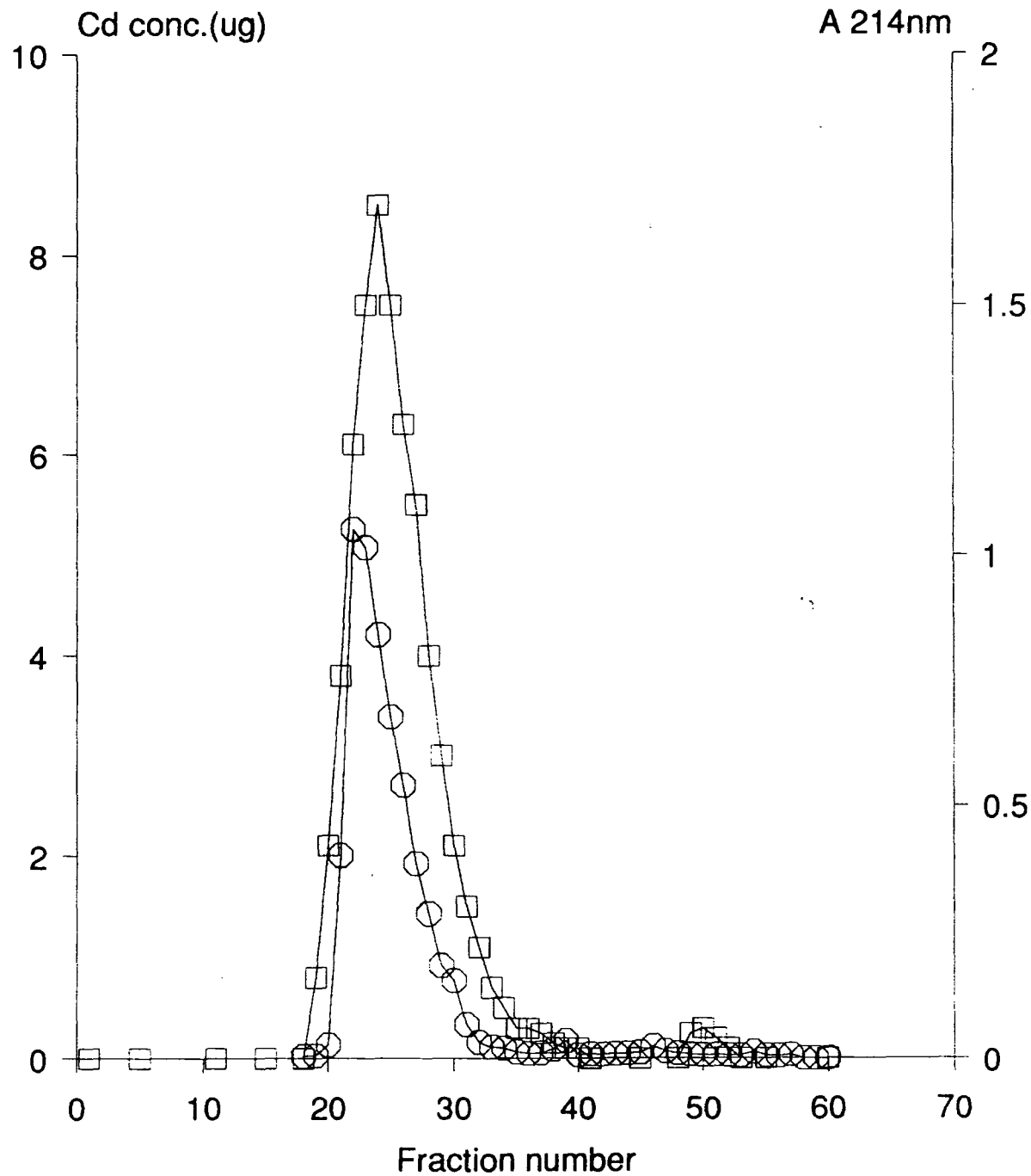

Cd conc.(ug)
A 214nm
10
8
6
4
2
0
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
Fraction number

Figure 10: HPLC analysis of cadmium-binding ligands within a cadmium-sensitive culture of *A. nidulans*. The cadmium (⊟-) is resolved within fractions containing a body of soluble protein, but behind that of the void proteins (⊖-). The cultures were exposed to 1μM cadmium for 20 days prior to extraction and HPLC analysis.

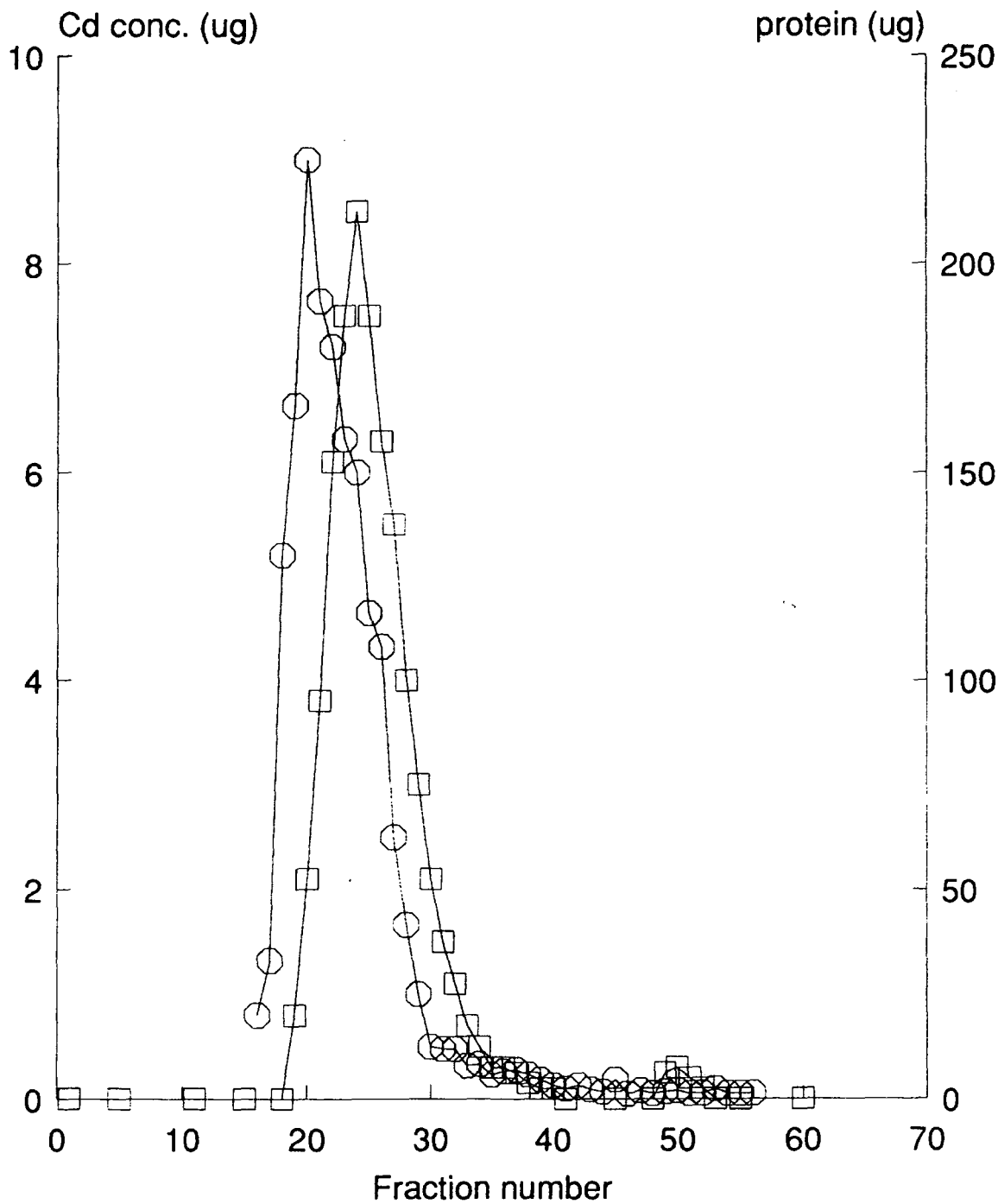

Cd conc. (ug)
protein (ug)
10
8
6
4
2
0
250
200
150
100
50
0
0
10
20
30
40
50
60
70
Fraction number

Figure 11: Ultraviolet absorption spectrum of the *Anacystis nidulans* MT in 10 mM Tris-HCl. Displacement in the spectrum was acheived through changes in absorbance by the mercaptide bond chromaphore with decreasing pH. pH 6 (⊟-); pH 3.5 (◇-) and pH 1.5 (▽-).

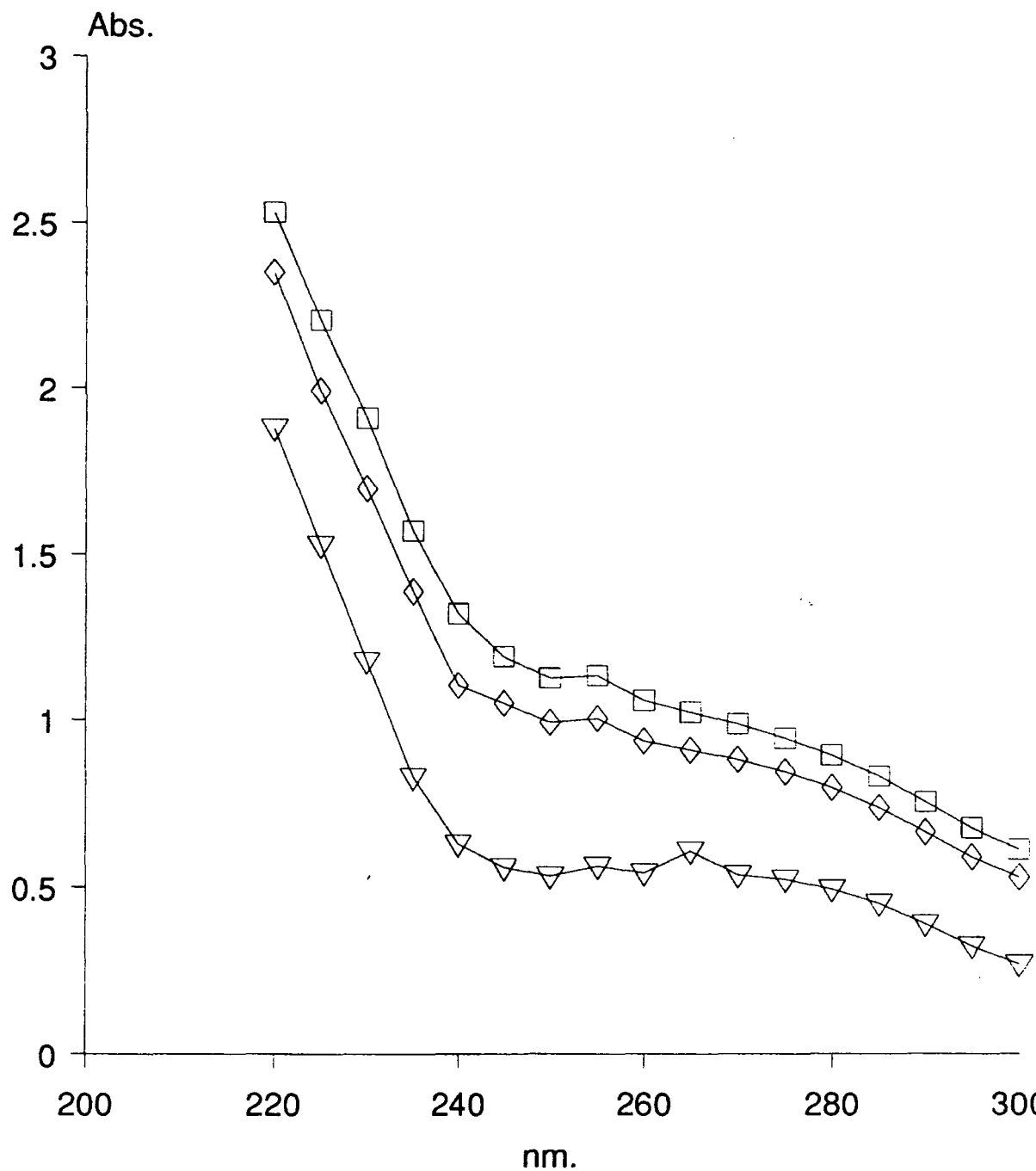

Abs.
3
2.5
2
1.5
1
0.5
0
200
220
240
260
280
300
nm.

Figure 12: Acid titration of the putative *A. nidulans* MT monitored as absorbance at 255 nm (mercaptide bond). The pH of half-displacement value is seen to occur at around pH 3.5 and is similar in value to other previously isolated metallothioneins.

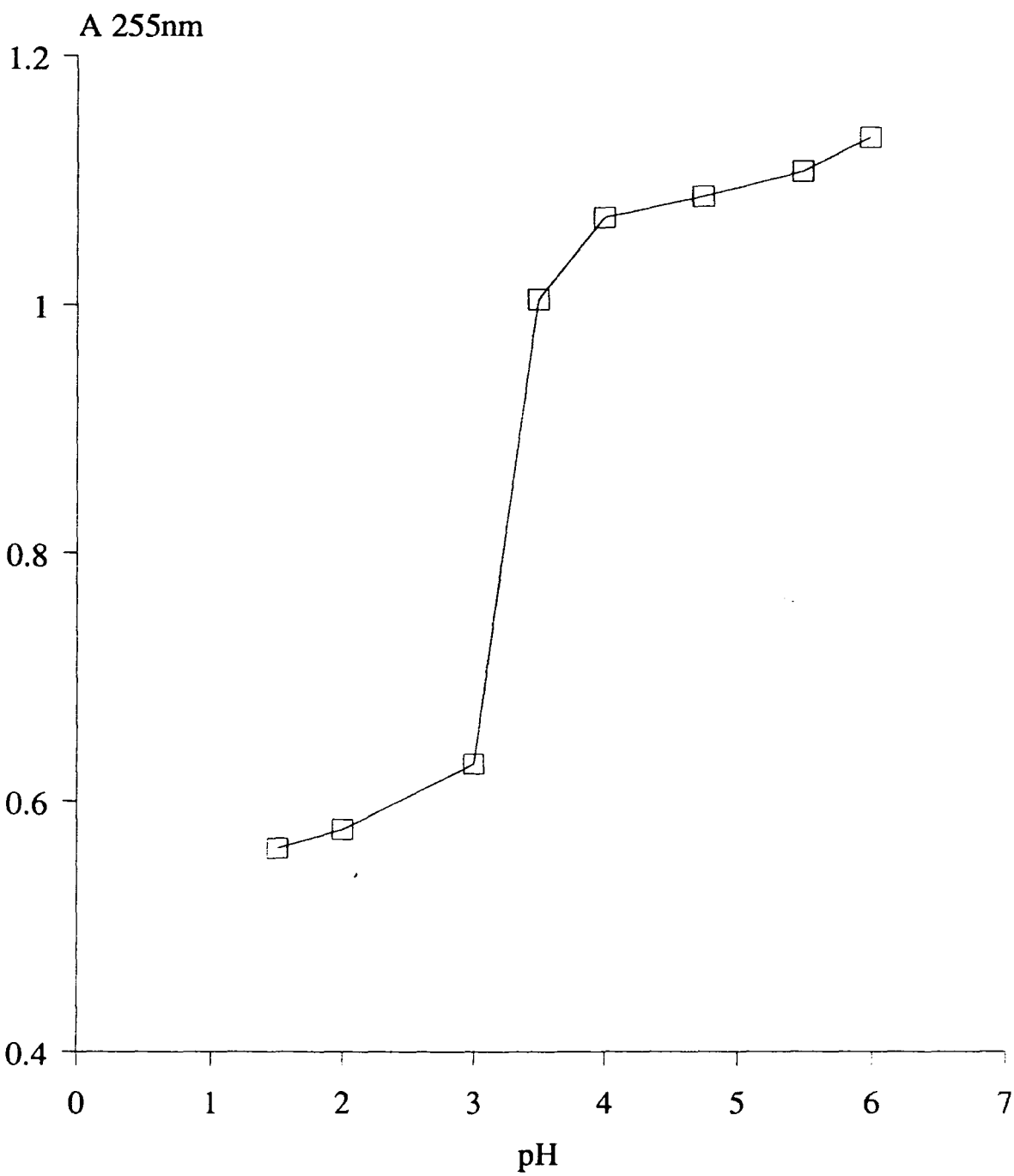

A 255nm
1.2
1
0.8
0.6
0.4
0
1
2
3
4
5
6
7
pH

Figure 13a: DEAE-cellulose ion exchange chromatography of a cadmium-binding ligand from *A. nidulans*. Cell cultures were grown for 20 days in 1 μM cadmium before extraction and analysis. The first peak eluted marks the start of the linnear gradient followed by a small peak of the MT.

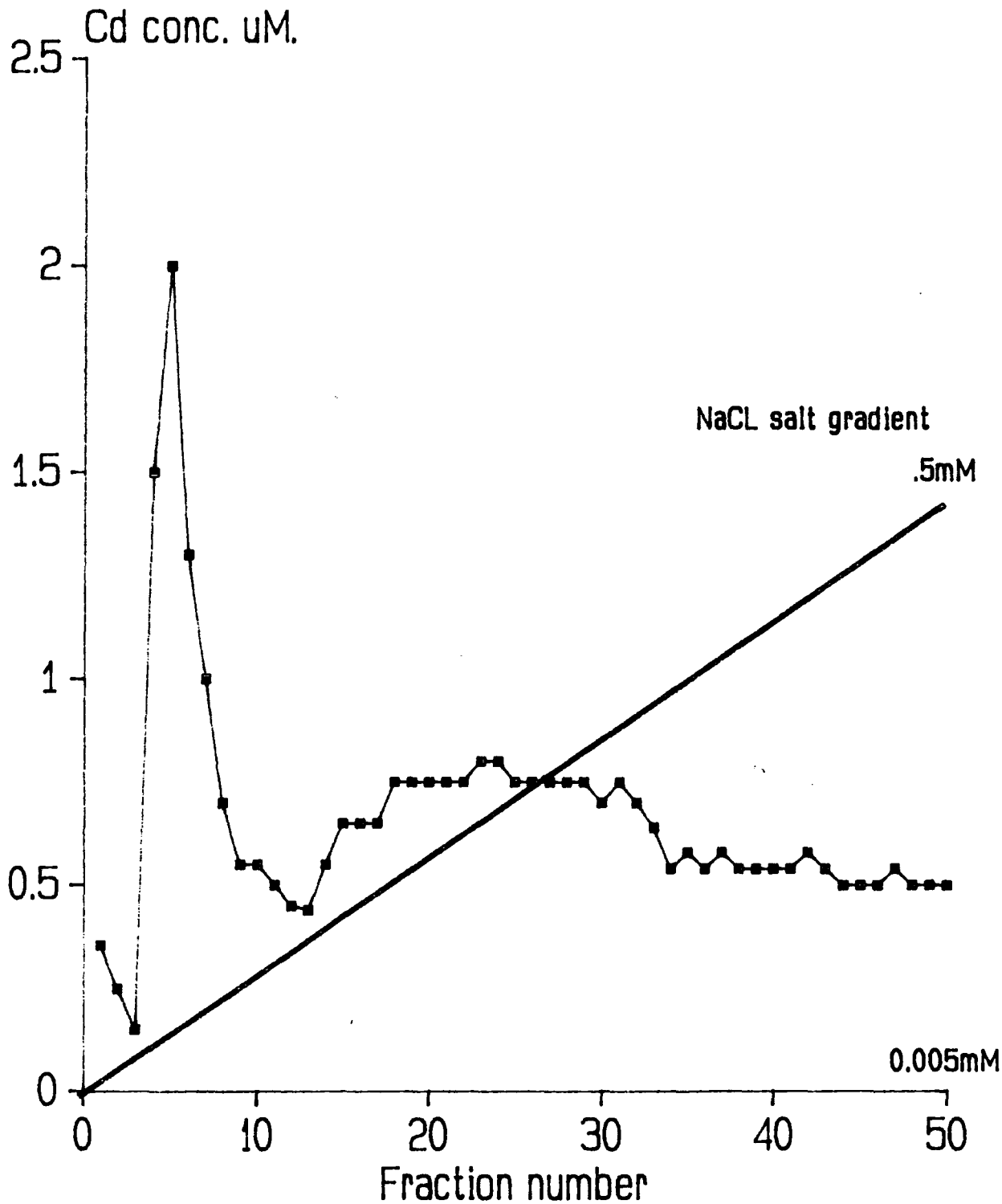
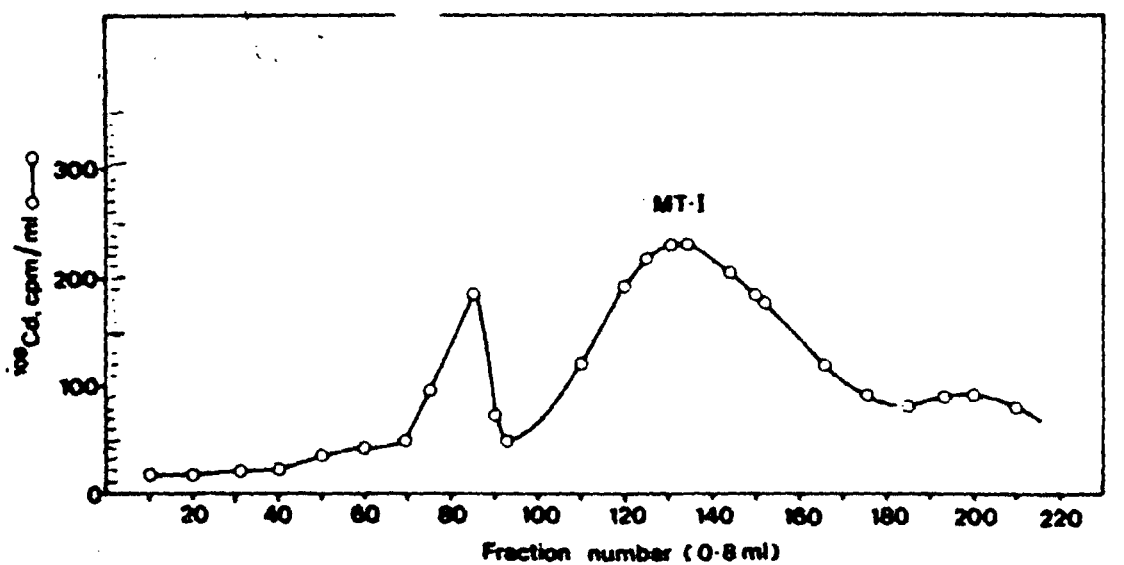

Cd conc. uM.
2.5
2
1.5
1
0.5
0
NaCL salt gradient
.5mM
0.005mM
0
10
20
30
40
50
Fraction number

Figure 13b: DEAE-cellulose ion exchange chromatography of pooled *Synechococcus* sp. MT fractions isolated by isoelectric focusing. The linear gradient (0.05-0.5M) was developed with Tris/HCl buffer, pH 6 in a total elution volume of 2 litres. The *Synechococcus* is apparently identical to *Coccochloris elabens* and was designated *Synechococcus* sp. Naegeli (strain RRIMP NI). In subsequent work, Olafson has purified an equivalent MT from *Synechococcus* TX20, using other seperation techniques (Olafson *et al.*, 1979).



DEAE-cellulose ion exchange chromatography of pooled Synechococcus sp. metallothionein fractions isolated by isoelectric focusing. The linear gradient (.05 - .5 M) was developed with Tris/HCl buffer, pH 8.6 in a total elution volume of 2 l.

3.0.3 PCR amplification of the *A. nidulans* MT gene

Initially the PCR reaction was run for 30 cycles overnight at 44^0C (95^0C for 5 min, then 30 cycles: 44^0C for 3 min, 72^0C for 9 min, 91^0C for 2 min; then a final 72^0C for 18 min). This cycle had previously been successfully used for the amplification of fragments over 1 Kb in size and allowed sufficient time for the complete transcription of the locus by the Taq polymerase (Fordham-Skelton, pers comm.). Examination under uv light revealed a band of DNA running in front of the 226 bp digested pBR322 + Alu 1 marker fragment but behind the 136 bp marker (Plate 1). Further analysis indicated that the band size was approximately < 150 bp (Plate 2). The control a distantly related *Synechococcus* sp. amplified under similiar conditions with the same two extention primers, had not worked. Due to the size of the expected reaction product it was considered unnecessary to have a 9 min polymerase transcription period and this was reduced to 30 cycles of 45^0C for 3 min. In latter trials the amount of oligonucleotide per reaction was reduced from 30 μl to 12 μl. A problem of overheating within the heating block was also corrected, the reduced amplification of the initial reaction when compared to latter experiments may have been due to Taq Polymerase inactivation. The reaction was repeated at 47^0C and the products analysed. The presence of several other DNA bands suggested that the primers had annealed to several loci within the genome and that transcription fragments of differing bp size had been formed and amplified. DNA band sizes corresponding to 140, 215, 265 355 and 405 bp were found when analysed latter on a 3.75% NuSieve GTG agarose gel. Re-amplification of 5 μl of the reaction mixture resulted in one band of 140bp being resolved (Plate 3). Analysis also revealed the presence of DNA fragments running between the primers and 140 bp fragment. To increase the stringency of the PCR, the annealing temperature was raised stepwise from 47^0C to 55^0C , 60^0C and 65^0C (Plates 4 and 5). The

size and position of the various fragments were recorded (Figs 14a, 14b, 14c and 14d). Increasing the reaction temperature reduced the number of fragments from nine at 47^0C , to four at 65^0C when analysed on 3.75% agarose.

The degree to which a fragment had been amplified was indicated by its flouresence under uv-light. Four DNA bands of approximately 600, 265, 215 and 140 bp were the predominant reaction products at 45^0C , although nine bands were recorded in total. At 55^0C three bands of 680, 265 and 140 bp were dominant. Whilst two dominant bands of 620 and 140 bp were recorded at 60^0C only the 140 bp potential MT gene product was predominant at 65^0C with three other faint bands (Table 10).

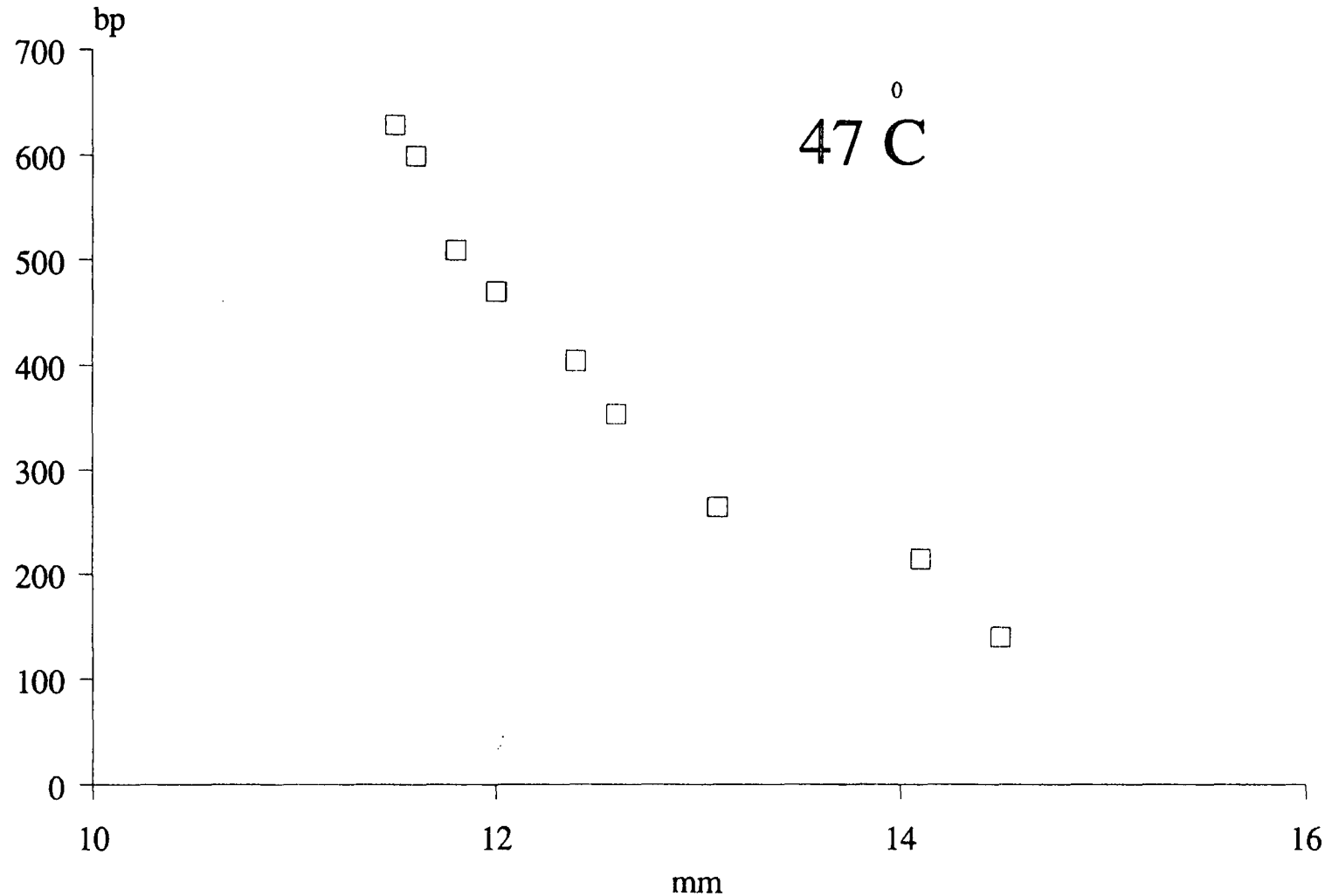
In conclusion, although the *Synechococcus* TX20 gene product is thought to be encoded by a 159 bp codon sequence. The size of the amplified gene product was not expected to exceed 150 bp due to the N-terminal annealing site of the $5' \mapsto 3'$ probe. In all the PCR reactions the dominant amplified fragment was approximately 140 bp in size. The effect of increasing the annealing temperature reduced the number of heterologous DNA fragments but did not inhibit amplification of the 140 bp fragment. The fact that a 140 bp fragment was recovered from the genomic DNA of *A. nidulans* but not from the DNA of a distantly related species adds further proof that *A. nidulans* is synonomous with *Synechococcus* TX20, that the MT isolated may be that reported by Olafson (1988) and that we may have identified the first prokaryotic MT gene. The non-amplification of DNA sequences from *Synechococcus* 562 raises the possibility that either this species does not contain the Class II MT or that variations within the MT amino acid/codon sequence may exist between species. The recovery of the fragment of 140 bp indicated that it may be the MT gene, only the isolation and sequencing of the fragment will determine if this is so.

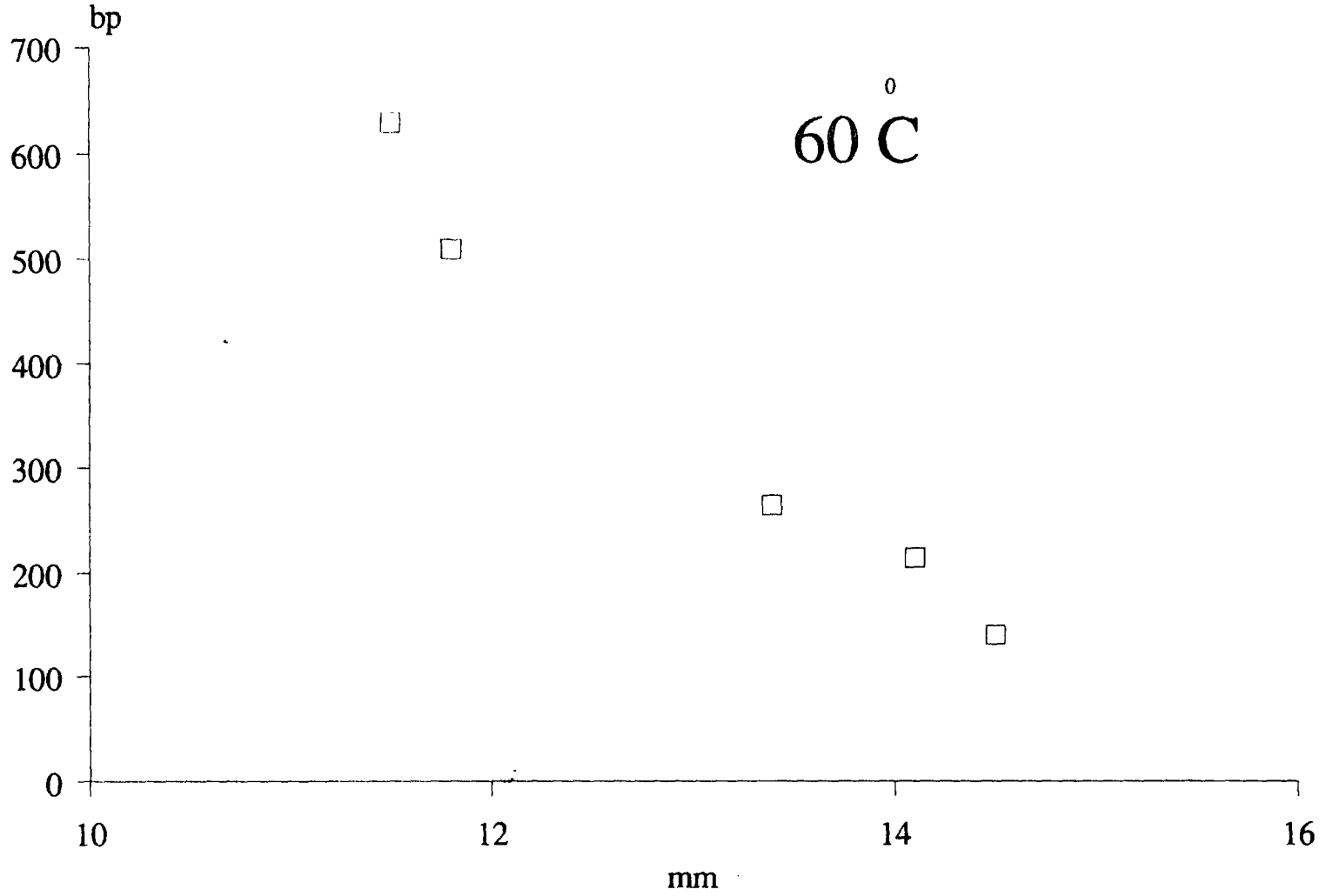
Table 10: The approximate base pair size of the reaction products obtained through the PCR amplification of the *A. nidulans* MT gene locus at different primer annealing temperatures.

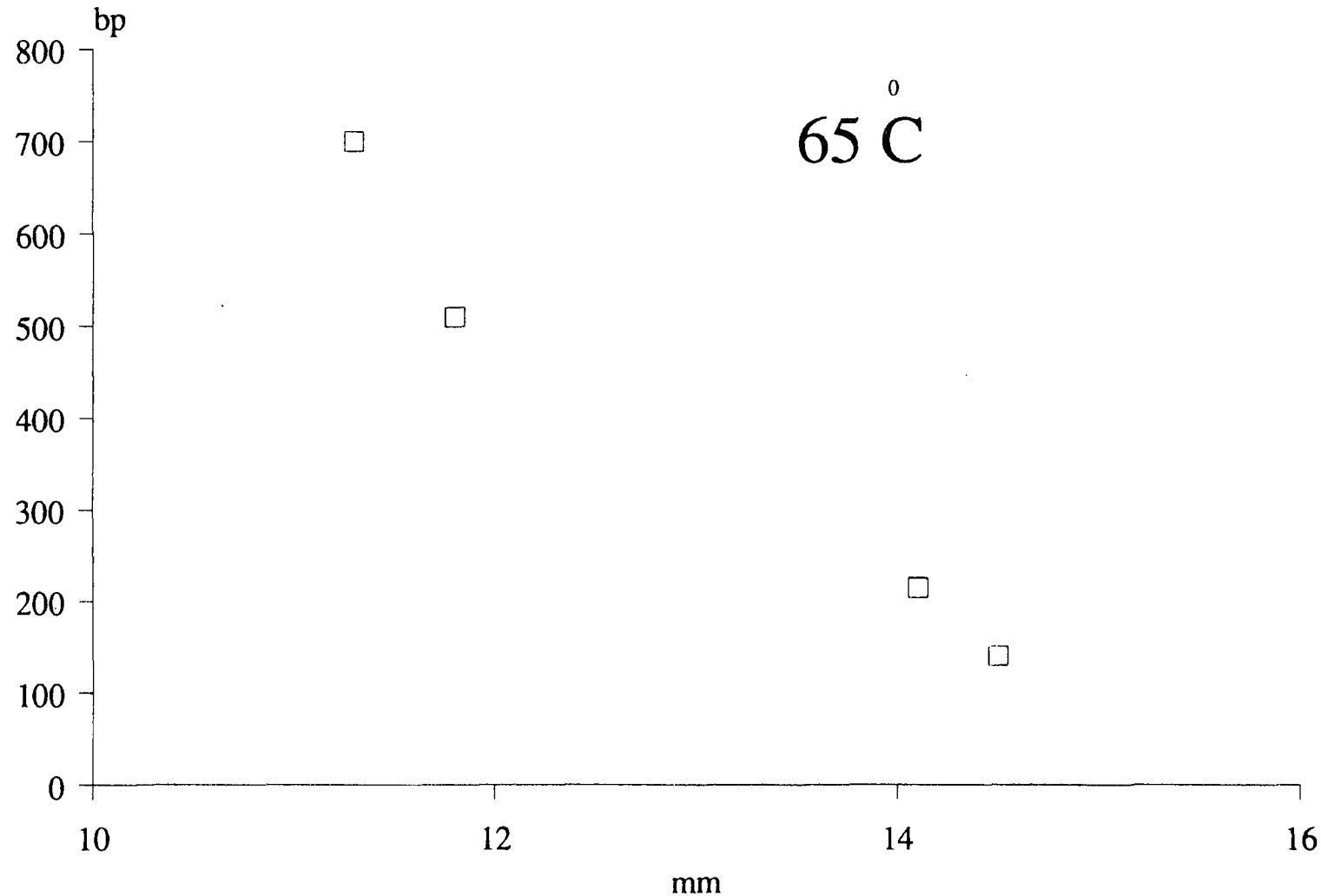
Temperature	base pair size						
	100-200	200-300	300-400	400-500	500-600	600-700	700-
47^0C	**140**	**215, 265**	355	405, 470	510, **600**	630	
55^0C	**140**	215, 225 **265**		405, 470		**680**	910
60^0C	**140**	215, 265			510	**620**	
65^0C	**140**	215			510		700

fragement sizes in bold type were the predominant reaction products at that temperature

Figures 14a, 14b, 14c and 14d: Electrophoresis of the reaction products obtained through the PCR directed amplification of the *A. nidulans* MT gene locus with two synthetic oligonucleotides based on the N and C-terminus amino acid sequence of the *Synechococcus* TX20 MT. The reaction temperature was raised from 47^0C , 55^0C , 60^0C and 65^0C respectively and the size of the electrophoretic fragments recorded against a pBR322 + Alu 1 restriction digest marker.


bp
700
600
500
400
300
200
100
0
47 °C
10
12
14
16
mm


bp
700
600
500
400
300
200
100
0
60 C
0
10
12
14
16
mm


bp
800
700
600
500
400
300
200
100
0
65 C
0
10
12
14
16
mm

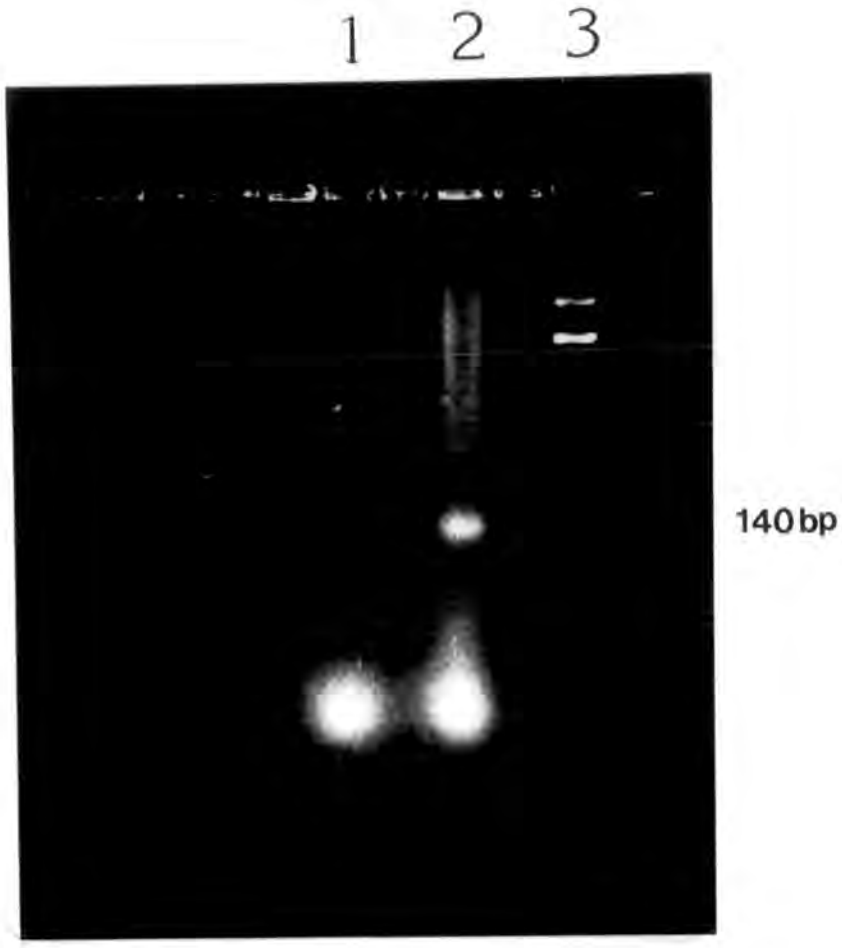


Plate 1: Taq Polymerase amplification of the *Synechococcus* TX20 gene with two synthetic oligonucleotides at 44^0C. Lane 1: *Synechococcus* 562 (Control); Lane 2: *Anacystis nidulans* : Lane 3; pBr322 + Alu 1 marker fragments. The approximate size of the *A. nidulans* reaction fragment is ¡ 150bp.

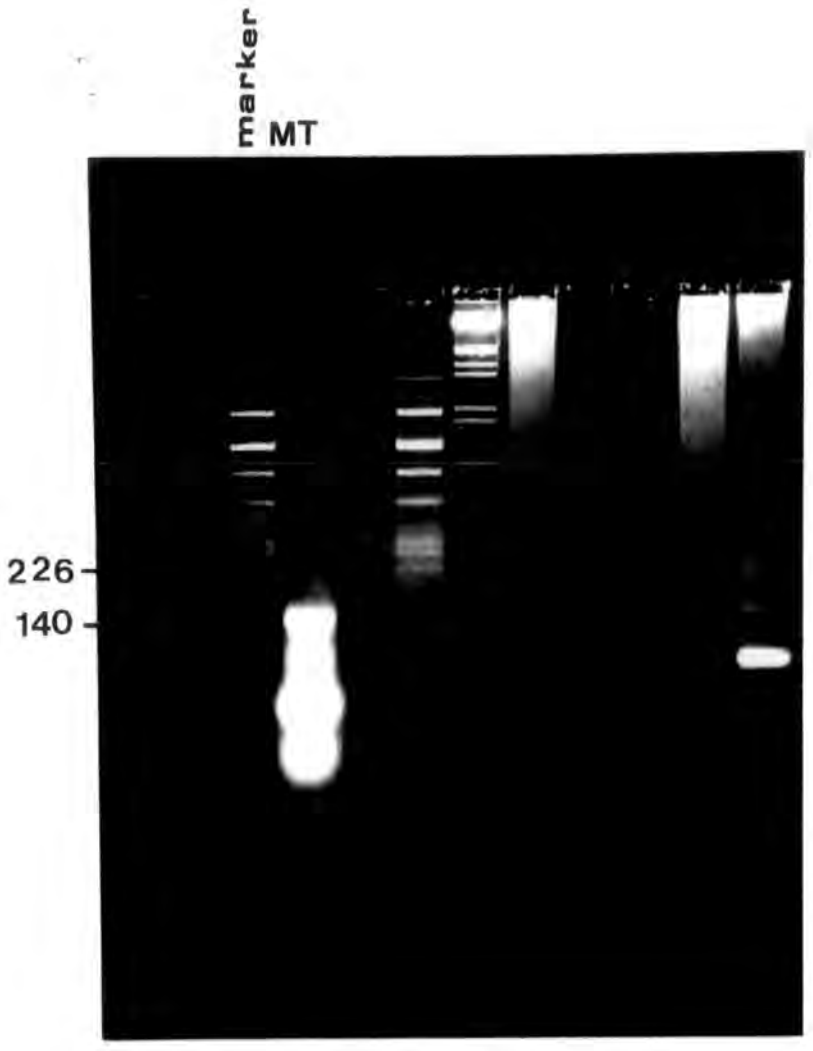


Plate 2: Analysis of the reaction products of the PCR amplification of the *Anacystis nidulans* MT gene locus with two N and C-terminal specific oligonucleotide probes. The reaction was run at 47^0C and analysed in 2% PAGE. The marker fragments are pBR322 + Alu 1.

LANES 4,5,6,9 & 10 ARE A SEPERATE EXPERIMENT.

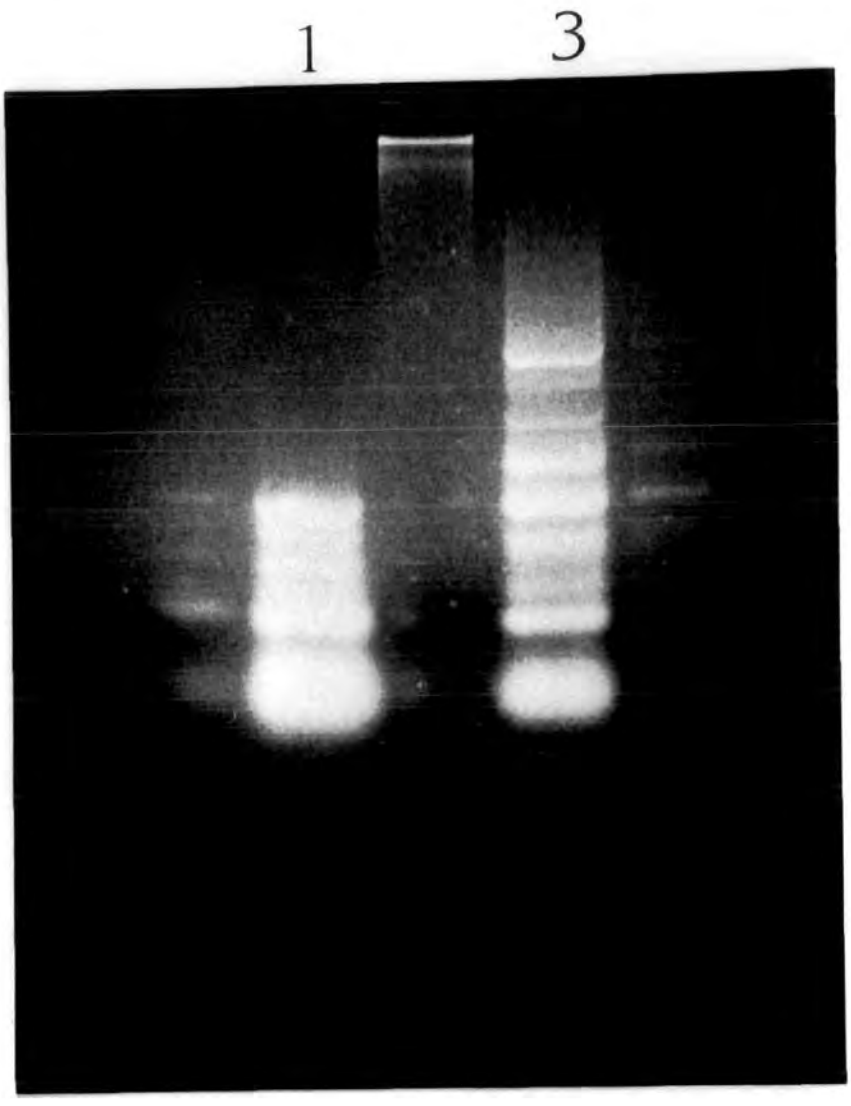
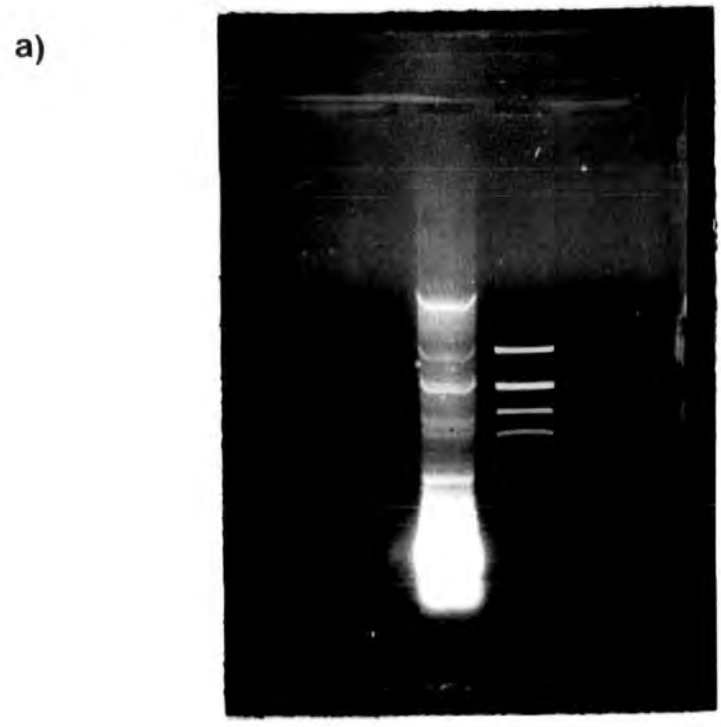
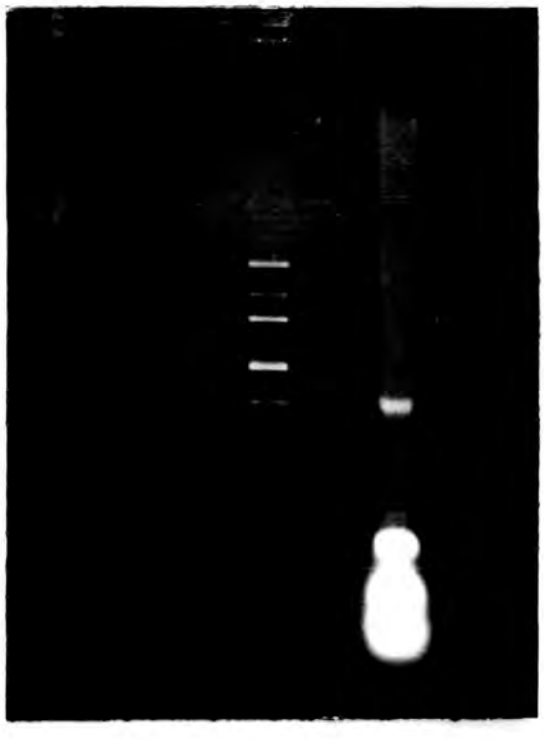


Plate 3: Analysis of the PCR reaction fragments of the amplified MT gene in *Anacystis nidulans* Lane 3: 47^0C PCR ; Lane 1: 47^0C reamplification of the products within Lane 1.





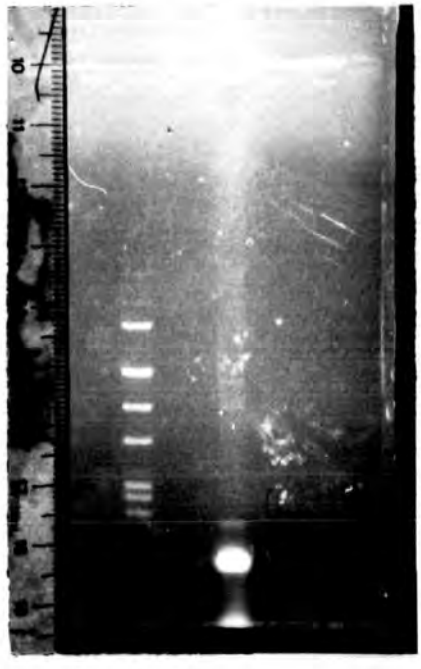


Plate 4: The effect of increasing the annealing temperature during the PCR amplification of the *A. nidulans* MT gen locus. a) 55^0C ; b) 60^0C ; c) 65^0C .

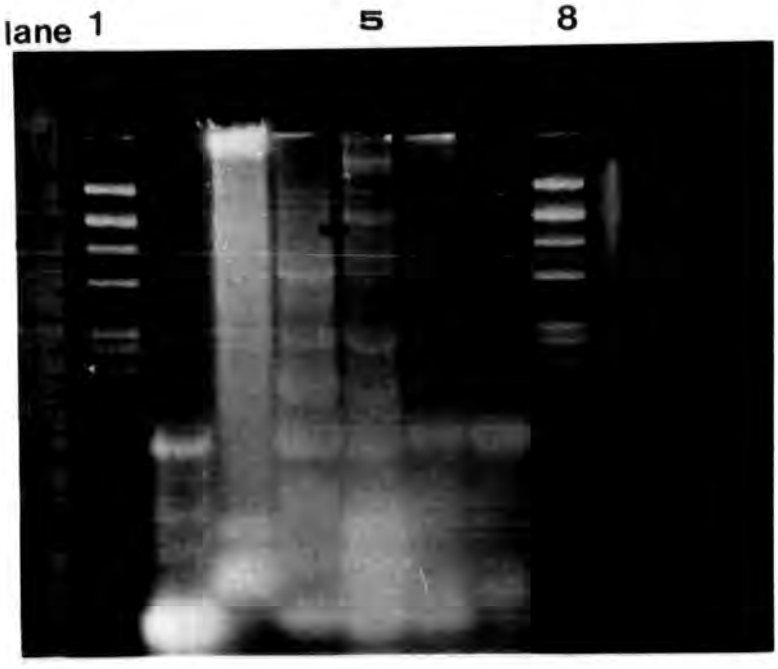


Plate 5: The effect of increasing the annealing temperature during the PCR amplification of the *A. nidulans* MT gene locus. Lane 1: marker (alu 1 + pBR322 digest fragments; Lane 2: 47^0C Re-Amplification; Lane 3: *A. nidulans* genomic DNA (control); Lane 4: 47^0C ; Lane 5: 55^0C ; Lane 6: 60^0C ; Lane 7: 65^0C ; Lane 8: marker.

3.0.4 PCR fragment recovery and insertion into pUC18

The bands relating to the 140 bp putative MT gene product were isolated. The DNA fragments were purified, treated with polymerase and ligated into a blunt ended site within pUC18. Three attempts at transformation into *E. coli* yielded a total of 12 isolates, these were given the designation ANMT 1-12. Analysis of cut and uncut plasmid recovered from the twelve isolates by agarose gel electrophoresis, when run against pUC18 did not reveal the presence of an insert. No difference between the control and plasmids were observed with regard to size (Plate). The 12 ANMT strains were also transferred onto nitrocellulose filters and screened with a [^{32}P]-labelled probe. Although a positive result was obtained with regard to the twelve isolates and the positive control, the fact that the negative control of purified pUC18 was blotted directly onto the nitrocellulose, whilst the pUC18-putative insert plasmids were lysed onto the filter within their bacterial hosts, meant that the result had to be treated with caution. Subsequently another transformation has yielded twelve more isolates and these are currently undergoing investigation.

In conclusion, to date, attempts at cloning the PCR 140 bp fragment into *E. coli* have been unsucessful.

Chapter IV

DISCUSSION

4.0.1 Prokaryotic metallothioneins

The isolation of class I and II MT within many higher organisms and $(gammaEC)_nG$ only within higher plants, eukaryotic algae and some yeast, has led to the suggestion that an evolutionary divergence has occurred within plants and animals (Gekeler *et al.*, 1988). Animals respond to toxic trace metal stress by induction of MT gene expression, plants by induction of $(gammaEC)_nG$ formation via enzymatic polymerisation of peptidic precursors. Two copper-induced MTs and a cadmium-induced $(gammaEC)_nG$ have been isolated recently within *Candida glabrata* (Mehra *et al.*, 1988) and within the last few months a class I MT has been isolated and sequenced within *Pisum*, a species known to possess $(gammaEC)_nG$ (Evans *et al.*, pers. com.). The identification therefore of putative $(gammaEC)_nG$ within *A. nidulans*, a prokaryotic organism in which a class II MT protein has previously been identified (Olafson, 1984; Olafson, 1988) casts doubts on the strictness of this divergence and suggests that some prokaryotes and eukaryotes may contain two classes of MT. The identification of putative $(gammaEC)_nG$ is significant in that, if it is confirmed, *A. nidulans* will be the first prokaryotic organism in which they have been identified.

To date $(gammaEC)_nG$ have been recorded within a wide range of plant species (Grill *et al.*, 1987: Jackson *et al.*, 1987) and they are believed

to be ubiquitous within the Angiosperms and Gymniosperms. Of greater relevance to this study, (gammaEC)$_n$G complexes have been reported in several representative genera of eukaryotic algae; *Rhodophyta, Phaeophyta, Xanthophyta, Chlorophyta, Bacillariophyta, Chysophyta and Euglenophyta* (Gekeler *et al.*, 1988, Shaw *et al.*, 1988). In addition they have been isolated within *S. pombe* (Musasugi *et al.*, 1981; Kondo *et al.*, 1985). Putative (gammaEC)$_n$G was induced readily within *A. nidulans* on exposure to cadmium, with induction of putative (gammaEC)$_n$G synthesis occuring within 48 hr of exposure. Within *S. pombe* and several eukaryotic algal species the synthesis of (gammaEC)$_n$G has been induced by copper, cadmium, bismith, lead, silver, zinc and arsenic (Grill *et al.*, 1986; Gekeler *et al.*, 1988). Direct detoxification and the formation of metal-(gammaEC)$_n$G complexes has only been demonstrated in the case of copper and cadmium (Jackson *et al.*, 1987), although indirect evidence has been presented for the occurrence of silver-(gammaEC)$_n$G complexes. No other metal associated complexes have been demonstrated to occur *in vivo*. The existence, however, of these complexes cannot be discounted. Other previously reported copper and cadmium binding peptides (CdBPs) occuring within algae (Stokes *et al.*, 1977; Hart and Bertram, 1980; Nagano *et al.*, 1984; Gingrich *et al.*, 1986) are potential (gammaEC)$_n$G complexes. These CdBPs were inducible and represented the main trace metal-complexing agent. Amino acid analysis of the CdBPs of *C. fusca* and *E. gracilis* indicated a high proportion of glycine, cysteine and glutamic acid (Nagano *et al.*, 1984; Gingrich *et al.*, 1986). (gammaEC)$_n$G are composed of the repeating dipeptide unit gammaglutamylcysteine with a single carboxy terminal glycine residue, so the results suggest that these are related (gammaEC)$_n$G complexes. The presence of other amino acids , although regarded as contaminants, may have indicated the low level presence of other MTs. The occurrence of free sulfide within the *A. nidulans* putative (gammaEC)$_n$G complex, a phenomena

previously confirmed for the Cd-binding complexes of *S. pombe*, *E. gracilis* and *C. fusca* (Musasugi *et al.*, 1983; Hayashi *et al.*, 1986; Weber *et al.*, 1987; Gekeler *et al.*, 1988) suggests that the phenomena may be widespread amongst the $(gammaEC)_nG$ complexes of lower organisms.

Anacystis nidulans is the only prokaryote known to possess a class II MT. The MT consists of fifty three residues, the peptide sequence of which bears little homology to those of mammalian or invertebrate MTs, hence its classification as a class II MT. Although a conserved stretch of 6 residues (Lys-Lys-Ser-Cys-Cys-Ser) is thought to resemble a region separating the two metal-binding domains within mammalian MT. The *Synechococcus* MT is thought to possess only a metal-thiolate cluster structure similiar to that found in eukaryotic MTs but in a single domain, has a lower cysteine content and is comparatively hydrophobic when compared to the *S. cerevisiae* and mammalian MTs (Olafson., 1988). In comparison to the single-binding domain Class I MTs isolated from *N. crassa* and *A. bisporus* (Munger *et al.*, 1987; Kagi and Kojima, 1987), the algal MT is approximately twice the molecular weight and again shows little sequence homology. The high degree of sequence homology (80%) between the *A. bisporus* and *N. crassa* MTs, and a stretch of 11 amino acids at the amino terminus homologous to mammalian MT, suggest a common ancestry separate to that of the *Synechococcus* TX20 (*Anacystis nidulans*) MT. The algal MT thus appears unique, for the moment, amongst MT in terms of amino acid sequence.

Olafson through, through reverse phase HPLC of the Synechococcus MT, isolated eight potential metal-binding isoforms. Seven fractions were found to contain MT isoforms, whilst the last fraction containing the greatest absorbance at A_{250} was contaminated with pigments and remained unresolved. Although no isoforms were identified within *A. nidulans* they may have been present. The high molecular weight fraction analysed within *A. nidulans* displayed a similar

charge density to *Synechococcus* MT on DEAE-Sephadex, the characteristic mercaptide chromophore in association with cadmium, the displacement spectrum of a MT when expossed to increasing proton concentration and a pH of half displacement similar to other MT. For mammalian MT the pH of half displacement is pH 3.2 for cadmium-MT and pH 4.5 for zinc-MT (Gingrich *et al.*, 1986). An estimated pH of half displacement of 3.5 for the *A. nidulans* MT is therefore closer to these values, than for the *E. gracilis* $(gammaEC)_nG$ which releases 50% of *in vivo* cadmium bound at between pH 5-5.6. Further evidence that the Synechococcus TX20 MT was present within *A. nidulans* was provided by the use of oligonucleotide probes and the PCR reaction.

Resistance of *A. nidulans* to the presence of cadmium within the media seemed to be related to increased $(gammaEC)_nG$ production. Between unadapted and adapted strains shocked for 2 days with a lethal dose of cadmium, the percentage of cadmium bound to the low molecular weight putative $(gammaEC)_nG$ pool rose from 50% of all intracellular cadmium to 80%. It was also notable that when the adapted strain was grown in the presence of a low dose of cadmium for an extended period, 55% of all cystolic cadmium was within the putative $(gammaEC)_nG$ fraction. This raises an important question regarding cadmium tolerance. Is metal resistance the result of increased selection for improved synthesis of $(gammaEC)_nG$? The results seem to suggest that within *A. nidulans* increased $(gammaEC)_nG$ synthesis was the path towards tolerance. If this is so, what effect will increased $(gammaEC)_nG$ synthesis have on metal homoeostasis? These results, and the work with *Euglena gracilis* and *S. pombe*, suggest that cadmium induces metallothionein synthesis and that the metal is strongly associated with $(gammaEC)_nG$. Synthesis of zinc-$(gammaEC)_nG$ was not induced by exposure to zinc in *E. gracilis* (Weber *et al.*, 1987; Shaw *et al.*, 1989) and Reese and Wagner (1987) have proposed that zinc and $(gammaEC)_nG$ are likely to

be only weakly associated within the natural cell enviroment. Therefore, if $(gammaEC)_nGs$ are unlikely to reduce the intracellular availability of free zinc ions within the cytoplasm. The important cellular processes of DNA replication, RNA transcription, energy metabolism, protein synthesis and degradation, which all require zinc-metalloenzymes, are all unlikely to be severely disrupted. If this is true, $(gammaEC)_nG$ induction and homoeostatic control of cadmium and copper ion levels within algal cells, could provide intracellular protection against the adverse effects of these ions without disrupting metallothionein regulated zinc homoeostasis. The level of metal required to induce synthesis of the two MTs is an important consideration. From the results obtained from the exposure of adapted and unadapted strains it could be proposed that class II MT synthesis is the primary route to metal homoeostatic regulation within *A. nidulans* until a threshold is reached upon which $(gammaEC)_nG$ synthesis is induced to reduce intracellular levels of cadmium. It would also be of interest to determine the preferred route of homoeostasis in *A. nidulans* strains adapted to high zinc concentrations (Fahmi *et al.*, 1982).

4.0.2 Adaptive mechanisms involving $(gammaEC)_nG$

The association of $(gammaEC)_nG$ with acid-labile sulphur has raised speculation that these peptides are involved in assimilatory SO_4^{2-} reduction (Steffens *et al.*, 1986) and a theoretical cycle of assimilatory sulphate reduction and $(gammaEC)_nG$ biosynthesis has been proposed (Appendix). Furthermore, the association of S^{2-} with cadmium-$(gammaEC)_nG$ complexes leads to a greater affinity and capacity for cadmium. Several possible ways by which adaption of the existing $(gammaEC)_nG$ synthesis pathway could lead to increased metal tolerance have been suggested (Robinson, 1989):
1) Modifications in the activity of pathway enzymes, such as γ-glutamyl

cysteine synthetase or GSH synthetase, leading to increased $(gammaEC)_nG$ biosynthesis; 2) Increased activity of enzymes reponsible for S^{2-} saturation of metal-$(gammaEC)_nG$ complexes; 3) Modified rates of $(gammaEC)_nG$ turnover; 4) Modified compartmentation of one of the components, $(gammaEC)_nG$, S^{2-}, or metal.

4.0.3 Amplification of the *A. nidulans* MT gene

The two oligonucleotides used to probe the *Anacystis* genome both contained a sixteen fold redundancy, three inosine bases were also included to minimise redundancy. The successful amplification of genomic DNA of the correct size even at higher annealing temperatures, indicated that the probes were annealing to two domains within a MT gene locus and that inosine was not inhibiting the formation of primer-genomic DNA duplexes. Both probes had been designed to include the Cys-X-Cys metal-binding sequence characteristic of MTs. The non-amplification of DNA from *Synechococcus* 562 is therefore intresting as it was possible that both these regions would form conserved domains within the MT molecule. It is notable that four of the seven *Synechococcus* MT isoforms isolated previously contained no valine molecules within their amino acid structure (Olafson *et al.*, 1988), the remaining three isoforms were found to contain between one and three valine molecules. This Indicated that within the *Anacystis* genome being probed isoforms were likely to be present. Closer examination of the probe sequence revealed that the first three nucleotides of the 5'↦3' N-terminus primer coded for valine. Therefore, if a substitution for valine had occured within an isoform at this position, the primer would be reduced from a twentymer to an eighteenmer.

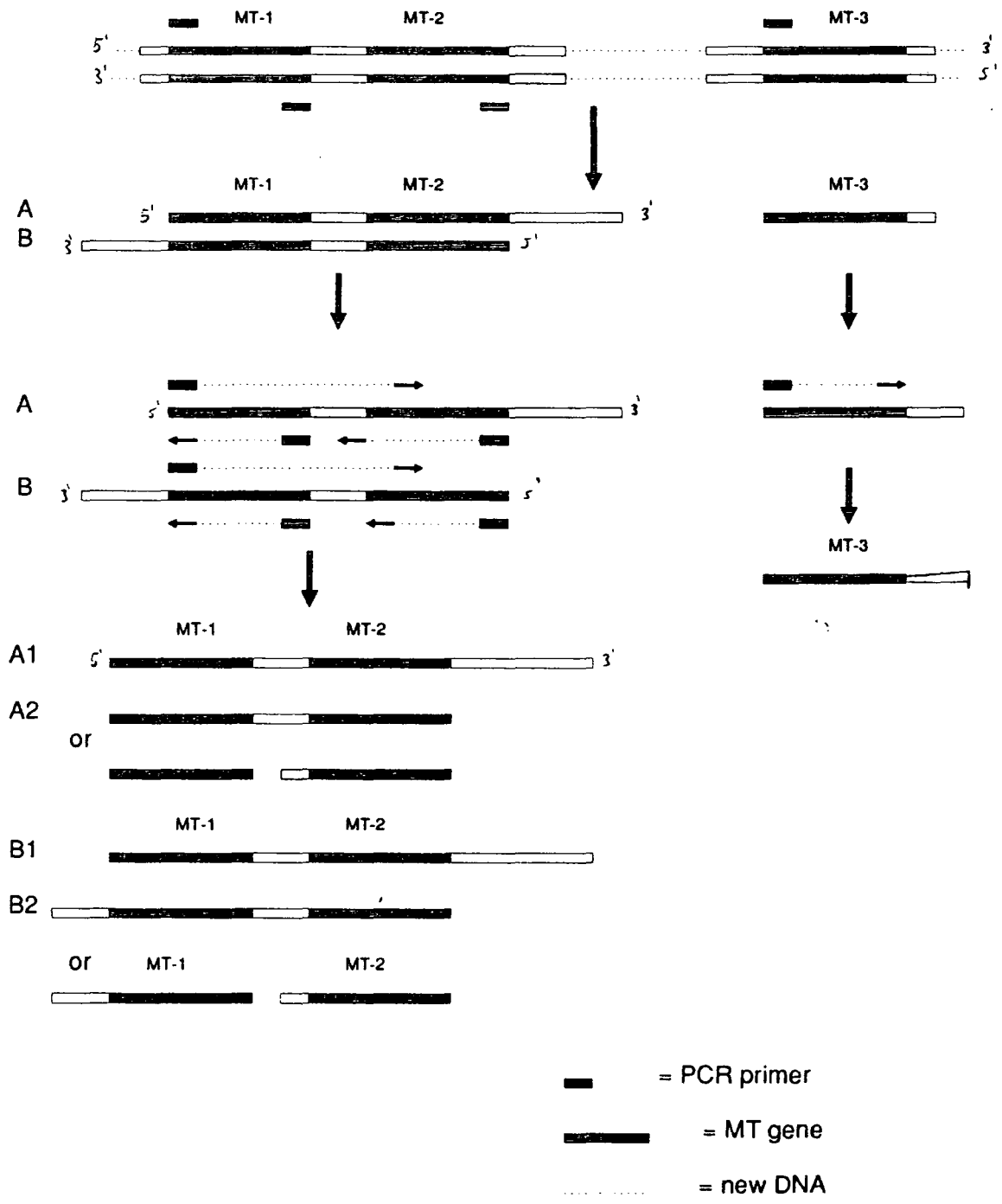
The presence of different fragment sizes was unexpected. During the early cycles of the PCR reaction when single stranded genomic DNA-primer duplexes

are formed, polymerase transcription of the genomic strand may cause the formation of a ssDNA fragment that extends beyond the annealing sites of the 3'↦5' and 5'↦3' primers. The rate of *Taq* polymerase transcription is estimated at approximately 1000 bp/min (Davies, 1988). The subsequent cycle of primer annealing, transcription and disassociation will produce one ssDNA corresponding to the original overtranscribed strand (the long product) and a second the length of which will depend on the distance between the two primers (the short product). The next round of the PCR cycle will produce one ssDNA of the long product and three of the short product. During further cycles the long product will continue to act as a template for the production of correctly sized fragments (Oste, 1988). Fragment extensions are thus quickly diluted out of the mixture. During the final cooling period these fragments will either form an imbalanced double strand with a primer or remain as ssDNA fragments. During electrophoresis they should run behind the primers but in front of the PCR product. Examination of all the gels revealed that these fragments may have been present. The presence of DNA fragments running behind the main PCR fragment therefore raised the possibility that the primers were annealing to several different sites within the genome. The occurrence of multigenic MT families is not unknown amongst eukaryotes. Within Man, MTs comprise a multigene family of about nine members. However only four of these genes appear to be expressed at the protein level; hMT-I_A, hMT-I_E, hMT-1_F and hMT-2_A. The remaining five MT genes are thought to be pseudogenes and are not transcribed; $\Psi MT - I_C, \Psi MT - I_D, \Psi MT - 1_G, \Psi MT - 1_H$ and $\Psi MT - II_B$ (Karin and Richards, 1982; Richards *et al.*, 1982).

Metal tolerance within other organisms has been linked to gene copy number. Crawford and co-workers have reported that within Chinese Hamster cells cadmium resistance resulted from the co-ordinated amplification of the MT-1 and MT-2 genes, to between 3- to 60-fold above the basal gene dosage of Cd-

sensitive cells (Crawford *et al.*, 1985). In *S. cerevisiae* gene amplification has been observed, with copper resistant ($CUP1^R$) strains containing ten or more tandem duplications of the CUP1 locus (Fogel and Welsh, 1982). Within this study the genomic DNA used throughout the PCR amplification originated from a cadmium-sensitive culture. Therefore it was unlikely that tandem duplications of the MT gene locus were present. Any result with the PCR amplification would therefore be expected to reflect the basal gene number. Within *Synechococcus* TX20, the reported presence of isoforms, may either be due to gene amplification as a result of cadmium selection, or MT species with distinct intracellular roles as suggested by Karin (1985). Each isoform may carry zinc to different intracellular compartments or else interact with different classes of enzyme. Olafson (1988) demonstrated that the isoforms varied in respect of elution time from reversed phase HPLC and metal composition. If variations within the isoforms are encoded by different gene sequences, it is possible that specific isoforms may anneal to only one or neither of the two primers. If only one primer was annealing to the 5'$\mapsto$3' or 3'$\mapsto$5' strands of a specific MT gene, each amplification would produce only one extended ssDNA fragment at that locus. If however the MT genes are clustered within a locus, mis-primer annealing could lead to transcription between the 3' and 5' ends of two MT genes (Figure 15).

Figure 15: Schematic diagram of a PCR in which primer annealing within a MT multigene family is unequal. Only two cycles are shown, in both the fate of the original DNA strand is omitted. MT-1 and MT-2 are in tandem, MT-2 has an amino acid change at the N-terminus that inhibits annealing of the $5' \mapsto 3'$ primer. It is assumed there is a 50% possibility that the MT-2 $5' \mapsto 3'$ polymerase will encode through to MT-1. MT-3 is situated upon the same DNA strand but is amplified seperately. A codon change within the C-terminus inhibits primer annealing. Note that the longer primer extensions (A1, B1) can increase additively with each cycle. In contrast strands A2 and B2 will give rise to an assortment of fragments. Short discrete, primer terminated copies of the MT-1 gene will proceed to double with each subsequent cycle and become the predominant form. The remaining fragments will continue to increase additively with each cycle.


MT-1
MT-2
MT-3
5'
3'
A
B
A1
A2
or
B1
B2
= PCR primer
= MT gene
= new DNA

Chapter V

FUTURE STUDIES

(1) To determine the differences in the regulation and synthesis of the two cadmium-binding ligands between cadmium-sensitive and tolerant cultures. Competition may exist between the two types of regulatory molecules for a particular metal ion and the affinity and co-ordination preference of the molecules may dictate whether MT or $(gammaEC)_nG$ are synthesized.

(2) More detailed structural characterization of the two ligands, an example of this would be an indication of the pH of half displacement for the putative $(gammaEC)_nG$.

(3 Use of the PCR reaction products as probes to determine the position of the MT locus within the *A. nidulans* genome. Once the site is identified to screen and isolate the upstream regulatory elements.

(4) Once the upstream region has been placed inside a suitable vector to sequence the region and assess for the presence of any prokaryotic MREs.

(5) Use the MT gene and probe MREs to engineer cyanobacteria which are highly cadmium-resistant and accumulate cadmium from solution.

(6) Use of any putative MREs to regulate the activity of other genes, and use of the cyanobacterial MT as a selectable marker (e.g. selection for cadmium-resistance).

Chapter VI

MAJOR CONCLUSIONS

(1) The results indicate that the cyanobacteria *A. nidulans* contains two cadmium-binding ligands. The first resembles the class II MT previously isolated by Olafson and the second to the class III MTs, or $(gammaEC)_nG$, commonly associated with plants and lower eukaryotes. It is believed that this is the first evidence of two classes of metallothionein within a prokaryotic organism and that $(gammaEC)_nGs$ may be present in non-eukaryotic organisms.

(2) Although both classes of MT were induced by the addition of cadmium. Differences between their regulation and induction were noted. Selection for a cadmium-tolerant strain resulted in cultures in which $(gammaEC)_nG$ was the predominant cadmium-binding ligand following exposure to sublethal doses of metal. $(gammaEC)_nG$ was also the predominant ligand in cultures exposed to lethal cadmium levels. Putative class II MT was only found in cadmium-sensitive cultures exposed to sublethal levels of cadmium.

(3) Analysis of the two molecules indicated characteristic MT properties. The presence of sulfide within the lower molecular weight species, an HPLC elution time similar to $(gammaEC)_nG$ previously resolved from *D. innoxia* was suggestive of putative $(gammaEC)_nG$. The higher molecular weight ligand contained a mercaptide bond chromophore and a pH of half displacement similar to other class I and II MT MTs.

(4) Data suggests that the two extension primers corresponding to the genes encoding the N and C-terminus of *Synechococcus* TX20 MT successfully annealed

to the MT gene locus within *A. nidulans*. Increasing the annealing temperature of the polymerase chain reaction did not reduce the transcription of the correctly sized reaction product. The results confirm previous work by Olafson into the occurence of a class II MT and confirm the similarity between *A. nidulans* and *Synechococcus* TX20.

(5) The presence of other polymerase chain reaction products indicated that the *A. nidulans* MT may be present within isoforms or gene duplications of the main MT gene sequence. Differences within the annealing sites of the extension primers were demonstrated through the reduction in number of fragments with increasing temperature.

(6) The project accomplished the first four objectives set out in the introduction, with the fifth still remaining to be carryed out.

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Chapter VII

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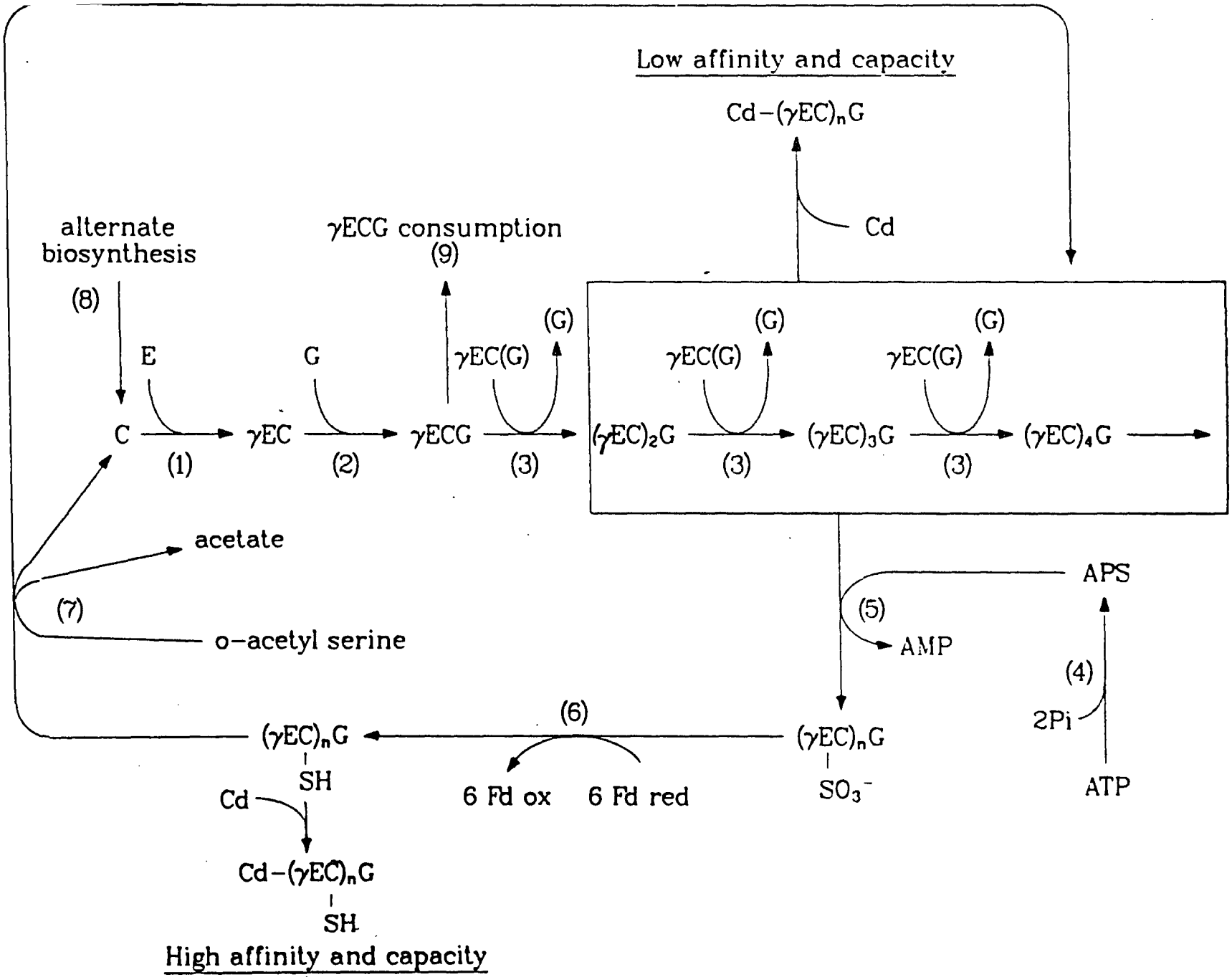
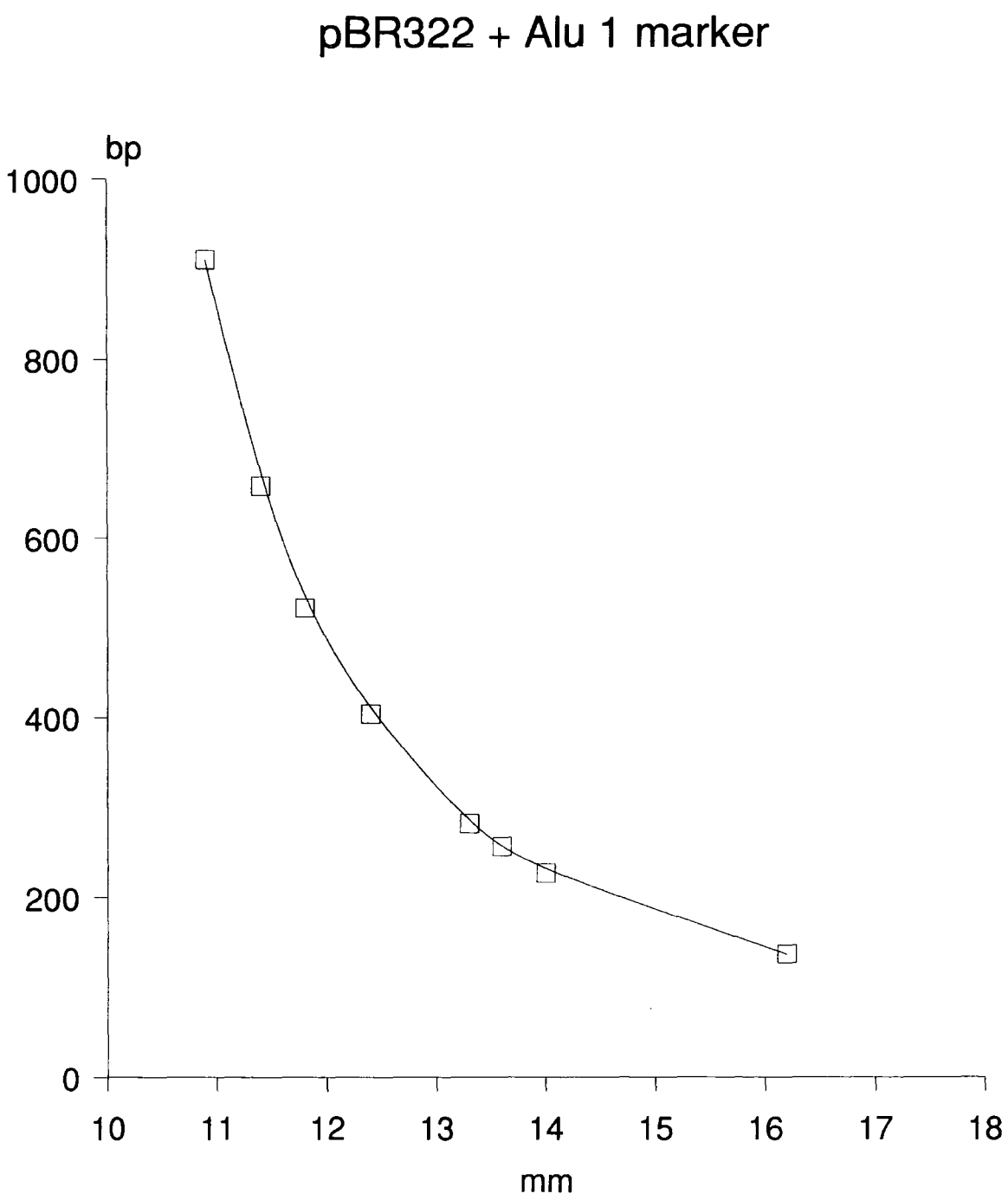
Table 11: Total cadmium fractions recovered after analysis by AAS

Fraction no.	Expt.A	Expt.B	Expt.C	Expt.D	Expt.E	Expt.F
1	0.5			0.75		0.8
2	0.5			0.7		
3	0.5			0.6		
4	0.5			0.7		
5	0.5			0.7		
6	0.5			0.7		
7	0.5			0.7		
8	0.5			0.7		
9	0.5			0.7		
10	0.5			0.7		
11				0.7		
12	0.5			0.7		
13	0.5			0.7		
14				0.7		
15				0.7		
16	0.5			0.7		
17	0.5			0.7		
18	0.5			0.7		
19	0.5		1.2	0.8	0.8	1.2
20	3.5	0.5	5	1.2	2.1	2
21	15		17.5	1.5	3.8	2
22	10.5		40	1.5	6.1	1.8
23	7		48	1.2	7.5	1.6
24	3		38.5	1	8.5	1.5
25	1.3		24	1	7.5	1.3
26	1.2		15	0.8	6.3	1.2
27	2		9	0.8	5.5	1
28	1.5		2 7	0.8	4	0.9
29	3		3.8	0.8	3	0.8
30	3		2	0.8	2.1	0.9

Fraction no.	Expt.A	Expt.B	Expt.C	Expt.D	Expt.E	Expt.F
31	3.1	0.5	1.5	0.8	1.5	0.9
32	3.2	0.5	1.4	0.8	1.1	1
33	2.5	2	0.9	1.1	0.7	1.25
34	0.5	3	0.8	1.2	0.5	1.4
35	3.1	3.5	0.8	1.5	0.3	1.5
36	4.3	4	0.7	1.6	0.3	1.55
37	5.1	4.5	0.8	1.7	0.25	1.6
38	8.5	5	0.8	1.8	0.15	2.2
39	9.6	7.6	0.6	1.8	0.1	6
40	11.7	12	0.5	2	0.1	12
41	26.9	19	0.5	3		17
42	50	25	0.5	3.5		12
43	65	20	0.5	3		7.2
44	22.8	13.5	0.45	2.9		7
45	10.5	10.5	0.1	2.8		4
46	8.5	7	3	3.6		3.4
47	4.4	4	5.1	14		2.3
48	11	4	7	33		2
49	50	7	9.5	35	0.25	1.9
50	86	8	11.8	20	0.3	1.8
51	53	4.5	7	10	0.2	1.8
52	10.5	2.2	2.5	3		1.5
53	4.4	1	0.6	2		1.4
54	1.25	0.5	0.5	1.5		1.4
55	1.2			1.1		1.3
56	1.1			1		1.3
57	0.9			0.9		1
58	0.8		3	0.9		1
59	0.9			0.7		0.9
60	0.8			0.9		0.8
Total	455.5	168.5	216	181.7	63.05	135.1

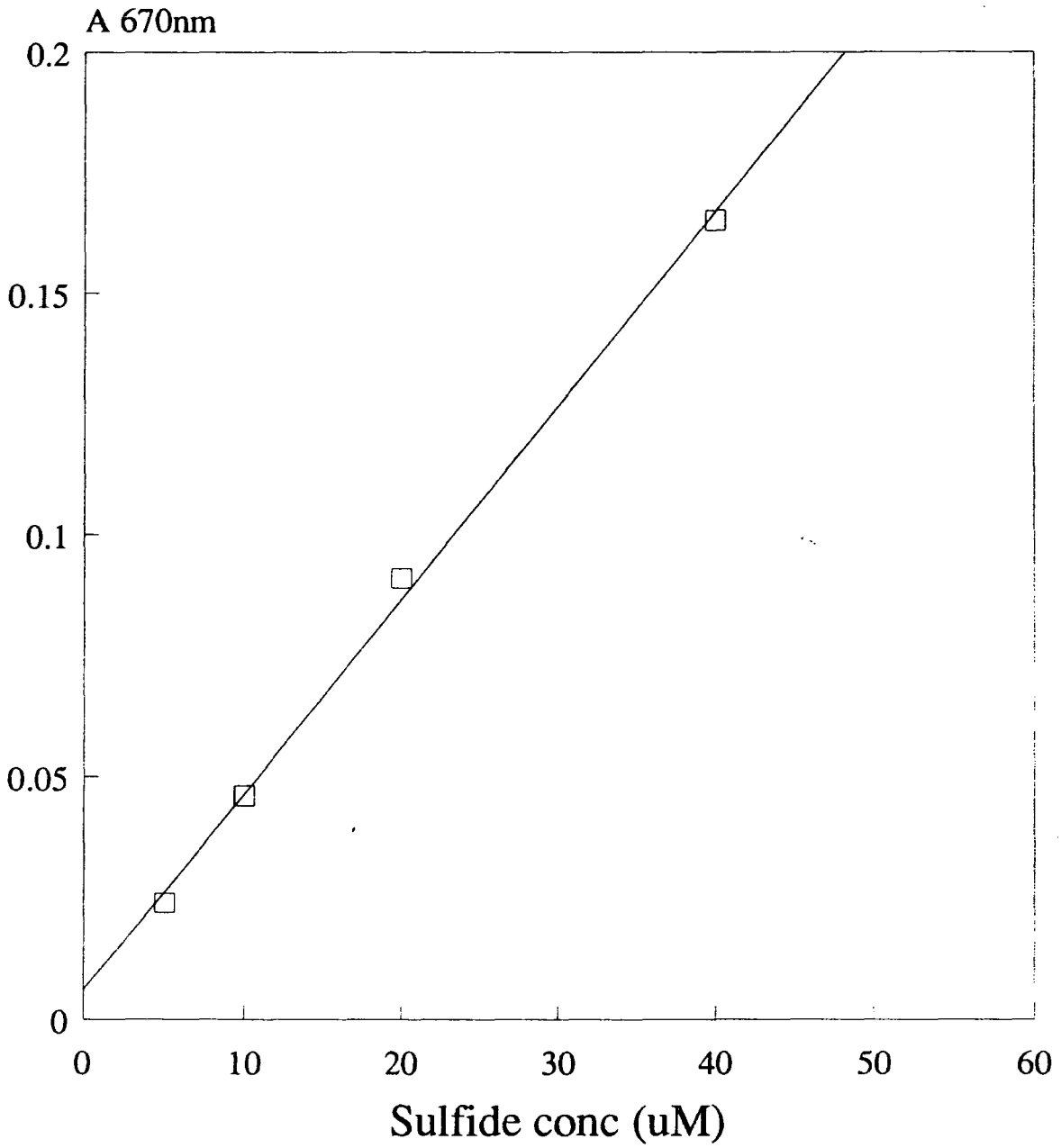
Appendix 1: A theoretical unified scheme linking assimilatory sulphate reduction and $(gammaEC)_nG$ biosynthesis as proposed by Robinson (1989). A possible alternative source of S^{2-} to that shown above for the formation of Cd-S^{2-}-$(gammaEC)_nG$ aggregates, is generated by the action of non-organic sulphate reductase. The enzymes involved in this theoretical cycle : 1, gamma-glutamylcysteinecynthetase (EC6.3.2.2.),2,glutathione synthetase(EC63,2,3,),3,uncharacteri enzyme(s) assumed to be $(gammaEC)_nG$ synthetase,4,ATP sulphurylase (EC2,7,7,4), 5, APS sulphotransferase, 6, ferredoxin dependant organic thiosulphate reductase (EC 1.8.7.1),7, 0-acetyl L-serine sulphydrolase (EC4,2,99,8), 8, alternative pathways for cysteine biosynthesis, 9, pathways that consume glutathione.


Low affinity and capacity
Cd–(γEC)nG
Cd
alternate biosynthesis
(8)
γECG consumption
(9)
E
G
γEC(G)
(G)
γEC(G)
(G)
γEC(G)
C
(1)
γEC
(2)
γECG
(3)
(γEC)2G
(3)
(γEC)3G
(3)
(γEC)4G
acetate
(7)
o-acetyl serine
APS
(5)
AMP
(4)
2Pi
ATP
(6)
(γEC)nG
SH
Cd
6 Fd ox
6 Fd red
(γEC)nG
SO3-
Cd–(γEC)nG
SH
High affinity and capacity



The restriction of pBR322 with Alu 1 was used as a size marker for the amplified products of the PCR reaction.

Sulfide Assay (standard)



Bradford assay (standard)