Expression of recombinant metal-binding proteins in E. Coli and in Synechococcus PCC7942: examination of metal binding in vivo and vitro

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EXPRESSION OF RECOMBINANT METAL-BINDING PROTEINS IN *E. COLI* AND IN *SYNECHOCOCCUS* PCC7942: EXAMINATION OF METAL BINDING *IN VIVO* AND *IN VITRO*.

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ABSTRACT.

EXPRESSION OF RECOMBINANT METAL-BINDING PROTEINS IN *E. COLI* AND IN *SYNECHOCOCCUS* PCC7942: EXAMINATION OF METAL BINDING *IN VIVO* AND *IN VITRO*.

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Metallothioneins (MTs), cysteine-rich proteins and polypeptides, are proposed to detoxify excess intracellular metal ions via sequestration. Three genes, each encoding a protein related to this group of molecules, were expressed in *Escherichia coli* and in *Synechococcus* PCC7942 (variant PIM8) in order to examine the metal binding properties of their products. Phenotypic alterations, in terms of metal-tolerance and -accumulation, were assessed in cells expressing these genes.

The genes which were expressed were: (1) *smt*A from *Synechococcus* PCC7942, which is designated to be the first isolated prokaryotic MT gene; (2) $PsMT_A$, a gene from pea (*Pisum sativum* L) which encodes a protein with similarity to class I MT; and (3) a synthetic gene encoding $(Glu\text{-}Cys)_3Gly$, an analogue of the phytochelatin (PC; class III MT) molecule $(\gamma Glu\text{-}Cys)_3Gly$.

The protein encoded by *smt*A was shown to have high affinity for metal ions (Hg, Cd, Cu, Zn), supporting the designation of *smt*A as a prokaryotic MT gene. Comparison with mammalian MT revealed that the affinity of the product of *smt*A for Zn was higher than that of the mammalian protein, suggesting a role for this protein in Zn homoeostasis and/or detoxification in *Synechococcus* sp. *E. coli* cells expressing *smt*A exhibited increased accumulation of Zn and Cd (3-fold and 1.4-fold respectively relative to control cells), but no increase in tolerance toward Zn, Cd or Cu.

Comparison of the metal-affinity of the product of $PsMT_A$ with that of mammalian MT revealed that this protein also has high affinities for Cu, Cd and Zn. These data support the hypothesis that $PsMT_A$ is a higher plant MT gene. Affinity of the product of this gene for Cu was higher than that of mammalian MT, suggesting a role for this protein in Cu homoeostasis and/or detoxification. Expression of $PsMT_A$ in *E. coli* resulted in increased accumulation of Cu, Cd and Zn. Cu accumulation was increased more substantially than either Zn or Cd accumulation in cells expressing $PsMT_A$. No increase in tolerance toward any of these metals was observed in *E. coli* expressing this gene.

There is evidence that PCs are involved in Cd detoxification in higher plants. Genes encoding enzymes involved in the synthesis of these molecules have not been isolated, precluding gene transfer experiments for investigation of their function. Expression of a gene encoding $(Glu\text{-}Cys)_3Gly$ in *E. coli* resulted in increased tolerance toward Cd, but not Cu or Zn. Thus, a predicted function of a secondary metabolite (PC) was observed when a gene product based on the structure of this molecule was expressed in a heterologous system. No significant increase in accumulation of Cd, Cu or Zn was detected in cells expressing this gene.

*smt*A transcripts were shown to increase in abundance in response to elevated concentrations of Cd in *Synechococcus* PCC7942 (variant PIM8). Sequences derived from the *smt* locus were fused to a synthetic gene encoding $(Glu\text{-}Cys)_3Gly$, and introduced into *Synechococcus* PCC7942 (variant PIM8). Transcripts encoding $(Glu\text{-}Cys)_3Gly$ increased in response to exposure of these cells to Cd. Cells containing this construct exhibited increased tolerance toward Cd. Data concerning expression of $(Glu\text{-}Cys)_3Gly$ in *E. coli* and Synechococcal cells support the hypothesis that PCs may have a role in detoxification of excess intracellular Cd.

Comparison of the data obtained in these studies has been used to assess the factors affecting metal-accumulation and -tolerance as a result of expression of heterologous metal-ligands in microbial cells.

MEMORANDUM.

Part of the work presented in this thesis has been presented in the following publication:

Tommey, A.M., Shi, J., Lindsay, W.P., Urwin, P.E. and Robinson, N.J. 1991. Expression of the pea gene *PsMT*$_A$ in *E. coli*: Metal-binding properties of the expressed protein. FEBS Letters 292 (1,2) 48-52.

STATEMENT.

No part of this thesis has been previously submitted for a degree in this or any other university. I declare that, unless otherwise indicated, the work presented herein is entirely my own.

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ABBREVIATIONS

A	-	adenine
AAS	-	atomic absorption spectrophotometry
ATP	-	adenosine-5'-triphosphate
Ap	-	ampicillin
BSA	-	bovine serum albumin
BSO	-	buthionine sulphoximine
bp	-	base pairs (of DNA)
C	-	cytosine
CD	-	circular dichroism
cDNA	-	copy DNA
cm	-	centimetres
cpm	-	counts per minute
cps	-	counts per second
Ci	-	curies
Cm	-	chloramphenicol
Da	-	daltons
DMSO	-	dimethylsulphoximide
DNA	-	deoxyribonucleic acid
DNase	-	deoxyribonuclease
DTT	-	dithiothreitol
EDTA	-	ethylenediaminetetraacetic acid
EXAFS	-	extended X-ray absorption fine structure
G	-	guanine
g	-	grams
GST	-	glutathione-S-transferase
h	-	hours
HPLC	-	high performance liquid chromatography
IPTG	-	isopropyl-2-D-thiogalactoside
IUPAC	-	International Union of Pure and Applied Chemists
Kb	-	kilobases
Km	-	kanamycin
min	-	minutes
MOPS	-	3-(N-Morpholino)propane-sulphonic acid
Mr	-	molecular weight (in daltons)
mRNA	-	messenger RNA

MT	-	metallothionein
NMR	-	nuclear magnetic resonance
OD	-	optical density
ODU	-	optical density units
PAC	-	perturbation of the angle of correlation of γ-rays
PEG	-	polyethylene glycol
PC	-	phytochelatin
PCR	-	polymerase chain reaction
PPO	-	2,5-diphenyloxazole
RNA	-	ribonucleic acid
RNase	-	ribonuclease
rRNA	-	ribosomal RNA
s	-	seconds
SDS	-	sodium dodecyl sulphate
SSC	-	saline sodium citrate
T	-	thimine
tris	-	tris(hydroxymethyl)methylamine
v	-	volume
w	-	weight
Xgal	-	5-bromo-4-chloro-3-indolyl-2-D-galactoside

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This is dedicated to my family and to Fiona, with thanks for their love and support throughout my studies.

CHAPTER 1 GENERAL INTRODUCTION.

This general introduction is presented as an overview of the broad subject of metal ion homoeostasis and detoxification, with an emphasis on mechanisms involving proteins and polypeptides which are proposed to detoxify metal ions via sequestration. For clarity, results sections are presented as separate chapters which include introductions to the relevant areas of research and details of specific experimental methods. The introductions to chapters 3, 4 and 5 provide details regarding the particular ligands with which the research is concerned and the biological systems with which they are associated. Chapter 6, which involves experiments designed to achieve metal-regulated expression of a novel metal ligand in *Synechococcus* PCC7942, provides a review of the mechanisms involved in metal-regulation of gene expression.

1.1 Occurrence and mobilisation of metals.

The d-block metals, with which this research is primarily concerned, generally occur naturally as ores, from which they can be mobilised into the wider environment by mining and subsequent processing. Emission from metal processing and related industries, has led to the dispersion of metals throughout the biosphere (Nriagu and Pacyna 1988). Metal-contaminated wastes are often mixed with municipal wastes prior to treatment, a practice which can result in high levels of metal in the output from some waste water treatment plants. This has led to potentially dangerous levels of toxic metals in agricultural land surrounding industrial areas via the application of these materials (Andriano 1986).

1.2 Bulk, trace and non-essential metals.

Metals can be classified as bulk, trace or non-essential in relation to their interaction with living organisms. Those often classified as bulk metals (Na, Mg, K and Ca) are present in high concentrations in biological systems, for example making up 99 % of all metal ions found in man (Hughes 1981). One of the major functions of these ions is the formation of extra- to intra-cellular ionic and charge gradients which are essential to generate ATP via membrane-associated ATPases. Such gradients also allow the generation of trigger and activation

mechanisms such as the transmission of nerve impulses, muscle contraction, and hormone secretion. Bulk metals are also found as cofactors to many enzymes, and are often important structurally in the stabilisation of large polymerised complexes and in hard tissues.

Metals which are essential for most living systems, but are present at much lesser concentrations, are generally referred to as trace (or ultra-trace) metals. These comprise the d-block metals V, Cr, Mn, Fe, Co, Ni, Cu, Zn and Mo. Fe and Cu, two of the more abundant of the trace metals, have several well-characterised functions. As well as transfer of respiratory gases (Fe in haemoglobin; Cu in haemocyanin), these ions are often involved in redox reactions (as is Co and to a lesser extent Cr), and hence are involved in the large number of biochemical processes which involve electron transfer. Trace metals (especially Cu and Zn) are also found as cofactors to a wide variety of enzymes. Zn is found as cofactor to representatives of all six classes of enzyme (IUPAC classification), and is important in maintaining the structure of proteins which interact directly with DNA to control gene transcription (Vallee et al 1991).

The essential metals described above can exert toxic effects if present at sufficiently high concentrations. Metals such as Cd, Hg, Pb, Sn, As and Ag, do not have defined biological functions, and are associated with detrimental effects on living organisms. However there is some evidence that As, Cd and Pb, may be essential for animal life (Schwartz 1977). Thus, the distinction between essential and non-essential metals is not absolute.

1.3 Basis of toxicity of metal ions.

Metal ion toxicity can be attributed to a number of effects. The properties which give metal ions useful biochemical functions are often those which render them potentially toxic. As summarised by Ochiai (1987), toxicity can be due to:

(1) Liganding to, and thereby affecting the function of, biologically important molecules. Such effects may lead to inactivation of molecules such as enzymes via conformational alteration of the molecule, or displacement of essential metal ions. Such interactions may also lead to disruption of cellular or organellar membrane integrity.

(2) Participation in oxidation and reduction reactions, causing interferance with cellular metabolism.

The mutagenic (hence carcinogenic and teratogenic) effects of Cd are attributed to direct interaction with DNA (Koizumi and Waalkes 1989).

1.4 General characteristics of metal-binding sites of biological molecules.

Metal-binding sites must have the following characteristics. (1) a region which has a high concentration of metal-liganding atoms (oxygen, nitrogen or sulphur), (2) a sufficient number of such atoms to stabilise the metal (from 2 to 8 depending on the metal ion), and (3) these must be arranged in the correct three-dimensional configuration to allow space for the metal ion (Que 1988). Additionally, in biological systems metal-binding molecules must have the correct affinity for the ion such that it is not stripped from the binding site by other molecules in the cell, but conversely in some instances may be released for donation to other ligands. Metal-binding molecules have therefore necessarily evolved specific configurations to allow binding of metal ions with varying affinities.

Research into the nature of metal-coordination sites of proteins has been concentrated on several areas. The cysteine-rich molecules thought to be involved in metal detoxification (metallothioneins [MTs] and phytochelatins [PCs]) have been studied to give insight into how they function in metal sequestration. These molecules, and their interaction with metal ions is discussed in detail in the following sections. Similarly, the metal-binding sites of metal-dependent transcription factors have been studied in detail, providing information on how these proteins interact with DNA (Vallee et al 1991). Research into the interaction of proteins with metal ions has included the analysis of the metal-binding pockets of enzymes (e.g. experiments involving alteration of, and creation of, metal-coordination sites in enzymes have been performed in order to attempt to change the catalytic activity and specificity of enzymes; Merkle et al 1991). Additionally, there has been considerable research into the use of metal-affinity chromatography for the purification of proteins containing metal-binding sites (Arnold 1991). The creation of novel metal-binding sites in proteins via genetic engineering (Suh et al 1991), has been used to allow one-step separation of the heterologous

protein using a metal-affinity column (Brewer et al 1991). Recent advances involving the use of computer-aided design, in conjunction with detailed comparison of the Cu-binding sites of a variety of proteins, has allowed the design of potential Cu-binding sites in proteins for which the 2° structure has previously been defined (Hellinga and Richards 1991). This approach has been successfully used to create a Cu-binding site in the *E. coli* thioredoxin protein via site-directed mutagenesis of the gene encoding this molecule (Hellinga et al 1991). These lines of research have provided considerable information regarding the nature of metal-binding sites.

Metal-binding sites of proteins are formed from the side chains of amino acids (although other areas of the molecule such as the N-terminus, and the amide and carboxyl groups of the protein backbone can interact with metal ions). The amino acids which are most important in this regard are: glutamate, aspartate and tyrosine (providing available oxygen groups); histidine, lysine and arginine (nitrogen); and cysteine and methionine (sulphur). Of these, histidine and cysteine are most commonly found at the metal-binding sites of proteins (e.g. in MTs and Zn-finger proteins). Adjacent amino acids which do not play a direct role in metal coordination can contribute to the strength of the binding site. It is thought that the proximity of aromatic amino acids, for example, enhance binding to histidyl residues (Leporati 1986). Because of the complexity of protein molecules due to tertiary structure, and because of effects of residues not directly associated with metal coordination, binding sites in proteins are often not discernable purely by sequence analysis, but require more vigorous investigation (Berg 1986). The preferred associations of selected metal ions with liganding atoms is expressed in table 1.1.

Oxygen	Oxygen/Nitrogen/Sulphur	Nitrogen/Sulphur
Na K	Ga Cr Pb Fe Ag	Cu Zn
Mg Ca Al	Tl Cd Mo Mn V	Co Hg Ni

Table 1.1 The preferred ligand(s) for various metal ions. The table shows the ligand(s) with which various metals will associate. The partition of metal ions into such groups is not strict, as most metal ions will show some affinity for all three ligands. Only Al shows complete specificity (from Hughes and Poole 1989).

Knowledge concerning the nature of metal-coordination sites may, in the long term, allow the design of sites capable of selective binding of metal ions. This will involve detailed analysis of natural molecules and also, as described above, the design and characterisation of novel molecules. Considerable research has been focused in this area, and novel metal-binding proteins and peptides have been synthesised (both chemically and by expression in *E. coli*). Research involving synthetic analogues of MT and PC is discussed in chapter 5.

1.5 Metallothionein.

Metallothionein (MT) was first isolated from equine renal cortex by Margoshes and Vallee (1957) who were attempting to identify a biological role for Cd. Subsequent biochemical analyses of the protein (Kägi and Vallee 1960; Kägi and Vallee 1961) revealed that it was of low molecular weight, had a high content of sulphur (due to cysteine residues), high cadmium and zinc content, and was low in aromatic amino acids. Since this initial description of MT, similar proteins have been isolated from a variety of sources, and biochemical and genetic analyses have provided a wealth of information about these molecules. Several reviews give an exhaustive examination of the biochemistry and molecular genetics of MT (Hamer 1986; Kägi and Kojima 1987; Kägi and Schäffer 1988; Waalkes and Goering 1990; Andrews 1991). A summary of the information contained in these reviews, and of other recent research, is presented below.

1.5.1 Classification and occurrence of MT.

The characterisation of MT by Kägi and Vallee (1960; 1961) has served as the basis for classification of MT molecules. Thus, MTs are defined as low molecular weight, cysteine- and metal-rich proteins and polypeptides, having few aromatic or hydrophobic amino acids, and with demonstrated structural or functional homology to mammalian MT. Class I MTs are defined as proteins with locations of cysteine residues closely resembling those of equine MT, whereas class II MTs are proteins in which the positions of the cysteine residues are not closely related to those of equine MT. Additionally, all MTs isolated from vertebrates (class I MT) can be sub-divided further (on the basis of ionic charge differences) into isoforms MT-I and MT-II, each isoform potentially comprising several isoproteins. Phytochelatin [or $(\gamma Glu\text{-}Cys)_nGly$], have been termed class III MTs. These molecules differ from classes I and II in terms of structure, synthesis and occurrence, and are therefore discussed separately (section 5.1).

MTs have been isolated from a wide range of vertebrate and invertebrate animals (cited in Hamer 1986). They are isolated in association with metal ions (generally Cd, Zn and Cu), the composition of which varies dependent on the organism, tissue and history of metal exposure. For example, MT isolated from human liver contains almost exclusively Zn, whereas that from kidney normally contains both Cd and Cu. MTs which are exclusively Cu-thioneins have been isolated from the lower eukaryotes *Candida glabrata* (Mehra et al 1988), *Saccharomyces cerevisiae* (Karin et al 1984) and *Neurospora crassa* (Lerch 1980). Various prokaryotes have been shown to produce MT-like proteins (cited in Silver and Misra 1991). One such protein has been purified and sequenced (Olafson et al 1988), and the corresponding gene from the cyanobacterium *Synechococcus* PCC7942, has been isolated (Huckle et al 1992; chapter 3). Additionally genes capable of encoding proteins with homology to class I MTs have recently been discovered in higher plants (de Miranda et al 1990; Evans et al 1990), but the putative products of these genes remain to be isolated (reviewed in chapter 4).

1.5.2 Structure and metal binding characteristics of MT.

Most research concerning the structure of MT relates to mammalian MT, which has served as a model for MTs in other systems. Mammalian MT consists of 61 or 62 amino acids, of which 20 are cysteines. A

consensus sequence derived from those of 14 mammalian MT sequences is shown in figure 1.1.

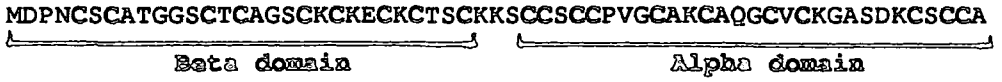


Figure 1.1 A consensus sequence for mammalian MT derived from the sequences of 14 mammalian MTs. Invariant cysteine residues are shown in bold type. [Reproduced from Hamer (1986), in which original references are cited].

In native MT isolated from mammalian tissue all cysteine residues are deprotonated and participate in metal ion binding. A variety of different biochemical and spectroscopic techniques have revealed the metal binding characteristics of the protein. In native MT from non metal-stressed renal tissue, the alpha and beta domains bind 4 and 3 divalent metal ions respectively. Data, initially from comparison of the low energy bands in the far UV absorption spectra of MT with those of tetrahedral halide complexes, revealed that the metal ions in MT were coordinated tetrahedrally (Vasák et al 1981). Confirmation of this has come from various spectral techniques, for example; EXAFS (Hasnain et al 1987), PAC (Vasák and Bauer 1982) and NMR (Schultze et al 1988). The alpha and beta domains can be separated by digestion of the linking amino acids using subtilisin, and each domain is found to bind metal ions in the same way as in the complete molecule (Winge and Miklossy 1982). Thus, the 11 cysteines of the alpha domain coordinate 4 metal ions through 5 bridging and 6 terminal sulphur ligands. The beta domain has 6 terminal and 3 bridging thiolate ligands.

In addition to binding Zn and Cd, mammalian MTs also bind Cu(I) *in vivo*, and by pre-administration can be isolated in association with various other metals (section 1.5.4). In the case of the mono-valent metal ions Au(I), Cu(I) and Ag(I), 12 g atoms of metal are bound per mole of MT, six ions in each of the two domains. Although less spectroscopic data for such complexes is available, EXAFS measurements on Cu-MT suggest that the 12 Cu ions may each be coordinated to three cysteines in a trigonal structure (Winge 1987). Using *in vitro*

substituted MT, Nielson et al (1985) were able to determine the relative binding affinities for metal-MT complexes to be in the order: Hg(II) > Cu(I) > Cd(II) > Zn(II) > Ni(II),Co(II).

The cysteine residues of the Cu-MTs isolated from *N. crassa* (Beltrami and Lerch 1983) and *Agaricus bisporus* (Munger and Lerch 1985) align perfectly with those of the N-terminal region of mammalian MTs, which results in these proteins being termed class I MTs. The MT-like proteins which have been isolated from other lower eukaryotes (e.g. the Cu-MTs of *S. cerevisiae* and of *C. glabrata*), and the Cd/Zn MT of the cyanobacterium *Synechococcus* PCC7942, however, not only diverge from vertebrate MTs, but generally show little sequence similarity to one another. They do, however, contain the typical cys-cys, cys-X-cys and cys-X-X-cys sequences which are characteristic of MTs.

Metal coordination characteristics of the Cu-MTs from lower eukaryotes have been shown to be similar, with data from EXAFS and NMR suggesting Cu-clustering in each case. A trigonal arrangement for Cu/S coordination has been proposed for both the *S. cerevisiae* MT (CUP1) and its regulator protein, CUP2 (cited in Nakagawa et al 1991). Smith et al (1986) examined the Cu-MT of *N. crassa* and found Cu-S distances suggestive of Cu clustering. They postulate that a Cu-S core is surrounded by a second shell of S atoms from surrounding cysteine residues.

1.5.3 Molecular genetics of MT.

The organisation and regulation of MT genes has been the subject of much research, and has been reviewed from several perspectives (Andrews 1991; Palmiter 1987; Hamer 1986). Mammalian MT genes are encoded by three exons interrupted by two large introns. The third exon encodes the entire alpha fragment of the molecule. Transcription is controlled by a number of upstream activator and enhancer sequences which regulate transcription of MT genes in reponse to various inducers (Karin et al 1987, see section 1.5.5). Current research is focussed on isolation of the *trans*-acting transcription factors which associate with these regions (Andersen et al 1990). The organisation of lower eukaryotic MT genes is considerably less complex than mammalian systems. The *trans*-acting factor (CUP2) which controls activation of the *S. cerevisiae* MT gene (CUP1) has been isolated

(Welch et al 1989), and its Cu-dependent interaction with CUP1 upstream activation sequences studied (Nakagawa et al 1991).

All MT genes examined show increased mRNA accumulation via increased transcription in response to administration of metal ions. Mammalian MT genes respond both to the essential trace metals Zn and Cu, and to a range of toxic metals. The Cu-thionein of *N. crassa* has been shown to respond only to Cu (Munger et al 1989), and whereas the CUP1 gene of *S. cerevisiae* is activated by both Cu and Ag, the Ag-induced protein does not bind Ag in *S. cerevisiae* cells (Hamer 1986). Induction of MT genes, and other metal-responsive genes, is discussed in greater detail in chapter 6.

1.5.4 The role of MT in metal detoxification.

Whether or not detoxification of supra-optimal concentrations of metal ions is the only function of MT in animal cells is a subject of debate (discussed in section 1.5.5). That MTs have a major role in the detoxification of metal ions is indicated by several lines of evidence, as described below.

Mammalian MT synthesis is induced by metal ions (Zn, Cu, Cd, Hg, Pb, Bi, Ag, Au and Pt), and the protein is found in association with the inducing metal ion (Webb 1987). Cd detoxification by MT molecules has been studied in many species, and the link between MT induction by Cd and high-affinity sequestration of Cd by the induced protein is well established (Kägi and Kojima 1987).

The role of MT in metal detoxification has been further established by examination of cells in which MT production is altered. Mammalian cells in culture in which MT gene transcription was decreased, exhibited decreased tolerance to Cd (Compere and Palmiter 1981). These cells, in which MT transcription was depleted via altered methylation of the gene, were normal in all other respects. Similarly, Hamer et al (1985) reported that *S. cerevisiae* cells in which CUP1 was deleted via replacement with a heterologous marker gene were Cu-hypersensitive. In all other aspects of cellular metabolism (cell doubling time, sporulation, germination, cell mating and diplophasic growth) the CUP1-deleted cells were normal. In addition, transfection of the CUP1-deleted cells with plasmids which contained either the CUP1 gene or monkey MT-I gene under the control of CUP1 regulatory sequences, protected the cells from Cu toxicity (Theile et al 1985).

In contrast to MT-deleted cells, several authors have reported that amplification of MT genes results in increased tolerance to metal ions. Amplification of the CUP1 gene in *S. cerevisiae* resulted in increased resistance to Cu ions (Karin et al 1984). Similarly, Mehra et al (1990) selected *C. glabrata* cells for increased Cu resistance, and demonstrated that these cells had a stable chromosomal amplification of the MT-II gene to around 30 copies. These cells were resistant to a concentration of Cu 14 times that required to inhibit growth of wild-type cells. Beach and Palmiter (1981) demonstrated MT gene amplification in mammalian cell lines which were selected for increased Cd resistance.

The protective effect of MT against Cd toxicity in whole animals has been demonstrated by examination of tissues in which MT synthesis is not inducible. Mouse testes are more highly susceptible to the toxic effects of Cd than other tissues (Rehm and Waalkes 1988; Waalkes et al 1988), an effect which has been attributed to the lack of MT gene inducibility in this tissue (Durnam and Palmiter 1981). A low molecular weight Cd-binding protein distinct from MT has been isolated from mouse testes following pretreatment with Cd. The low cysteine content of this protein may imply reduced Cd affinity, and hence result in increased Cd toxicity (Waalkes and Goering 1990).

1.5.5 Proposed alternative functions of MT.

Although MT is involved in detoxification of excess of certain free metal ions in animals and fungi, alternative functions in cellular metal metabolism not directly related to metal ion detoxification have also been proposed (discussed by Karin 1985). Several lines of evidence point to alternative roles for MTs. Firstly, the fact that the protein can be isolated from healthy mammalian liver and is generally 100 % Zn-MT, indicates that the protein in this tissue is unlikely to be involved in detoxification. Secondly, the long list of inducers of MT genes, which includes UV or X-ray irradiation, infection, starvation and administration of substances as diverse as chloroform and glucocorticoids, strongly suggests that MTs are not concerned solely with detoxification of excess metal ions via sequestration (Kägi and Shäffer 1988). A third line of evidence comes from studies in which the temporal and spatial expression of MT genes has been examined. Animal MT expression has been shown to vary widely

the organism. Of particular interest is the programmed regulation of MT genes in rat foetal development (Kern et al 1981). Recent studies have also shown changes in sub-cellular localisation of MT in cultured hepatocytes, a clear shift from cytoplasm to nucleus being observed in early S-phase (Tsujikawa et al 1991). Some authors have speculated that Zn-MT may act as a specific activator of those enzymes requiring Zn. Activation *in vitro* of such enzymes by Zn-MT has been demonstrated, however the significance of these experiments is called into question by the fact that inorganic Zn salts activate these enzymes equally effectively. Similarly, Zeng et al (1991a; 1991b) were able to inhibit DNA binding of the Zn-dependent transcription factors Sp1 and TFIIIA *in vitro* by the addition of apo-MT, thus abolishing transcription activation in an *in vitro* assay. These authors postulate that similar activity *in vivo* could potentially act as a control mechanism for modulation of a large subset of genes whose transcription is activated by Zn-dependent transcription factors.

As summarised by Hamer (1986), the sole function of the Cu-MT of *S. cerevisiae*, is likely to be detoxification of excess intracellular Cu. Mammalian MT, however, in addition to a role in sequestration of excess intracellular metal ions, may have alternative roles involving storage and transport of essential metals such as Zn and Cu.

1.6 Phytochelatins [$(\gamma Glu\text{-}Cys)_nGly$].

The molecules referred to in this report as phytochelatins (PCs), having the general structural formula $(\gamma Glu\text{-}Cys)_nGly$, were first isolated by Murasugi et al (1981) from Cd-resistant *Schizosaccharomyces pombe* cells and named cadystin. Molecules of identical structure were subsequently isolated from several species of plant, yeast and algal cells. They have variously been referred to as PCs, poly(gammaglutamylcysteinyl)glycines and class III MTs (cited in Kägi and Kojima 1987). These molecules are discussed in detail in chapter 5.

1.7 Potential applications of metal-binding molecules.

Metal-binding molecules have potential applications, which were summarised in relation to the *S. cerevisiae* MT by Butt and Ecker (1987). The use of MTs in metal recovery, either for scavenging of precious metals or for removal of toxic metals from wastes prior to

precious metals or for removal of toxic metals from wastes prior to dumping, have received some attention. At present, however, the use of either the isolated proteins, or of organisms engineered to express these proteins and hence bioaccumulate metals, is exclusively experimental.

1.7.1 The use of metal-binding molecules for removal of metals from solution.

The ability of MTs and PCs to bind a variety of metal ions has led to investigation into the use of these molecules for the removal of metals from metal-contaminated solutions. Legislation in the UK, which places Cd on a list of ten substances for which landfill and sea dumping is restricted, has led to increasing pressure to develop effective methods for dealing with Cd-contaminated wastes at source. Several methods using MTs and related molecules have been successfully patented. Jackson et al (1990) patented a system in which immobilised PC molecules were used to strip metals from solution. A similar metal-chelating matrix, in which the structure of PC molecules was built into the design of a chemically synthesised resin was made by Yin and Blanch (1989). In both cases removal of metal ions from solution was demonstrated, and the metal could subsequently be stripped from the matrix using low pH buffers.

Similar systems have been developed in which immobilised biomass (both eukaryotic and prokaryotic cells) have been used to bioaccumulate metal ions from metal-containing solutions. Macaskie et al (1986) developed a method which utilised *Citrobacter* sp. immobilised on glass beads or in polyacrylamide gels. The immobilised cells were found to be capable of stripping Cd from solution. Up to 70 % of Cd could be removed from 16 l of a 200 µg/ml solution at a flow rate of 70 ml/h. It was found that the metal was removed via the formation of a metal-phosphate precipitate on the cell surface (Macaskie et al 1987). Pb, U, and Ag could also be accumulated by the immobilised cells. Several other systems involving the use of immobilised biomass for the removal of metal ions from solution have been described, although in most cases the ligand responsible for binding metal ions has not been elucidated. Brierley et al (1989) used immobilised non-living microbial biomass of mixed populations to remove Ag, Cd, Cu, Zn and Pb from dilute solutions. Au could also be accumulated from solutions of

$AuCN_3$. Greene and Darnall (1989) developed the use of algal and cyanobacterial cells immobilised in a silica polymer matrix for the accumulation of Au from dilute solutions. The matrix could also be used for removal of Cd, Cu, Pb and Hg from contaminated wastes. Systems such as those described above may have commercial potential in removal of metal ions from contaminated wastes prior to dumping, or to recovery of precious metals from dilute solutions.

1.7.2 The use of MT genes to alter metal-tolerance and -accumulation.
An alternative strategy to that outlined in section 1.7.1 is to use whole organisms expressing MT genes to accumulate metal ions. Bacteria harbouring heterologous MT genes are capable of sequestration of metal ions from their growth medium. As described in section 1.8, for example, *E. coli* expressing MT genes are capable of increased metal accumulation. Similar results are likely to be obtained with other species. Butt and Ecker (1987) reported that *S. cerevisiae* cells selected for increased gold tolerance accumulated the metal in association with MT, indicating that precious as well as toxic metals could potentially be recovered using whole organisms expressing MT genes.

In some situations, it may be desirable to increase metal resistance of a particular species. Said and Lewis (1991) reported that microbial degradation of organic chemicals was affected by relatively low concentrations of metal ions (Hg, Zn, Cd, Cr and Cu). It may be possible in the future to introduce MT genes into biodegrading organisms to increase metal tolerance, hence allowing the degradation of metal-contaminated organic wastes.

Misra and Gedamu (1990) expressed the human MT-II gene in both tobacco (*Nicotiana tabacum*) and oilseed rape (*Brassica napus*), and reported increased metal tolerance in both cases. Similar results were reported by Lefebvre et al (1987) for the expression of human MT-II in *N. tabacum*. As well as potentially generating plants capable of reclamation of metal-contaminated land, the use of MT genes in this way may allow tissue-specific sequestration of metal ions in non-consumed parts of edible crops.

1.8 Expression of MT genes in *E. coli*.

Although there have been some difficulties reported in the expression of MT and related genes in *E. coli*, either due to low protein stability or to assumed toxicity to the cell (Berka et al 1988; Hou et al 1988; Kay et al 1991), various MT genes have recently been studied in this way. MT genes from diverse sources such as yeast, monkey and rainbow trout (see below) have been successfully expressed and have been found to assume the correct conformation to bind metal ions *in vivo*. Such studies have examined alterations in the uptake and detoxification of metals by *E. coli* which over-express MT, in order to assess potential biotechnological uses of these genes, and to provide information concerning the metal binding characteristics of the expressed proteins *in vivo*.

Romeyer et al (1988) and Jacobs et al (1989) reported the expression of human MT-II as a fusion protein both to AraB and to the membrane protein Lpp. In the latter case, the fusion protein was directed to the outer membrane. Both proteins were found to bind Cd, and their expression increased Cd and Cu accumulation from medium supplemented with these ions. Kille et al (1990) reported that the expression of rainbow trout MT resulted in increased Cd, but not Cu or Zn accumulation by *E. coli* cells. Expression of the *S. cerevisiae* MT (Berka et al 1988) and of the *N. crassa* MT (Romeyer et al 1990), both resulted in increased Cd and Cu accumulation, and, in the case of the *N. crassa* MT, Cd and Cu were accumulated selectively from a mixture of 16 different metal ions. Hou et al (1988) expressed mouse MT-II in *E. coli*, and reported that the induced protein led to increased tolerance to a variety of metal ions (Hg, Ag, Cd, Pb, Cu and Zn). To date, this is the only report of increased metal tolerance brought about by the expression of a heterologous metal-binding protein in *E. coli*. Induction of the MT gene also resulted in increased Cd accumulation by the cells (accumulation of other metals was not analysed).

There is an apparent anomaly in the observation that the expression of several metal-binding proteins in *E. coli* which, though resulting in increased accumulation of specific metals, show no apparent alteration of the host cell's tolerance to the accumulated metals. It is also observed that proteins which have demonstrated ability to bind specific metal ions *in vitro* do not necessarily accumulate these ions *in vivo* when expressed in *E. coli*. The possible reasons for observed

phenotypic alterations in *E. coli* expressing heterologous metal binding proteins are discussed in detail in chapter 7 in relation to results obtained during the course of this research.

Uptake and accumulation of metal ions by *E. coli* cells expressing metal ligands will be affected by the endogenous ligands present in the cell. As has been noted in several studies, *E. coli* cells are naturally resistant to relatively high concentrations of metal ions (Romeyer et al 1988), however the mechanisms or physiological characteristics responsible for this high level of resistance are currently poorly characterised. Recent data has been presented concerning the genes involved in copper uptake and transport (*cut* genes; Rouch et al 1989), however only preliminary data has been reported concerning the proteins involved in this system (section 3.1). Less information is available on Zn uptake and homoeostasis in *E coli* cells, though it is assumed that a system analogous to that identified for Cu must exist. Rouch (1986) noted that mutations in the genes involved in Cu metabolism also caused alteration in the response of *E. coli* to Zn, indicating that these ions may share some components of a transport system. Cd, being non-essential, must enter the cell via non-specific uptake. The mechanism by which Cd enters the *E. coli* cell has not been elucidated. It is possible that uptake may occur via the transport system of an essential metal, as is the case in *Staphylococcus aureus* in which the Mn transport system is involved in the uptake of Cd. Several attempts have been made to isolate endogenous *E. coli* ligands concerned with Cd tolerance (section 3.1), and evidence has been presented that such proteins are induced in response to Cd stress (Khazaeli and Mitra 1981). Since no data is available concerning the affinities of these endogenous proteins for metal ions, their effect on metal coordination by heterologous MTs is difficult to assess, however such ligands will compete *in vivo* with heterologous MTs for intracellular metal ions. Additionally, the high degree of tolerance exhibited by *E. coli* cells to toxic metals, may conceal any additional protective effect of expressed proteins.

1.9 General aims of the research.

The research described in this thesis involves the expression of several metal-binding proteins in *E. coli* and in the cyanobacterium *Synechococcus* PCC 7942. This was undertaken in order to gain

information regarding the metal binding properties of these molecules, and to examine changes in the host cell's metal metabolism brought about by their expression. Such information will assist in clarifying the biological role of the molecules concerned, and also allow assessment of the their potential biotechnological applications, as outlined in section 1.7. The results obtained are presented in four separate chapters, each with an introduction to the subject area and an outline of the specific aims of the research.

CHAPTER 2 GENERAL MATERIALS AND METHODS.

2.1 Materials.

2.1.1 *E. coli* strains.

The *E. coli* (K12) strains used in this research were JM101 [*sup*E, (*lac-pro*AB), {F'*tra*D36, *pro*AB, *lac*I^{q}Z M15}, (r_{k+}, m_{k+}), *mcr*A(+)] and DH5α {F'/*end*AI, *hsd*17, (r_{k-} m_{k+}), *sup*E44, *thi*1, *rec*A1, *gyr*A, (Nal^{r}), *lac*ZY$_{A}$, -*arg*^{F}, U169, 80d*lac* (*lac*Z M15)}. Both strains were obtained from Northumbria Biologicals Ltd., Cramlington, Co. Durham.

2.1.2 Plasmids.

The following commercially supplied plasmids were used: pUC18, pUC19, pGEM4z, pGEX3x, and Bluescript KS^{+}. pUC18, pUC19 and Bluescript KS^{+} were obtained from Boehringer Mannheim, Lewes, UK. pGEM4z was obtained from Promega Ltd., Enterprise Rd., Southampton, UK. Full descriptions of these plasmids are given in Sambrook et al (1989), in which original references are cited. pGEX3x (Smith and Johnson 1988) was obtained from Pharmacia LKB Biotechnology, and is described in section 3.2. Other plasmids used during the course of this research were pJHNR4.9 (Huckle et al 1992; described in section 3.1), pGP1.2 (Tabor and Richardson 1985; section 5.2), pUC105 (Kuhlemeier et al 1981; section 6.2) and pHP45Ω (Prentki and Krisch 1984; section 6.2).

2.1.3 Chemicals, reagents and laboratory consumables.

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

Taq polymerase; Perkin-Elmer/Cetus, ILS Ltd., Newbury St., London.

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

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

Ficoll-400, Sephadex gel permeation products, PD-10 (G-25 pre-packed Sephadex) columns; Pharmacia LKB, Milton Keynes, UK.

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

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

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

PPO; Koch-Light Ltd., Colnbrooks, Berks., UK.

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

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

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

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

Scintillation fluid (Ecoscint A); National Diagnostics, Mannville, New Jersey.

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

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

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

2.1.4 Metal salts.

Metals were used as the following salts:

$CdSO_4.8H_2O$; $NiCl_2.6H_2O$; $(C_2H_3O_2)Pb.3H_2O$; $ZnCl_2$; $CuCl_2.2H_2O$; $CrCl_3$; $CoCl_2.6H_2O$; $HgCl_2$.

2.2. Media and buffers.

2.2.1 Buffers used in DNA and RNA manipulations.

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

2.2.2 *E. coli* growth media.

Luria-Beltrami (LB) medium and M9 minimal medium were as described in Sambrook et al (1989).

TSS (transformation and storage solution for preparation of competent cells); LB broth at pH 6.5 containing 10 % w/v PEG 8000 and 50 mM $MgSO_4$. 5 % DMSO v/v was added to this prior to use.

2.3 Methods.

2.3.1 Atomic absorption spectrophotometry (AAS).

Atomic absorption analysis for measurement of the concentration of metal ions in solution was performed using a Perkin Elmer Model HGA spectrophotometer. Analyses were performed according to manufacturer's protocols. Five replicate readings each of 3 sec duration were taken automatically for each sample, and the results averaged. Metal was quantified via the construction of a calibration graph, which was re-plotted for each set of measurements.

2.3.2 The use of dithizone to detect Hg.

It was not possible to measure Hg by AAS. Instead, Hg was measured using the metal-responsive chemical dithizone. A micro-titre plate test for the analysis of Cd has been described (Lindsay 1988). It was found that Hg could be measured using the same method, as outlined below.

1) 200 µl of freshly prepared dithizone solution (0.1 mg/ml in 1 N NaOH) was added to the wells of a micro-titre plate.

2) 100 µl of test solution was added. (Tests were performed in duplicate).

3) The resulting colour change was measured using a Titretek (Flow Laboratories) micro-titre plate reader by absorbance at 540 nm.

Concentrations were estimated by reading from a standard curve measured using the same methods. Hg could be quantified in this way to a minimum concentration of 1 µM.

2.3.3 General molecular biology methods.

(Those methods not described in detail in this section or in the separate methods sections presented for each chapter were performed as described by Sambrook et al 1989).

2.3.3.1 Plasmid mini-preps from *E. coli* by alkaline lysis.

(Modified from Birboim and Doly 1979).

1) *E. coli* cultures (5 ml) were grown in LB broth for 16 h at the appropriate temperature and with appropriate selection, dependent on the plasmid concerned.

2) An aliquot (1.5 ml) was removed to an Eppendorf microfuge tube, the cells harvested by microcentrifugation (MSE microcentaur microcentrifuge), and the supernatant removed.
3) The cells were resuspended in 100 µl of an ice cold solution of 50 mM glucose, 10 mM EDTA, 25 mM tris-Cl (pH 8.0).
4) After 5 min at room temperature, 200 µl of a solution of 0.2 N NaOH, 1 % SDS, was added, the contents mixed by inversion, and the tube placed on ice for 10 min.
5) 150 µl of an ice cold solution of potassium acetate was added, the contents mixed, and the tube incubated on ice for a further 10 min.
7) After microcentrifugation at high speed for 5 min, the supernatant was removed to a fresh tube, RNAse A was added to a final concentration of 20 µg/ml, and the tube incubated at 37 °C for 30 min.
8) 500 µl of phenol/chloroform (1 : 1) was added, the contents mixed, centrifuged at high speed, and the aqueous phase transferred to a fresh tube.
9) 2.5 volumes of 100 % ethanol was added, and the tube left at -80°C for 10 min.
10) Plasmid DNA was rescued by microcentrifugation at high speed for 5 min, the pellet washed using ice cold 70 % ethanol, and dried briefly under vacuum.
11) Plasmid DNA was resuspended in 16 µl of water, 4 µl of 4 M NaCl added, followed by 20 µl of 13 % polyethylene glycol, and the tube incubated on ice for 30 min.
12) Purified plasmid DNA was recovered by microcentrifugation, washed using 70 % ethanol, and dried under vacuum. The pellet was finally resupended in 20 µl of water.

2.3.3.2 Plasmid maxi-preps from *E. coli*.

Large-scale preparations of plasmid DNA from *E. coli* were performed essentially as a scale up of steps 1 to 10 of the mini-prep protocol as given in section 2.3.3.1. 500 ml of culture was used (collected by centrifugation at 4000 rpm; Beckman centrifuge). Further centrifugation steps were performed using 30 ml glass corex tubes at 10 000 rpm using a Beckman JA-20 rotor. When greater purity of plasmid DNA was required, the following steps were included:

tubes, and the tubes heat-sealed.
3) Centrifugation was performed at 50 000 rpm for 16 h using a Sorval OTD65B ultracentrifuge.
4) Nucleic acid bands were visualised under UV illumination, and the plasmid band removed using a 10 ml syringe.
5) Ethidium bromide was removed by extraction with isoamyl alcohol saturated with $CsCl_2$, and salts removed by dialysis against TE buffer (pH 8.0).
6) Plasmid DNA was precipitated using 2.5 volumes of 100 % ethanol.

2.3.3.3 Preparation of RNA from *E. coli*.

(Kornblüm et al 1988)
1) 1.5 ml of culture was removed to a microfuge tube, and the cells harvested by microcentrifugation at 1600 rpm for 5 min.
2) The supernatant was removed, and the cells resuspended in 100 µl of TES-sucrose (20 % sucrose, 10 mM EDTA, 50 mM NaCl, 20 mM tris; pH 7.6) containing 1 mg/ml lysozyme, and incubated on ice for 15 min.
2) 100 µl of 2 % SDS was added, followed immediately by 10 µl of 10 mg/ml protease K, and the tube shaken at room temperature for 15 min.
3) The lysate was frozen (in liquid nitrogen) and thawed at 45 °C three times, prior to addition of 50 µl of loading dye (40 % glycerol, 100 mM EDTA, 0.1 % bromophenol blue, 0.1 % xylene cyanol).
The integrity of the RNA was checked, and its approximate quantity estimated by electrophoresis on a 1 % agarose mini-gel.
Prior to separation of the RNA by formaldehyde-agarose gel electrophoresis (section 2.3.3.10), the lysate was mixed in a solution containing 50 % formamide, and 1X MOPS (final concentrations), and incubated at 65 °C for 10 min.

2.3.3.4 Preparation and transformation of competent *E. coli* cells.

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

tube, mixed with plasmid DNA, and incubated at 4 °C for 30 min. The cells were then diluted by the addition of 0.9 ml of TSS, and incubated at 37 °C for 1 h. Aliquots of transformed cells were then plated onto LB agar containing the desired selective agent.

2.3.3.5 Agarose gel electrophoresis of DNA.

Agarose gel electrophoresis was performed as described by Sambrook et al (1989). Generally 0.8 % agarose gels were used. Higher concentrations up to a maximum of 2 %, as specified for each experiment, were used where small fragments (to a minimum of 100 bp) were to be separated. Maxi-gels were cast using tris-acetate buffer (40 mM tris-acetate [pH 7.7], 2 mM EDTA), and mini-gels using tris-borate buffer (89 mM tris-borate, 2 mM EDTA). DNA was loaded into the wells of the gel after addition of loading dyes (0.25 % each of bromophenol blue and xylene cyanol, 15 % w/v Ficoll 400). In all gels presented, the size markers employed were lambda phage DNA restricted with PstI. The restriction fragment sizes produced by PstI digestion of lambda DNA which are visible on such gels are as follows (in base pairs): 11581, 5077, 4749, 4507, 2838, 2540, 2459, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448, 339, 264, 247, 216, 211, 210, 164, 150 and 90.

2.3.3.6 Isolation of restriction fragments from agarose gels.

Gel slices containing the fragment to be cloned were cut from agarose or polyacrylamide gels using a clean scalpel blade, and purified from the gel block by electroelution (Sambrook et al 1989). The DNA was purified by phenol extraction and precipitated using 2.5 volumes of ethanol in a final concentration of 1 mM ammonium acetate. For fragments less than 1 Kb in size, 1 mM glycogen was added to assist precipitation. DNA was recovered by microcentrifugation, dried and resupended in water.

2.3.3.7 Cloning of DNA fragments.

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

2.3.3.8 Radioactive labelling of DNA.

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

2.3.3.9 Polyacrylamide gel electrophoresis of DNA.

DNA fragments less than 50 bp in size were separated on 20 % polyacrylamide gels. Gels were prepared as described by Sambrook et al (1989), using a Bio-Rad Protean II gel system. Sizes were estimated by running oligonucleotides of known size in adjacent lanes.

2.3.3.10 Formaldehyde-agarose gel electrophoresis of RNA.

Formaldehyde-agarose gels were prepared and run as described by Sambrook et al (1989). Ribosomal RNA bands were used as size markers for these gels. The rRNA bands produced by the electrophoresis of *E. coli* or *Synechococcal* RNA and their sizes are indicated where gels are presented.

2.3.3.11 Southern and northern blotting.

DNA and RNA was transferred to nylon hybridisation membranes essentially as described by Sambrook et al (1989). DNA was denatured prior to transfer by soaking in an excess of denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 1 h, followed by neutralisation of the gel by soaking in an excess of neutralisation solution (1.5 M NaCl, 0.5 M tris; pH 7.5) for 1 h. Gels were blotted for 16 h using 10X SSC, after which time complete transfer of nucleic acids had occurred. Filters were then placed under vacuum at 80 °C for 1 h prior to hybridisation to radioactive probes.

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

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

2.3.3.13 Separation of proteins by gel permeation chromatography.

Gel permeation chromatography was performed using Sephadex (Pharmacia) beads. Columns were run at 4 °C, and were calibrated prior to use using Blue Dextran and $ZnCl_2$ to establish the void and total volumes respectively.

2.3.3.14 SDS-PAGE analysis of proteins.

SDS-PAGE was performed using a Bio-Rad Protean II gel system. Gels were prepared exactly as described by Hames and Rickwood (1988). Size markers "SDS 5" were obtained from Sigma Chemical Co. The sizes of the marker proteins are shown for each experiment. Gels were stained using either Coomassie Brilliant Blue or by Silver staining (Hames and Rickwood 1988). Protein samples were prepared prior to loading on the gel by boiling for 10 min in a solution consisting of 10 mM $NaPO_4^{2-}$, 2 % SDS, 5 % 2-mercaptoethanol, 10 % sucrose and 0.002 % bromophenol blue (final concentrations).

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

PCR reactions were carried out essentially as described by Saiki et al (1988). Reaction conditions were as follows: 200 µM each of dATP, dTTP,

dGTP and dCTP, 50 mM KCl, 10 mM tris-Cl [pH 8.3], 1.5 mM $MgCl_2$, 0.01 % gelatin (final reaction volume 50 µl). The concentration of each primer and the amount of template DNA added varied according to the particular experiment concerned. Taq polymerase was added to the reaction last, and the contents mixed and overlaid with mineral oil. Reactions were carried out using a Hybaid Intelligent Heating Block. Details of temperatures and time settings used to control denaturing, annealing, extension, and of the number of amplification cycles, are stated for individual reactions.

2.3.3.16 Automated DNA sequencing.

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

2.3.3.17 Synthesis of DNA oligonucleotides.

Oligonucleotides were synthesised using an Applied Biosystems 381A DNA synthesiser operated using standard protocols. Modification to this protocol for production of "trityl on" oligonucleotides to allow further purification is described in section 5.2.2.

CHAPTER 3 EXPRESSION OF A CYANOBACTERIAL GENE, *smt*A, IN *E. COLI*.

3.1 Introduction.

The Synechococcal gene *smt*A encodes a protein which has been termed a class II metallothionein (Robinson et al 1990b). Certain features of the protein, as discussed below, have indicated that it may be involved in detoxification of elevated concentrations of intracellular Cd and Zn, in a manner analogous to MTs in other systems. This is the only characterised protein to be ascribed such a function in a prokaryotic cell.

3.1.1 Metal tolerance mechanisms in prokaryotic cells.

Prokaryotes have evolved a wide range of defences against the toxic effects of metal ions. The existence of plasmids, and the relative ease of lateral transfer of genetic information has, however, resulted in the widespread occurrence of similar types of detoxification systems with related genes being found in several different species. The many metal detoxification systems found in prokaryotes are reviewed elsewhere (Silver and Misra 1991). Three basic types of mechanism have been described: (1) efflux pumping of the metal ion out of the cell; (2) redox conversion of the metal ion to a chemically less toxic form; and (3) sequestration of the metal by metal-binding molecules. The better characterised examples of each of these mechanisms are briefly discussed below, and examples of analagous systems are noted.

3.1.1.1 Cd efflux in *Staphylococcus aureus*.

Plasmid pI258, isolated from *S. aureus* contains genes which encode a system for energy-dependent Cd-efflux from cells exposed to toxic concentrations of this metal. This system is dependent on the production of a 79 KDa protein, the product of the *cad*A gene, which acts as a Cd pump in a similar fashion to the hydrogen ion transferring Na,K ATPases found in animal cell plasma membranes (Silver et al 1989). Analagous efflux mechanisms have been found to be responsible for metal ion detoxification in the following instances: (1) Cd, Zn and Co resistance in *Alkaliginese eutrophus* (Neis and Silver 1989), (2) arsenate resistance in *S. aureus* and *E. coli* (Silver and Keach 1982; Chen et al 1986) and (3) plasmid-encoded copper

resistance in *E. coli* (Rouch et al 1989). In the case of chromate resistance in *Pseudomonas fluorescens* (Ohtake et al 1987), which results in reduced accumulation, the mechanism (reduced uptake or increased efflux) has not been elucidated.

3.1.1.2 Hg detoxification by redox conversion.

The mechanism of resistance to Hg and organomercurial compounds is by chemical detoxification. Eight related Hg detoxification systems from both Gram positive and Gram negative cells have been studied, the best characterized being that found in *Bacillus* species (O'Halloran et al 1989). The Hg resistance operon typically consists of six to eight genes which act in the uptake, intracellular transport and chemical modification of the Hg or organomercurial compound. Organomercurial compounds are first cleaved by the enzyme organomercurial lyase, to give less toxic Hg^{2+} ions, which are converted to Hg° by mercuric reductase. Hg° is then volatilised from the cell. Other systems in which metal ions are detoxified by redox shift include: (1) Chromate reduction to Cr^{3+} by *Pseudomonas* sp. (Ishibashi et al 1990) and *Alkaliginese eutrophus* (Neis et al 1990); and (2) the oxidation of arsenic As^{3+} to As^{5+} by *Enterobacter cloaceae* (Wang et al 1989).

3.1.1.3 Intracellular metal-binding proteins.

The Cd- and Zn-binding protein isolated from *Synechococcus* (TX-20) by Olafson et al (1979), represents the only metal-sequestering protein so far isolated from a prokaryote which has been sequenced. This chapter involves characterisation of the metal binding properties of the protein via expression as a fusion protein in *E. coli*, and the known features of the protein are therefore discussed in detail in following sections.

Several other proteins which have been proposed to detoxify metal ions via sequestration in prokaryotic cells have been partially purified and characterised. Khazaeli and Mitra (1981) partially purified a Cd-binding protein of apparent molecular weight 39 KDa from *E. coli*. The protein was associated with greater than 60 % of the cytoplasmic Cd in cells which had been adapted to otherwise inhibitory levels of Cd. The protein was not detected in similar extracts from non-accommodated cells. This protein differs greatly in molecular weight to MT, and since no further characterisation was presented, its

relationship to MTs remains to be established. Similarly, several Cd-binding proteins were isolated from a Cd-resistant isolate of *Pseudomonas putida* by Higham et al (1984). Three distinct proteins were identified, having molecular weights from 3600 to 6900, and were shown to be capable of binding Cd, Zn or Cu. The proteins were shown to contain 10-22 % cysteine residues per molecule. NMR studies revealed a possible tetrahedral arrangement for binding of Cd ions. No further characterisation of these proteins has been reported, and so their relationship to MT molecules has not been established. A chromosomal gene in *E. coli* encoding a putative Cu-ligand involved in Cu metabolism, CutE, has been described by Rogers et al (1991). This protein appears to have functions other than in detoxification of free Cu ions, possibly involving cellular storage and transport of Cu.

3.1.2 Cyanobacterial MT from *Synechococcus* sp.

Olafson et al (1979) reported the isolation of a protein with similar properties to MT from the cyanobacterium *Synechococcus* TX-20. The protein was subsequently sequenced (Olafson et al 1988), and termed a class II MT (Kägi and Kojima 1987). It is the only prokaryotic MT which has so far been sequenced. The strain from which the protein was initially isolated (TX-20), is closely related to the strain designated PCC6301 by the Pasteur Culture Collection. A DNA fragment encoding part of the MT protein was amplified using DNA isolated from *Synechococcus* PCC6301 and cloned (Robinson et al 1990b). Subsequently, the analogous gene was isolated from a genomic library from a transformable variant of this organism, *Synechococcus* PCC7942 (Huckle et al 1992). Both strains PCC6301 and PCC7942 have also been referred to as *Anacystis nidulans*. The sequence of the genomic fragment from *Synechococcus* PCC7942 and of the encoded protein are presented in figure 3.1.

3.1.2.1 Primary structure of MT from *Synechococcus* sp.

The serine residue in position 32 of the protein sequence derived from the genomic DNA sequence (figure 3.1) was designated as a cysteine residue by Olafson et al (1988), however this residue was one of only two which was not confirmed in two independent protein sequence analyses. The DNA sequence derived by PCR from strain PCC6301 (Robinson et al 1990b) also had a serine residue at this position,

Figure 3.1 The sequence of part of the genomic fragment from *Synechococcus* PCC7942 contained in plasmid pJHNR4.9. The insert of plasmid pJHNR4.9 contains a 1.9 Kb fragment isolated from *Synechococcus* PCC7942, which includes the *smt*A coding region plus 650 bp and 1.1 Kb of sequence 5' and 3' to *smt*A respectively. All of the 5' sequence of the insert, and the 3' sequence comprising the transcript termination signal (underlined) is shown. Underlined in the 5' region is a dyadic sequence which may function in metal-regulated expression of *smt*A. The start and stop codons comprising a second open reading frame which is present in the region 5' to *smt*A, and reading in the opposite direction to *smt*A, are shown in bold type. The significance of these features is discussed in the text.

5' AAGCTTTACTACAACGAGCGCCGCTATCTACAGCA

ACTCGATCAAGAACGCTGCCTGAATCCCCAAGCATTCTTGGGCATGACAGAGCACGAT

GCTACTGCGATCGCCCCGACCACTCCCCAGCCGATTTCTGCCTAAGGTGCATCTCTAG

CGACACTCTTGTAAGTGATCGAGGGCGTTTTGATAAAGCGCCACAATGTGATGATCCT

GTAGCTGGTAGTAGACATGCCGCCCTTGCTTGCGATAGTCACCAGCCGCAGATTACGG

AGCGATCGCAATTGGTGAGACACCGCCGATTCGGAAACACCAATTGCCTGGGCCAAAT

CCCCAACACAGAGCTCCGATCGCGCTAACAGGGACAGCAACCGCAGTCGATTTGGATC

GGCCAGCACTGCAAAAAATTCGGCTAGCGATTGGGCAACTTCGGGTGCGATCGCTTGA

AGCTCCGAGGCGATCGCCGCATGAGTCCCTTGGCAGACTACCGTCTCTCCGTCCTGCA

GCACTGGTTTTGTCATGAGCCAATCACGGTTTGTCCACCCACCATACCTGAATCAAGA

TTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGATATTCAAAGGAGTTGCTGTC

met thr ser thr thr leu val lys cys ala cys glu pro cys leu
ATG ACC TCA ACA ACG TTG GTC AAA TGC GCT TGT GAG CCC TGT CTC

cys asn val asp pro ser lys ala ile asp arg asn gly leu tyr
TGC AAC GTC GAT CCC AGC AAA GCG ATC GAT CGC AAC GGT CTG TAC

tyr cys ser glu ala cys ala asp gly his thr gly gly ser lys
TAC TGC AGC GAA GCC TGT GCC GAT GGC CAC ACC GGT GGT AGC AAA

gly cys gly his thr gly cys asn cys his gly stop
GGC TGC GGC CAC ACC GGC TGT AAC TGC CAC GGC TAA TCAACTGTTTC

CCTGCTAATCCCCCATCAATCGAAACCGCTGGCTCCTCAATCATGGGCCAGCGGTTGAT

TATTTATAGGAGGTGCGATCGCGCAGCTTTACAACCCCTACTCGCCGGTGATCGAG 3'

suggesting that this designation is correct. Additionally, the sequence presented in figure 3.1 has two amino acids, histidine and glycine, at the carboxyl terminus which were not present in the original protein sequence.

There is no significant sequence homology between this protein and any other MT-like protein so far sequenced. Classification of the protein as a class II MT is therefore based on the arrangement of cysteine residues in motifs which are typical of MTs, and on observed association of the isolated protein with Cd and Zn ions. The sequence contains 8 hydrophobic residues, which makes this the most hydrophobic MT so far isolated.

3.1.2.2 Secondary structure of Synechococcal MT.

The studies so far reported on the secondary structure of the Synechococcal MT protein are by Olafson et al (1988). The native protein can be isolated in association with either Zn or Cd, dependent on the metal administered (see below). In addition, a small amount of Cu was found in association with the protein when induced with either ion.

Reverse phase HPLC analysis of the protein prepared from Cd-exposed Synechococcal cells resolved seven distinct peaks eluting at near isocratic conditions, all of which contained MT isoforms with ratios of Cd : protein of between 2.2 : 1 and 3.1 : 1. Only one of these peaks contained Cu in significant amounts. Zn was not detected in significant amounts in any of the MT peaks. Amino acid analysis of the peaks revealed no significant differences in the amino acid content of the various MT isoforms, indicating that the difference in retention observed during reverse phase HPLC analysis is more likely to be attributable to differences in metal speciation and disulphide aggregation than to differences in amino acid sequence. No evidence exists from genetic studies for more than one gene encoding the MT protein (Huckle et al 1992).

CD spectral analysis of the native Zn-protein exhibited some characteristics resembling mammalian Zn-MT (Olafson et al 1988). Addition of Cu to the metal-stripped apo-protein resulted in alteration of the CD spectrum to one with characteristics resembling *S. cerevisiae* MT, suggesting that the protein may be capable of binding Cu. As no further spectral analysis was performed, no

information on the spatial arrangement of the atoms has been published. In addition, no data has yet been presented regarding the relative affinity of the protein for metal ions. Such data would allow comparison of the metal binding characteristics of this protein with MT-like proteins from other systems, and thereby provide further information regarding the functions of the protein.

3.1.2.3 Induction of *smt*A expression.

Olafson et al (1980) reported that levels of Synechococcal MT were highly induced by supplementation of growth medium with either Zn or Cd (but not Cu), the protein being isolated in association with the inducing metal ion. Observation that MT induction is decreased by actinomycin D suggests that regulation is at the level of gene transcription. Studies on the induction of the *smt*A gene by various metal ions has indicated that several other metals (Hg, Ni, Cu and Co) result in increased transcript abundance (Huckle et al 1992).

3.1.3 Aims.

The aim of this research was to express *smt*A in *E. coli* in order to examine the metal binding characteristics of the expressed protein, both by *in vitro* analysis of the isolated protein, and by examination of the phenotypic effects of its expression in *E. coli*. Evidence that the product of *smt*A binds metal ions with a high affinity would support designation of this gene as the first isolated prokaryotic MT gene. The ability of the protein to bind metals other than Zn and Cd was examined (Hg, Cu, Ni, Cr and Co), in order to demonstrate whether or not the protein may be involved in homoeostasis and/or detoxification of these ions. Comparison of the metal binding characteristics of Synechococcal MT with equine MT was also undertaken to investigate the relationship between these molecules. Examination of the metal binding characteristics of the protein both *in vitro* and *in vivo* would also provide an assessment of the possible biotechnological applications of the *smt*A gene with regard to genetic alteration of other organisms for increased metal tolerance and/or accumulation.

3.2 Materials and methods.

3.2.1 Experimental design.

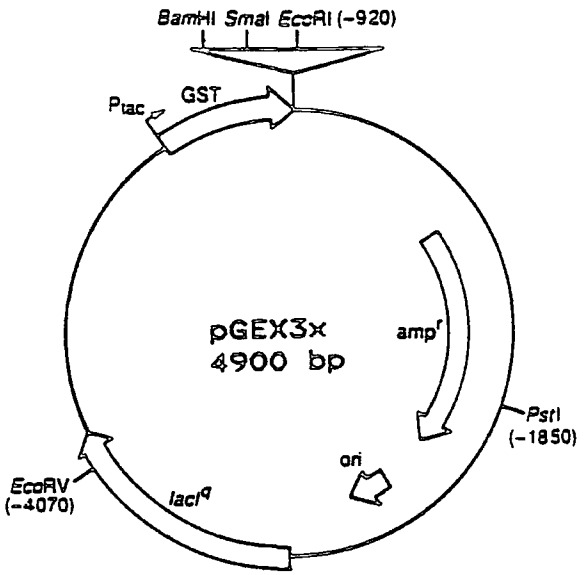
To gain expression of *smt*A in *E. coli*, the coding region of the gene was cloned via PCR amplification using the following primers: $^{5'}$GGCGGATCCCCATGACCTCAACAACCTTGGTC$^{3'}$ and $^{5'}$GGCGAATTCACTACAGTTGCAGCCGGTGTGGCC$^{3'}$. These anneal to the 5' and 3' ends of the *smt*A coding sequence respectively. The coding sequence was cloned into plasmid pGEX3x (section 3.2.2.1) to give plasmid pGPMT1. Vector pGEX3x gives high level of expression of cloned genes, and also allows the purification of preparative amounts of pure protein. The *smt*A coding sequence was expressed in this vector, and the protein isolated for *in vitro* analysis. The phenotypic effect of expression of the gene in *E. coli*, in terms of metal-tolerance and -accumulation was also assessed.

3.2.2 Materials.

3.2.2.1 *E. coli* expression vector pGEX3x.

Vector pGEX3x (figure 3.2) was obtained from Pharmacia LKB. Details of the construction and use of this vector are given by Smith and Johnson (1988), and in commercially supplied protocols and information. The plasmid allows cloning of DNA fragments to give carboxyterminal fusions to the glutathione-S-transferase (GST) protein encoded by vector sequences. Subsequent protein isolation is by association of the resulting fusion protein with glutathione-Sepharose using protocols as outlined below. The purified protein can subsequently be eluted from the glutathione-Sepharose matrix using reduced glutathione, which competes with the immobilised glutathione for binding to the GST active site. A DNA sequence encoding the amino acids Ile-Glu-Gly-Arg (the recognition sequence for serum cleavage factor Xa) is included just prior to the multiple cloning site of the vector. This means that treatment of resulting fusion protein with factor Xa will result in cleavage just before the start of the introduced protein. As described below, cleavage can be performed prior to elution from the glutathione-Sepharose matrix, and the cleaved fragment eluted free from the GST portion of the protein.

Figure 3.2 Plasmid pGEX3x. The design of the plasmid and polylinker sequence are shown. Factor Xa cleaves the resulting fusion protein after the arginine residue of the sequence Ile-Glu-Gly-Arg as indicated. Full details concerning the use of this clone are given in section 3.2.3.



Polylinker sequence:

```
                    __Factor Xa__
  P   K   S   D   L   I   E   G   R   G   I   P   G   N   S   S Stop
CCA AAA TCG GAT CTG ATC GAA GGT CGT GGG ATC CCC GGG AAT TCA TCG TGA
                                    BamHI  SmaI EcoRI
```

3.2.2.2 General materials.

Glutathione-Sepharose (Pharmacia LKB Biotechnology).

Factor Xa (Boehringer Mannheim).

Equine renal MT; this material was supplied by Sigma Chemical Co., and on analysis was found to comprise 5.2 nmoles Cd/nmole protein, and 1.8 nmoles Zn/nmole protein.

3.2.2.3 Buffers.

The following buffers, for use in pH displacement of metal ions from metal-binding molecules, were used to give a pH range from 1.0 to 9.5. (From Dawson et al 1990; pages 426-429):

pH range 6.0 to 9.4 - a 20 mM solution of K_2HPO_4 was titrated to the required pH using orthophosphoric acid.

pH range 3.8 to 5.5 - solutions of 200 mM NaOAc and 200 mM HOAc were mixed at the recommended ratios.

pH range 2.2 to 3.6 - solutions of 200 mM HCl and 200 mM glycine were mixed at the recommended ratios.

pH range 1.0 to 2.0 - solutions of 200 mM KCl and 200 mM HCl were mixed at the recommended ratios.

3.2.3 Methods.

3.2.3.1 DNA Manipulations.

The protocols used in cloning the *smt*A coding region were as outlined in chapter 2. Primers and reaction conditions for amplification of the coding region of *smt*A are described in section 3.2.1. and in the legend to figure 3.3 respectively. BamHI and EcoRI sites included in the sequence of the PCR primers were used to sub-clone the resulting PCR fragment into the pGEX3x polylinker as shown in figure 3.2.

3.2.3.2 Expression of GST or GST- fusion protein in *E. coli*.

For expression of GST or the GST-SmtA fusion protein in *E. coli*, cultures containing either pGEX3x or pGPMT1 (*smt*A-containing clone) were grown overnight in LB broth with 50 µg/ml Ap at 37 °C. These were diluted 1/10 in the same medium, and grown for 45 min under identical culture conditions. Metal was then added at the required concentration. (A non-inhibitory concentration, but one which was close to the inhibitory concentration, was chosen for each metal, as

judged using the method given in section 3.2.3.8.1. This was done to optimise the availability of the metal ion for association with the MT portion of the expressed protein. Thus, the levels chosen were: 0.5 mM Cd, 0.5 mM Zn, 2 mM Cu, 20 µM Hg, 1.8 mM Cr, 2.5 mM Co and 2.5 mM Ni. Addition of Pb at relatively low levels (500 µM) was found to result in precipitate formation, and association of the protein with Pb was therefore not tested). Incubation was continued for a further 15 min before addition of IPTG at a final concentration of 1 mM. Cells were then grown to an optical density of 0.5, and proteins extracted and purified as outlined below.

3.2.3.3 Purification of GST or GST- fusion protein from *E. coli*.

GST and GST-fusion proteins were extracted using the following method:

1) Cells were harvested by centrifugation at 3000 rpm, and resuspended in ice-cold PBS (150mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4; pH 7.3) containing 1 % Triton-X100.

2) Cell lysis was achieved by sonication (on ice) at full power for 6 x 30 sec with 30 sec between each burst of sonication.

3) Insoluble cell debris was removed by centrifugation at 10 000 rpm (Beckman JA-20), and the supernatant removed for purification of the protein of interest using glutathione-Sepharose.

4) A 5 ml Bio-Rad gel chromatography column containing 1 ml of glutathione-Sepharose beads was equilibrated in PBS, and the supernatant applied to th column.

5) 50 ml of PBS was passed through the column.

6) The purified protein was eluted from the column by application of 2 x 2.5 ml of tris-Cl (pH 8.0) containing 5 mM reduced glutathione.

7) The protein was desalted and the buffer changed by passage through a PD-10 column equilibrated in K_2HPO_4 (pH 9.4). After checking the quantity (by Bio-Rad protein assay) and purity (by SDS-PAGE) of the isolated protein, the amount of metal associated with the protein was measured by AAS and the material was used in further experiments as outlined below.

3.2.3.4 Metal substitution of MT.

Metal-substitution was performed using both GST-SmtA protein, purified as described above, and using commercially supplied equine renal MT. This was done by incubation of the Zn- or Zn/Cd-loaded protein in a 5

This was done by incubation of the Zn- or Zn/Cd-loaded protein in a 5 : 1 ratio of Cu : Zn or a 2 : 1 ratio of Hg : Zn for 16 h.

3.2.3.5 Estimation of the pH of half dissociation of metal ions.

To determine the pH of half dissociation of metal ions, the buffers described in section 3.2.2.3 were used. 0.35 ml of metal-loaded MT or GST-SmtA fusion in Na_2HPO_4 (pH 9.44) was mixed with 2.15 ml of each of a range of buffers between pH 1.0 and pH 9.4, and the mixture allowed to equilibrate for 1 h at room temperature. This was then applied to a PD-10 column equilibrated in the same mix of buffers. The pH of the mixture was recorded and plotted as the X-axis. Free and protein-bound metal ions were defined as those eluting in the total and void volumes of the column respectively. The proportion of metal bound to protein at each pH was determined by comparison of the amount of metal eluting in the void relative to the total volume of the column. This proportion was normalised in relation to the values obtained at pH 9.4. 100 % association at pH 9.4 was assumed. A small proportion of bound metal was found to elute at the total volume due to limited resolution of the column.

3.2.3.6 Cleavage of GST-SmtA fusion using factor Xa and purification of the cleaved fragment.

Cleavage of a Zn-loaded GST-SmtA fusion protein was performed while the protein was associated with the glutathione-Sepharose matrix. The procedure followed that outlined in section 3.2.3.3 up to step 5. Following passage of 50 ml of PBS through the column, the matrix was equilibrated with factor Xa cleavage buffer (1 mM $CaCl_2$, 50 mM tris-Cl, 150 mM NaCl [pH 7.5]), and factor Xa added at 1 % (w/w) of the total fusion protein (estimated by elution of an aliquot of the protein from the matrix, and Bio-Rad assay). This was left for 16 h at 25 °C, after which the cleaved SmtA portion of the protein was eluted using 50 mM tris (pH 8.0), and applied to a 1 cm (diameter) x 100 cm (length) Sephadex G-50 column. Fractions of 2.8 ml were collected and assayed for protein and Zn.

3.2.3.7 Protein sequencing.

The cleaved SmtA portion of the GST-SmtA fusion protein prepared as described above was subjected to amino acid sequence analysis using an

ABI model 477A gas-phase microsequencer. An aliquot (2 ml) of the Zn peak fraction from the Sepharose G-50-purified material was vacuum blotted onto an Immobilon-P membrane and vacuum dried prior to sequencing using standard operating procedures.

3.2.3.8 Examination of the phenotypic effects of the expression of heterologous MT in *E. coli*.

3.2.3.8.1 Selection of metal ion concentration for tolerance testing.

The concentrations of metal ion used to examine metal tolerance of *E. coli* cells were selected as (1) the lowest concentrations which arrested cell growth (subsequently referred to as the higher metal concentrations), and (2) levels allowing cell growth but with a significant decrease in cell viability (subsequently referred to as the lower concentrations). To determine these levels, cells were exposed to a range of metal ion concentrations essentially as described by Novick and Roth (1968). Triplicate cultures of LB were supplemented with metal ions over the following concentration ranges: Cd, 0 - 1.2 mM; Cu, 0 - 5mM; Zn, Co, Cr and Ni 0 - 3mM; Hg 0 - 100 µM (e.g. see figure 3.8), and growth of cells containing pGEX3x monitored using the procedure as outlined below for metal tolerance testing. Based on these results, the lower concentrations used in tolerance testing were as follows: 0.6 mM Cd; 1 mM Zn and 2mM Cu. The higher concentrations used were: 1 mM Cd; 1.4 mM Zn and 3.8 mM Cu.

3.2.3.8.2 Examination of metal tolerance of cells expressing heterologous MTs.

Metal tolerance of cells expressing heterologous MT, as compared to control cells (containing pGEX3x only), was determined as follows:

1) Cells containing either control or test plasmids were grown in LB broth containing 50 µg/ml Ap at 37 °C for 16 h.

2) Log phase cultures were set up from this, and growth standardised in both sets of cultures.

3) 2 ml cultures containing 1 mM IPTG were inoculated from these cultures. Two sets of triplicate cultures were set up for both control and test cells.

4) After 1 h, metal was added (at the levels indicated above) to one set of cultures, the optical density was read, and the cells incubated

for a further 5 h, with optical density readings being taken every 1 h.

3.2.3.8.3 Examination of metal accumulation by *E. coli* expressing heterologous MTs.

As with tolerance tests, cellular accumulation of metals from growth medium was performed using two concentrations of each metal ion: 0.3 and 0.6 mM Cd; 0.5 and 1 mM Zn; and 0.5 and 2 mM Cu. In each case, the former concentration was non-inhibitory to growth, and the latter inhibitory but non-lethal (figure 3.8). The following protocol was used:

1) Cultures were grown for 16 h under standard conditions, and used to inoculate log phase cultures.

2) The cells were grown to an optical density of 0.3, and all cultures diluted to give identical volumes and densities in all tests.

3) Metal was added at the levels indicated, and the cultures incubated for a further 3 h.

4) Cell density was re-checked, and standardised according to the least dense culture.

5) Cells were harvested by centrifugation, washed twice using fresh medium, and finally resuspended in 1 ml of 70 % (v/v) nitric acid.

6) The metal ion concentration was determined by AAS, and the amount of metal per 8 x 10^8 cells, calculated assuming 1 ODU = 8 x 10^8 cells/ml.

3.3 Results and discussion.

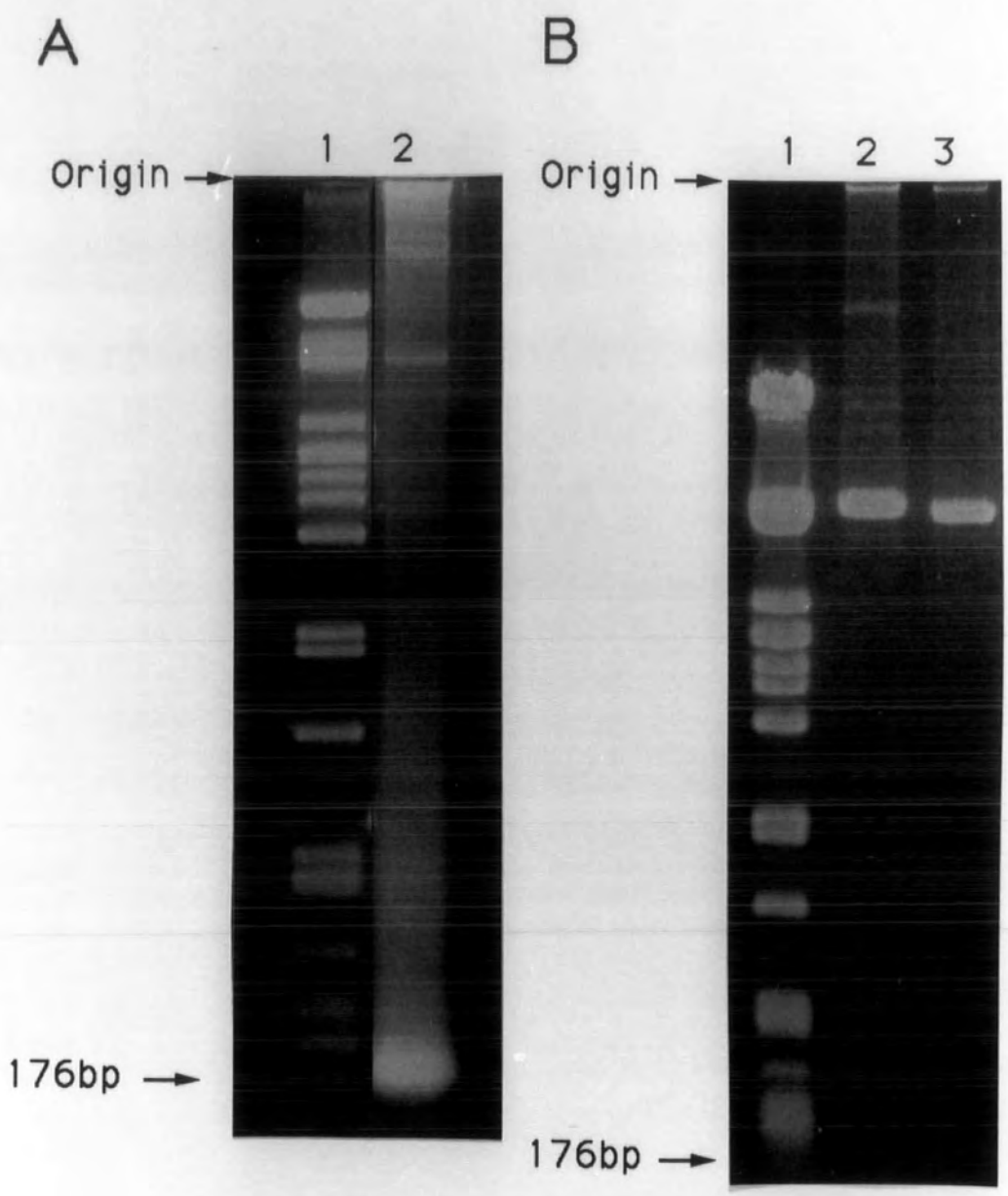
3.3.1 Amplification and cloning of the *smtA* coding region.

The PCR was used to amplify the *smtA* coding region to allow cloning into pGEX3x. Plasmid pJHNR4.9 (figure 3.1) was used as template in a reaction using primers described previously (section 3.2.1), and conditions as described in the legend to figure 3.3. Figure 3.3 shows the product of the reaction, and the subsequent clone isolated after restriction of the amplified sequence with EcoRI and BamHI and ligation into pGEX3x. This clone, named pGPMT1, was sequenced using primers which anneal to the pGEX3x vector, adjacent to the multiple cloning site. The DNA sequence, and its encoded amino acid sequence is shown in figure 3.4. As shown in the figure, the inserted sequence results in the *smtA* coding region being cloned in frame with the GST gene of the vector. Thus, SmtA is produced as a carboxyterminal fusion to GST. Since the GST translational termination signal is used to terminate the fusion protein, an additional seven amino acids (^{N}Ser-Glu-Phe-Ile-Val-Thr-Asp^{C}) are added to the carboxy terminus of the SmtA sequence.

3.3.2 Protein isolation and visualisation by SDS-PAGE.

Protein was isolated using methods outlined in section 3.2.3. To check purity, each batch of protein isolated was subjected to analysis by SDS-PAGE. Figure 3.5 shows typical protein isolates from pGEX3x- and pGPMT1-containing cells. The isolated protein samples shown in this figure reveal that a protein of the predicted size (Mr c.a. 35,000; figure 3.5 [A], lane 2) of the GST-SmtA fusion protein is successfully produced from clone pGPMT1 upon induction with IPTG. Also shown is an isolate of GST-SmtA fusion protein from cells which had been exposed to Cu (figure 3.5 [B], lane 2). In each case, proteins isolated from Cu-exposed cells were contaminated with several other protein species. This is of importance when considering the estimates of metal stoichiometry for the protein (see table 3.1), as it will result in under-estimation of the amount of Cu associated with the isolated protein.

Figure 3.3 Amplification and cloning of *smt*A. The *smt*A coding region was amplified using 200 ng of pJHNR4.9 (figure 3.1) as template, and 1 µg of each of the primers as described in the text. The PCR was performed using the following conditions: 96 °C for 1.5 min, 55 °C for 1.5 min and 72 °C for 4 min. The reaction was allowed to proceed for ten cycles, and an aliquot of the product (10 µl) separated on a 2 % agarose gel (figure 3.3 A). The DNA fragment was recovered and cloned into pGEX3x as described in the text. Figure 3.3 (B) shows the 176 bp fragment from the reaction described above excised from the resulting clone (pGPMT1). (A: lane 1, PstI-restricted lambda DNA; lane 2, PCR product as described above [for clarity, intervening lanes not relating to this part of the research were removed prior to photographing]. B: lane 1, PstI-restricted lambda DNA; lane 2, pGPMT1 restricted with EcoRI; lane 3, pGPMT1 restricted with EcoR1 and BamH1. The resulting excised band is barely visible due to the large size discrepancy between insert and vector, however a shift in the size of the double-cut vector is observed).


A
B
Origin
1
2
176bp
Origin
1
2
3
176bp

A (i)

5'GGCGGATCCCCATGACCTCAACAACCTTGGTC 3'

(ii)

3'CCGGTGTGGCCGACATTGACGTCACTTAAGCGG 5'

B

K S D L *I E G* R G I P M T S T T L V K C

AAATCGGATCTGATCGAAGGTCGTG*GGATCC*CC**CATGACCTCAACAACGTTGGTCAAATGC**

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

GCTTGTGAGCCCTGTCTCTGCAACGTCGATCCCAGCAAAGCGATCGATCGCAACGGTCTG

Y Y C S E A C A D G H T G G S K G C G H

TACTACTGCAGCGAAGCCTGTGCCGATGGCCACACCGGTGGTAGCAAAGGCTGCGGCCAC

T G C N C S E F I V T D

ACCGGCTGTAACTGCAGT*GAATTC*ATCGTGACTGACTGA

Figure 3.4 PCR primers and amplified sequence of *smt*A. (A) The 5' (i) and 3' (ii) primers used to amplify the *smt*A coding sequence. The primers contain EcoRI and BamHI restriction sites which facilitated cloning of the amplified product. (B) The DNA and encoded protein sequence of the product of the reaction performed using the primers described above after cloning in the expression vector pGEX3x (pGPMT1). Vector sequence is shown in normal type, EcoRI and BamHI recognition sites in italics, and the *smt*A gene and protein sequences in bold type. Also shown in italics is the recognition site in the GST protein sequence which allows cleavage of the fusion protein with factor Xa (IEGR).

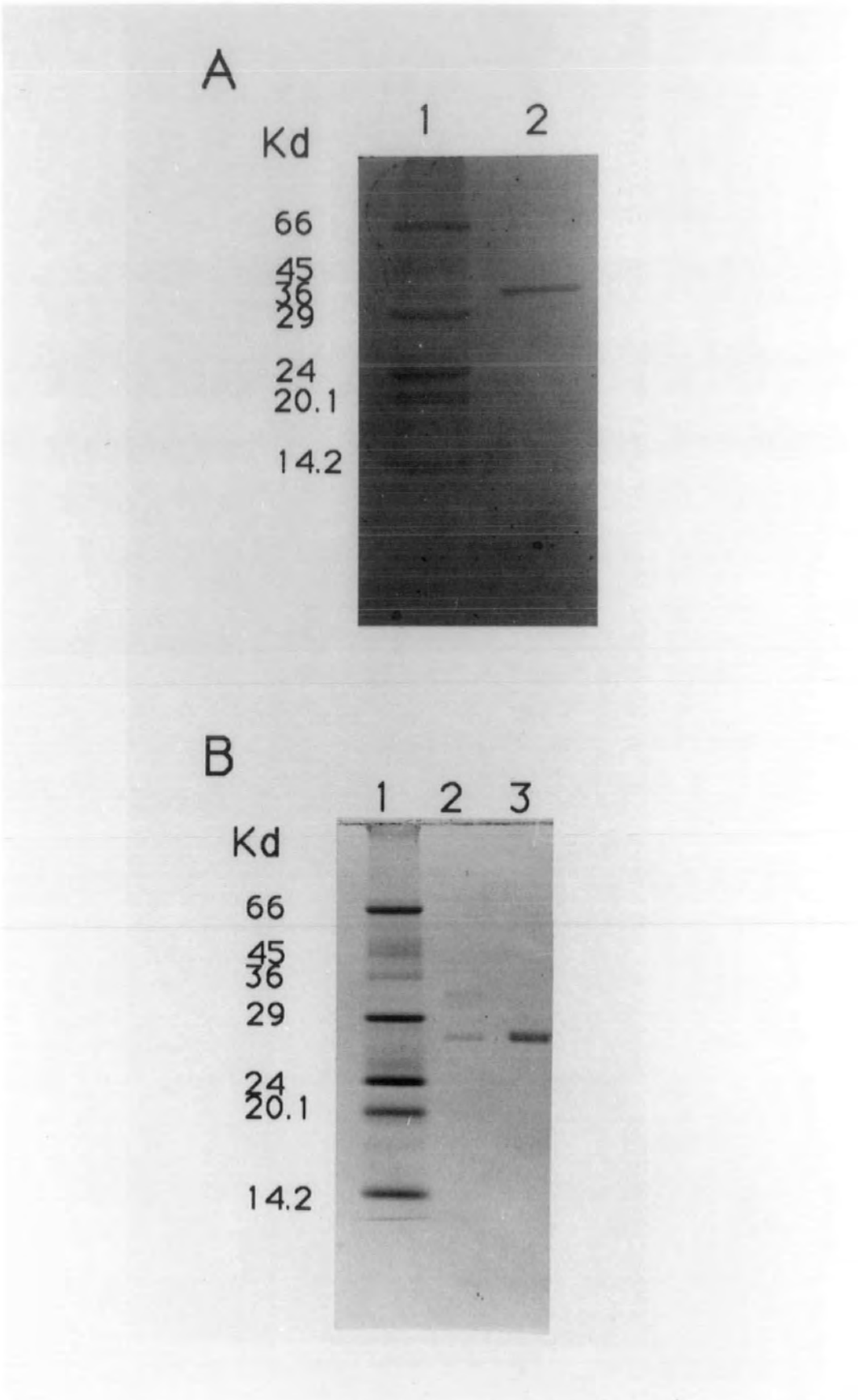

A
1
2
Kd
66
45
36
29
24
20.1
14.2
B
1
2
3
Kd
66
45
36
29
24
20.1
14.2

Figure 3.5 SDS-PAGE analysis of the protein products of pGPMT1 and pGEX3x. Protein isolated using a glutathione affinity column as described in section 3.2.3.3, was separated on a 12.5 % SDS-PAGE gel. A protein of the predicted size was purified in each case, except for GST-SmtA fusion protein purified from Cu-exposed cultures, from which several proteins co-purified.[(A) lane 1 - markers; lane 2 - GST-SmtA fusion protein purified from Zn-exposed cells. (B) lane 1 - markers; lane 2 - GST-SmtA fusion protein purified from Cu-exposed cells; lane 3 - GST protein purified from Zn-exposed cells].

3.3.3 Estimation of the stoichiometric ratio of metal to protein for heterologous GST-SmtA.

To provide an estimate of the relative amount of each metal ion (Cd, Cu, Zn or Hg) which associated with the expressed fusion protein *in vivo*, *E. coli* cells containing either pGEX3x (control) or pGPMT1 were exposed to metal ions using the method described in section 3.2.3.2. GST or GST-SmtA protein was purified, and the amount of protein, and metal associated with the isolated protein was assayed. The estimated molar ratio of metal ion to protein for Zn, Cd, Cu and Hg are shown in table 3.1.

	Zn : Protein	Cd : Protein	Cu : Protein	Hg : Protein
	1.20 : 1	0.87 : 1	0.80 : 1	1.02 : 1
(A)	0.87 : 1	0.70 : 1	0.12 : 1	0.82 : 1
	0.60 : 1	1.04 : 1	0.14 : 1	0.82 : 1
	4.57 : 1	4.77 : 1	2.60 : 1	6.44 : 1
(B)	3.96 : 1	5.12 : 1	2.30 : 1	7.30 : 1
	4.50 : 1	7.79 : 1	0.27 : 1	6.87 : 1

Table 3.1 The metal content of GST and GST-SmtA isolates. Protein was isolated from *E. coli* grown in metal-supplemented medium as described in section 3.2.3.2. Data is shown for protein isolated from three replicate cultures for each metal ion [(A) GST protein (B) GST-SmtA fusion protein].

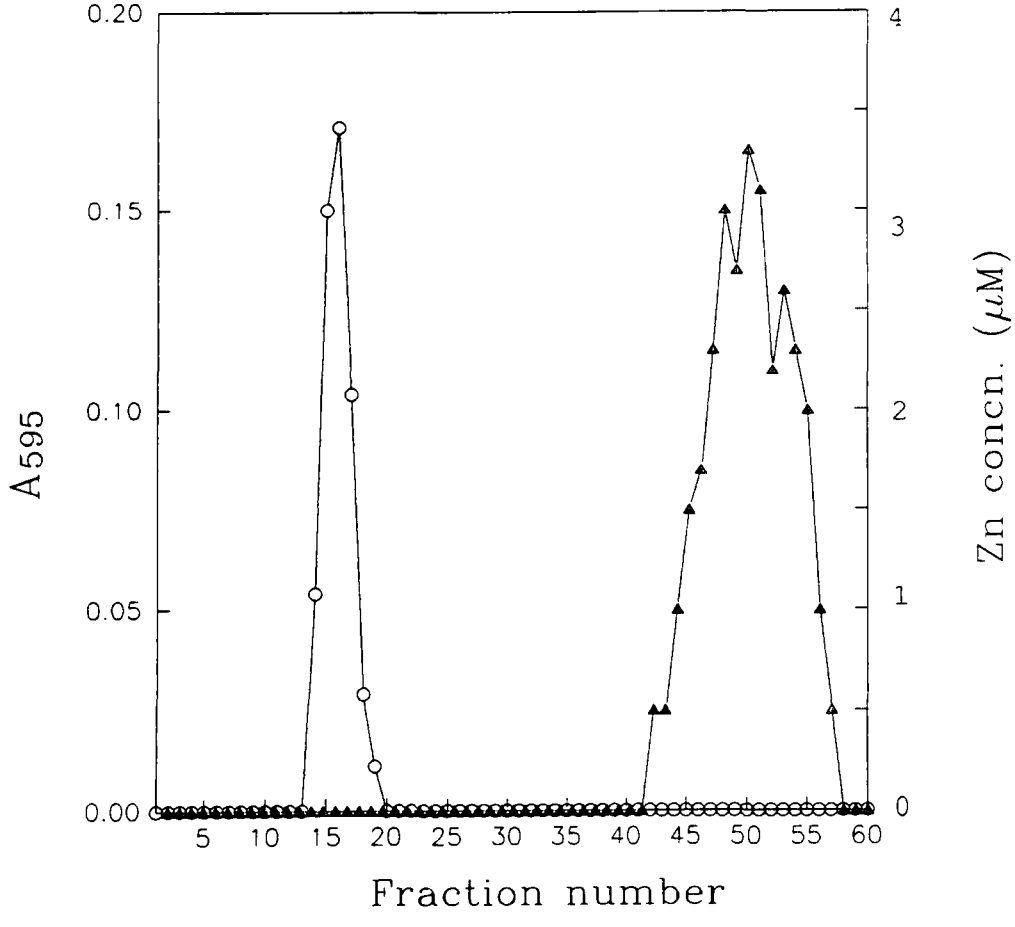
The data presented in table 3.1 indicates that there is very little association of metal ions with GST. The GST-SmtA protein, however, does associate with each of the metal ions tested, thus indicating that the SmtA portion of the protein binds metal ions in *E. coli*. This was confirmed (see section 3.3.4, below) by cleavage and separation of the SmtA portion from the GST portion of the fusion protein. The average estimated metal : protein ratios observed for each of the metal ions were 4.34, 5.89, 1.72 and 6.78 for Zn, Cd, Cu and Hg respectively. As discussed above, the relatively low value obtained for Cu may reflect the low purity of the isolated protein. The other values obtained are considerably higher than reported by Olafson

et al (1988), who reported Cd : protein ratios of between 2.2 and 3.1 : 1 for several protein peaks eluting from a reverse phase column, for MT isolated from Cd-exposed Synechococcal cells (the average ratio was 2.8 : 1). It is not known whether this represents complete saturation of the protein with Cd. No quantitative assessment of Zn binding to SmtA has been reported. Binding of other metals has not previously been reported. The higher Cd : protein stoichiometry obtained in these experiments is unlikely to be due to altered protein conformation. In all cases where recombinant MTs have been expressed in *E. coli*, and metal binding to the purified molecule examined, the results have suggested that the protein folded to accommodate metal ions with the same stoichiometries as observed in the natural system (cited in Kay et al 1991). One possible explanation could be that the fusion protein may bind a slightly higher quantity of metal ions to the natural Synechococcal protein due to metal association with the GST portion of the molecule, however the observed low metal : protein stoichiometries for the purified GST protein suggest that this is not the case. A higher Zn : protein ratio than that shown in table 1 was obtained when the cleaved and purified SmtA portion of the protein was analysed for Zn binding (see figure 3.6 and section 3.3.4). This is consistent with another possible reason why relatively high metal : protein ratios were obtained, namely that the Bio-Rad (Coomassie blue) staining reagent was not efficient in detection of the SmtA portion of the molecule. Amino acid analysis of purified protein (which was unavailable) would be required to define precisely the amount of protein present and hence the metal stoichiometries of the recombinant protein.

3.3.4 Protein cleavage, gel permeation separation and sequence analysis of SmtA.

In order to confirm that the SmtA portion of the fusion protein was responsible for metal binding, a Zn-loaded protein isolate was cleaved prior to elution from the glutathione-Sepharose column (section 3.2.2.6). The Sephadex G-50 profile of the material eluted from glutathione-Sepharose following factor Xa cleavage of the fusion protein is shown in figure 3.6. This figure clearly demonstrates that all of the Zn is associated with the major protein peak eluting from the column. The GST portion of the protein remains bound to the

(A)


0.20
0.15
0.10
0.05
0.00
A595
4
3
2
1
0
Zn concn. (μM)
5 10 15 20 25 30 35 40 45 50 55 60
Fraction number

(B)

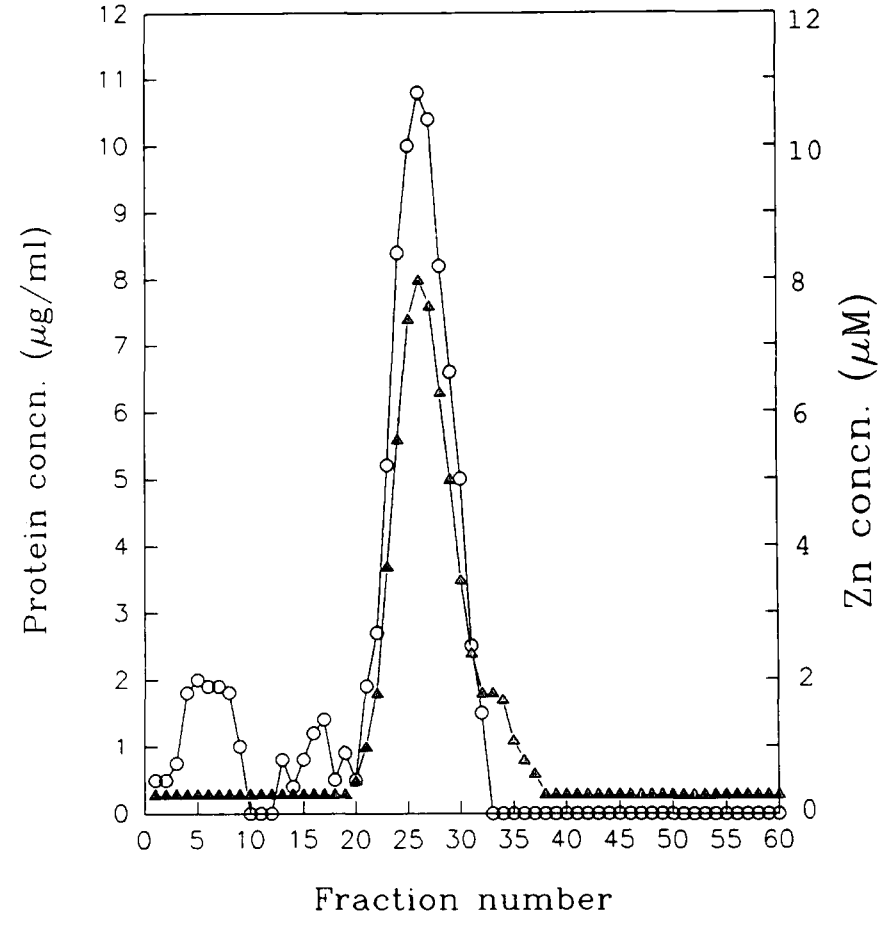

12
11
10
9
8
7
6
5
4
3
2
1
0
Protein concn. (μg/ml)
Zn concn. (μM)
0 5 10 15 20 25 30 35 40 45 50 55 60
Fraction number

Figure 3.6 Gel permeation separation of the SmtA portion of the GST-SmtA fusion protein following cleavage with factor Xa. GST-SmtA fusion protein was purified using standard methods, and cleaved using factor Xa while still associated with glutathione-Sepharose. The material eluting from the column following cleavage was separated using Sephadex G-50, as described in the text. Figure 3.6 (A) shows the void and total volumes of the G-50 column, as marked using Blue Dextran (O) and Zn (△) respectively. Figure 3.6 (B) shows the elution profile of both protein (O) and Zn (△) from the cleavage reaction described above.

glutathione matrix following cleavage, and so does not appear in the subsequent G-50 profile. An aliquot (1 ml) of the peak fraction of the G-50 profile (fraction 26) was subjected to amino acid sequencing, the result of which is presented in table 3.2. The major sequence obtained for the first 20 amino acids of the protein sequence shown in table 3.2 was identical to that predicted for the cleavage product of the GST-SmtA fusion protein (figure 3.4). A secondary sequence, which is present in much smaller amounts than the predicted sequence, indicates that some cleavage of the protein has occurred following the methionine residue of SmtA. No other amino acids are present in significant quantities at each cycle, demonstrating the purity of the isolated protein. The average Zn : protein ratio over the G-50 peak fractions (fractions 21-30) is 6.3 : 1, which is slightly higher than that shown in table 1 for GST-SmtA binding Zn. This may reflect increased protein purity, or as discussed above may reflect differences in the reactivity of the two proteins with the Coomasie blue staining assay. The data clearly demonstrates that the SmtA portion of the protein is responsible for binding metal ions. For the more detailed analyses of the metal binding properties of the protein presented in section 3.3.5, it was therefore possible to utilise GST-SmtA fusion rather than cleaved and purified SmtA. This allowed the preparation of relatively large amounts of protein for the analyses described below.

3.3.5 Determination of the pH of half dissociation of metal ions from GST-SmtA.

The pH of half dissociation of metal ions from metal-binding molecules is a useful representation of the binding affinity of the molecule for a particular ion, and has been used to distinguish MT from other cellular metal ligands (Vasàk and Armitage 1986). For this reason, sufficient GST-SmtA fusion protein was isolated to allow analysis of the degree of metal ion dissociation from the protein over a range of different pHs. As described above, GST-SmtA could be readily isolated in association with Cd, Zn, Cu or Hg by *in vivo* loading of the protein in *E. coli*. Due to the relatively low purity of Cu isolates, however, *in vitro*-substitution of Zn with Cu was used to confirm the validity of the data obtained using *in vivo*-loaded protein. *In vitro* exchange of Zn with Cu, as outlined in section 3.2.3.4, resulted in 100 %

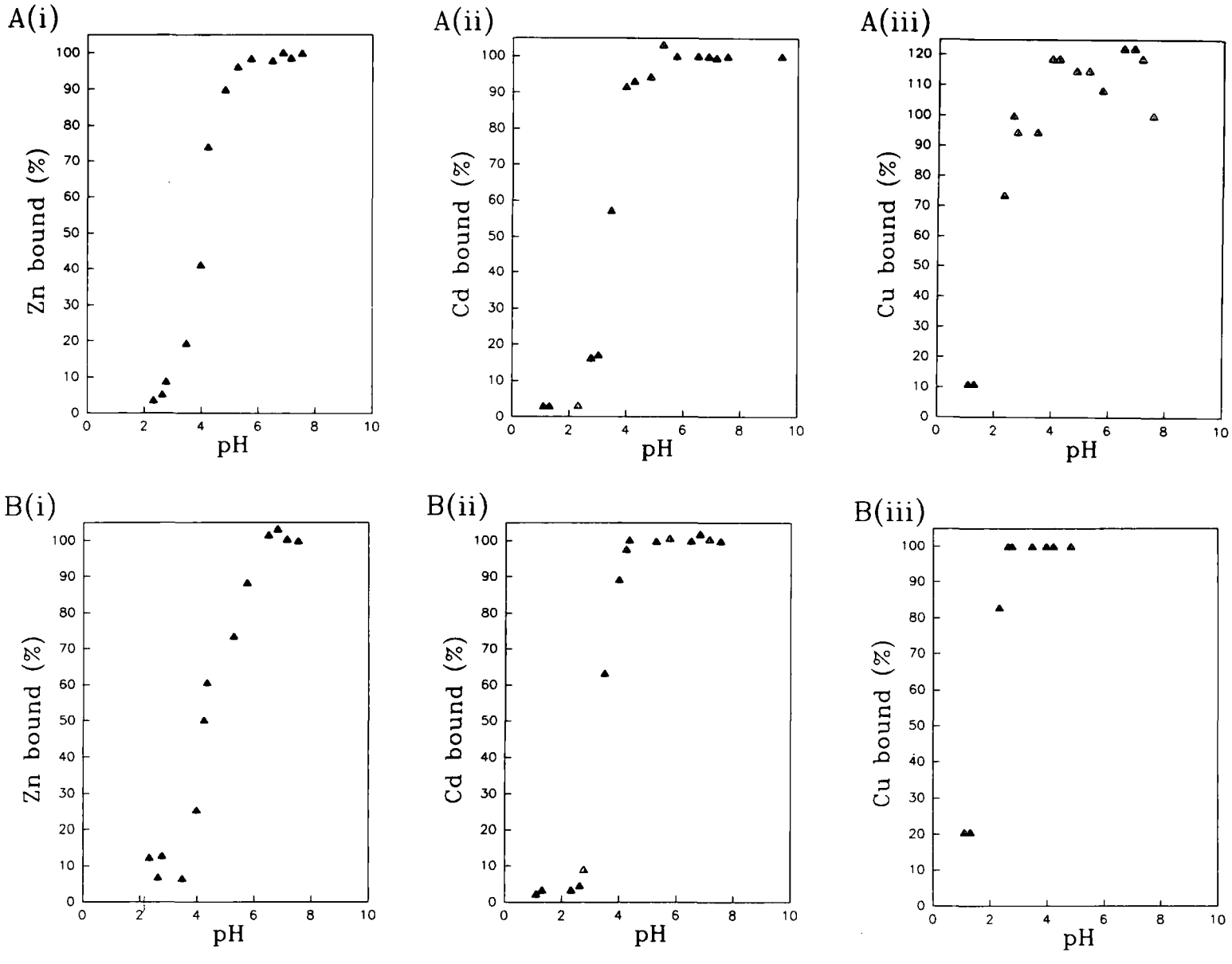
Table 3.2 The amino acid sequence of the cleaved portion of GST-SmtA fusion protein. An aliquot (1 ml) of peak 26 from the G-50 separation shown in figure 3.4 was subjected to amino acid sequence analysis using techniques described in the text. The sample was not derivatised prior to analysis in order to maximise yields at each cycle. The cycles at which no major peak was present in each case coincides with a predicted cysteine residue. The predicted sequence is shown in bold type. A secondary sequence (underlined) was identified as the SmtA sequence, and is consistent with a secondary cleavage product starting after the methionine initiation codon. The yield for each amino acid is given in pmoles.

Cycle	Ala	Arg	Asn	Asp	Cys	Glu	Gln	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
1	15.8	102.7	-0.2	53.4	-1.0	2.4	0.5	478.3	-0.4	-0.3	-1.2	21.2	4.9	0.4	0.3	1.9	76.2	0.7	3.3	1.3
2	0.0	0.0	0.3	0.0	-1.0	-1.5	0.2	0.0	0.7	528.8	46.7	0.0	-0.3	-0.1	7.6	23.1	-0.2	-0.5	-0.1	-0.8
3	-0.8	0.0	2.9	0.0	-1.0	-0.6	-0.1	0.0	1.7	0.0	0.0	0.0	0.0	-0.3	391.6	0.0	25.7	-0.4	-0.1	-2.0
4	-3.8	0.0	-0.4	-0.3	-1.0	33.4	-1.1	-1.4	0.1	0.0	-1.4	-1.0	433.6	-0.1	0.0	-2.1	25.6	-0.1	-0.3	-1.3
5	-0.7	-2.3	0.2	-1.3	-1.0	0.0	0.7	0.0	2.4	0.0	67.4	-1.9	0.0	0.4	0.0	0.0	116.0	-0.1	0.2	5.5
6	-0.4	0.0	0.4	-0.4	-1.0	0.0	-0.8	0.0	4.0	0.0	0.0	-0.3	0.0	0.0	0.0	48.6	0.0	0.1	0.3	84.0
7	0.1	36.9	-0.7	-0.6	-1.0	-1.5	-0.5	0.0	-0.9	-1.5	-0.3	51.4	0.0	-0.8	-11.3	0.0	51.6	0.3	-0.2	0.0
8	3.9	14.8	0.4	0.0	-1.0	-0.5	1.1	23.1	0.0	24.8	-6.3	0.0	0.0	-0.1	-3.8	4.2	110.2	0.1	0.5	14.8
9	83.9	9.5	-0.2	3.0	-1.0	-0.5	1.1	-1.1	8.3	14.8	169.7	16.1	0.4	0.2	2.5	0.0	22.7	0.2	-0.4	0.0
10	0.0	13.6	-1.0	0.0	-1.0	0.5	0.0	-0.5	0.0	0.0	92.2	0.0	10.8	0.2	11.3	0.4	2.1	-0.2	-1.0	174.7
11	15.6	0.0	1.5	1.0	-1.0	54.5	2.6	0.7	5.3	0.0	0.0	159.5	11.5	0.6	-5.8	-1.3	0.0	-0.2	-0.7	105.9
12	0.0	0.0	-1.1	0.0	-1.0	0.0	0.0	10.7	0.6	0.0	0.0	110.2	0.0	0.2	34.9	2.1	0.0	0.2	0.2	0.0
13	221.2	6.5	1.3	0.3	-1.0	9.6	0.0	9.3	3.7	1.5	-4.6	5.7	-0.2	-0.2	0.0	5.4	-2.1	-0.0	2.1	0.0
14	113.0	0.0	-1.6	-0.2	-1.0	0.0	-0.6	0.0	1.6	0.0	19.8	0.9	-0.5	-0.2	1.7	0.0	-2.9	-0.1	8.8	0.0
15	0.0	1.1	1.9	-0.2	-1.0	114.5	1.8	0.0	1.6	0.0	0.0	0.0	-0.3	-0.1	-7.2	1.1	-5.1	-0.1	3.6	-4.7
16	0.0	0.0	50.6	-0.3	-1.0	64.4	0.0	-2.3	0.0	-0.1	2.6	-0.9	-0.5	-0.3	95.5	0.0	20.0	0.3	0.0	-4.6
17	0.0	-3.0	0.0	-1.6	-1.0	0.0	0.0	1.5	0.0	-0.3	-9.4	-2.7	-0.5	0.3	64.1	0.0	0.0	0.2	0.0	24.8
18	-3.6	-2.3	4.3	29.9	-1.0	0.0	-0.2	0.0	-0.7	-0.2	68.3	3.1	-0.1	-0.0	0.0	-0.5	0.0	-0.2	-0.3	0.0
19	-5.3	-4.3	0.0	0.0	-1.0	0.0	-0.3	-1.9	0.4	0.4	56.7	-0.6	1.3	0.0	0.0	-0.3	0.0	-0.3	-0.7	0.0
20	2.5	-1.5	113.2	2.6	-1.0	-1.3	-0.3	3.4	0.0	-0.5	0.5	-1.9	0.6	0.3	0.3	3.8	2.5	0.0	0.6	-2.8

displacement of Zn, and replacement of Zn with an equimolar amount of Cu (as defined by partitioning of these ions into the void and total volumes of a G-25 [PD-10] column). This gave a ratio of Cu : protein of 6.08 : 1. A comparable stoichiometry (5.92 : 1) was obtained for exhange-bound Hg, using similar methods. The stoichiometry for Cu, being equivalent to that obtained for Zn, indicates that Cu was most likely to be coordinated to SmtA in the divalent form. This is in contrast to *in vivo*-loaded protein in which Cu is likely to be in the reduced (monovalent) form, since Cu(II) is rapidly reduced to Cu(I) during uptake by *E. coli* cells (Romeyer et al 1988). Attempts were made to isolate Co, Cr, and Ni in association with protein from cells grown in each of these metals, however in each case there was no more metal associated with the GST-SmtA fusion than with GST alone. Attempts at substitution of Zn with each of these metals also proved unsuccessful, as a ten-fold excess of each metal failed to displace any Zn from the protein. Protein precipitation was observed a higher concentrations.

The profiles for dissociation of Zn, Cd and Cu from SmtA are shown in figure 3.7. The data shown for Cu dissociation is for metals loaded *in vivo*. Identical results were obtained for *in vitro*-loaded protein. Under the conditions used in these experiments, no Hg was displaced at the lowest pH (pH 1.0). Also shown in figure 3.7 is the pH displacement curve for metal ions from commercially supplied equine renal MT, analysed using identical methods as those described for SmtA. As previously described, Cu ions were bound to equine Zn/Cd MT by incubation of the protein in a two-fold molar excess of Cu (relative to the total amount of Zn and Cd). A molar ratio of 7.12 : 1 for Cu : protein was observed following substitution, indicating that, as for SmtA, Cu had bound to the protein in the divalent form. The pH values obtained for half dissociation of metal ions from this molecule (4.5 and 3.0 for Zn and Cd respectively) are in good agreement with published values (Kägi and Kojima 1987), which validates the method for analysis of SmtA. Comparison of the estimates obtained for half dissociation of metal ions from SmtA (4.10, 3.50 and 2.15 for Zn, Cd and Cu respectively), shows that the protein has an extremely high affinity for Zn, the pH of half dissociation being lower than that of equine MT. Conversely, the pH values obtained for half dissociation of Cd and Cu show that SmtA has a lower affinity for each of these ions

Figure 3.7 The displacement of Zn, Cd and Cu from GST-SmtA and from equine renal MT by decreasing pH. The metal dissociation profiles of (i) Zn, (ii) Cd and (iii) Cu from *in vivo*-loaded GST-SmtA (A) and commercially supplied equine renal MT (B) are shown. Metal eluting in the void volume of a PD-10 column was assumed bound to MT, that in the total volume was assumed to be free metal. The amount of metal eluting in the void volume of the column at the highest pH was taken as the value for 100 % metal association with the protein, and the amounts dissociating at lower pHs normalised to this value.


A(i)
Zn bound (%)
pH
A(ii)
Cd bound (%)
pH
A(iii)
Cu bound (%)
pH
B(i)
Zn bound (%)
pH
B(ii)
Cd bound (%)
pH
B(iii)
Cu bound (%)
pH

than does equine MT. As has been observed in the analysis of other thiol-rich proteins, SmtA had a very high affinity for Hg. The lowest pH buffer used (pH 1.0) failed to displace any of the *in vivo*- or *in vitro*-loaded Hg from the protein. Hg is also a strong inducer of *smt*A transcription, indicating that *smt*A may be of importance in the detoxification of this metal ion as well as Cd and Zn in Synechococcal cells. The other metals examined (Co, Cr and Ni) could not be bound to the protein under the conditions examined. The failure of these metals to displace Zn *in vitro* indicates that the protein has a relatively low affinity for these ions under the conditions of the experiment.

3.3.6 Examination of the phenotypic effects of *smt*A expression in *E. coli*.

Information regarding alteration of metal tolerance and/or accumulation via expression of a metal ligand relates to the metal binding characteristics of the expressed protein *in vivo*, and hence gives further insight into the possible functions of the protein. Such studies also provide a useful assessment of the possible biotechnological applications of the gene, such as in engineering organisms for increased metal resistance.

The complex relationship which exists between factors which may affect metal binding to the expressed protein *in vivo*, and the resulting phenotypic effects of increased metal tolerance or accumulation is of relevance to results presented in other chapters of this thesis, and a detailed discussion of this subject is therefore reserved for the general discussion presented in chapter 7.

3.3.6.1 Examination of metal tolerance of *E. coli* cells expressing *smt*A.

Metal tolerance of cells expressing the GST-SmtA fusion protein from plasmid pGPMT1 was compared to that of cells which expressed only GST. The results of these experiments are presented in figures 3.9 and 3.10. Metal tolerance was tested at two concentrations of each metal, as defined previously (section 3.2.3.8.1). The graph shown in figure 3.8, which shows the growth of control cells (pGEX3x-containing cells) cultured in ranges of Cd, Cu and Zn, was used to select the concentration of each ion for tolerance testing. Two concentrations were selected; a higher concentration (the lowest resulting in

Figure 3.8 Exposure of *E. coli* containing pGEX3x to a range of concentrations of Cd, Cu, Zn and Hg. To determine inhibitory metal ion concentrations, *E. coli* cells containing plasmid pGEX3x were exposed to various metal ion concentrations as described in the text. Cells were incubated for 5 h, and the optical density at this time-point plotted against concentration for each metal ion. This allowed selection of concentrations at which to grow cells both to allow association of the metal ion with GST-SmtA for subsequent protein isolation and *in vitro* analysis, and for examination of metal tolerance and accumulation by *E. coli* cells expressing heterologous metal binding proteins (see section 3.2.3.8.1). The concentrations chosen for metal tolerance and accumulation testing and for metal exposure to bind metals to SmtA *in vivo* based on these results are as given in the text. (O - Cd; □ - Zn; ∇ - Cu; ■ - Hg).

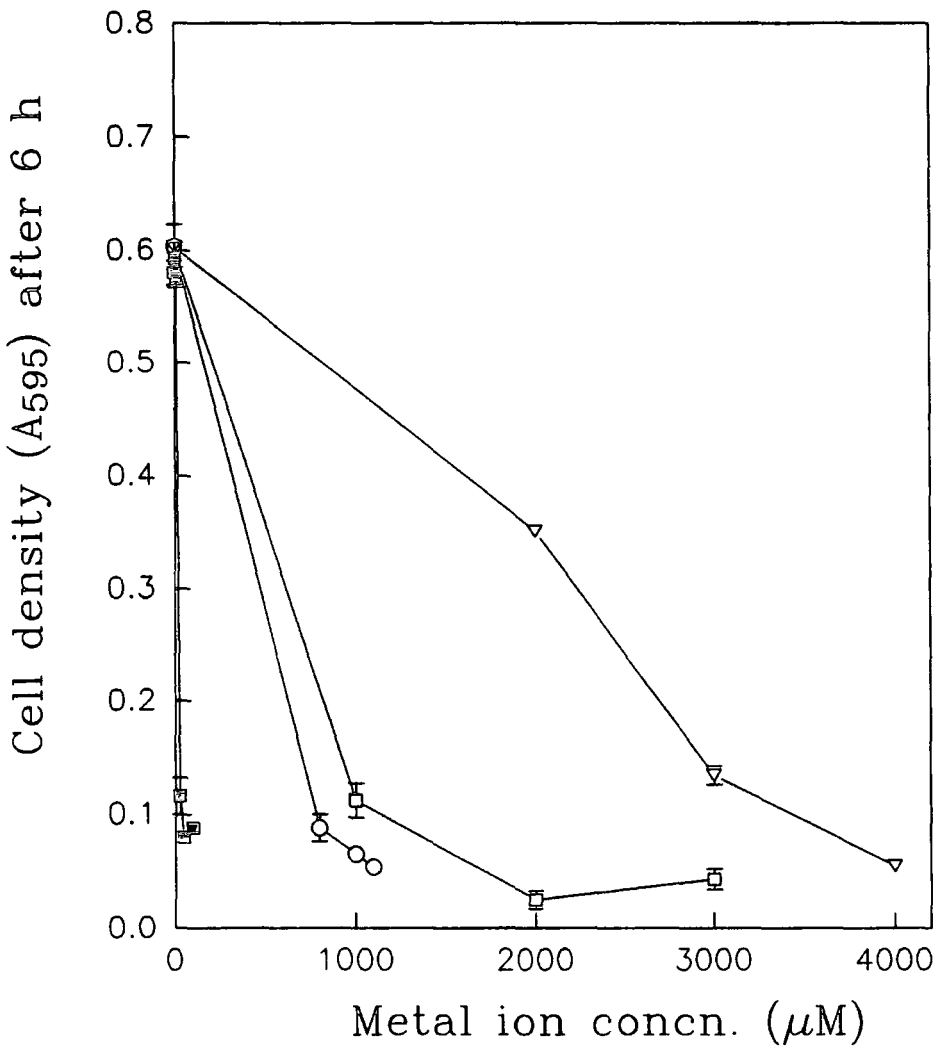
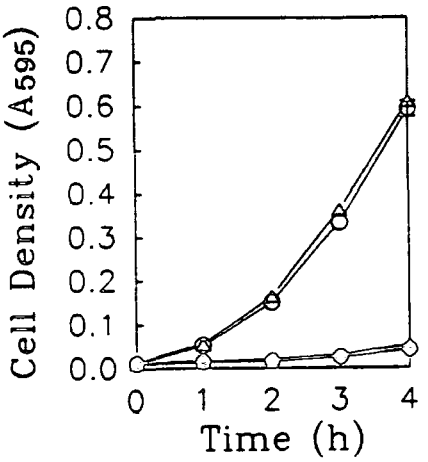
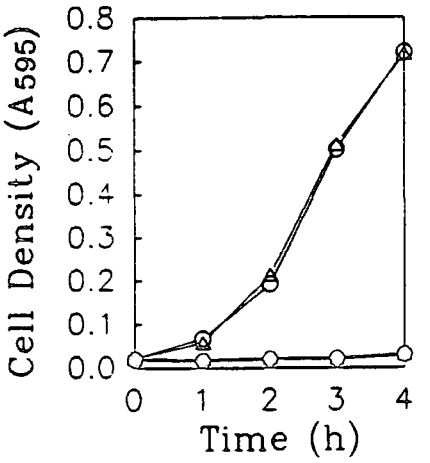

Cell density (A595) after 6 h
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1000
2000
3000
4000
Metal ion concn. (μM)

Figure 3.9 Exposure of *E. coli* cells containing either pGPMT1 or pGEX3x (control) to Cd, Cu and Zn (higher concentrations). Cells were exposed to metal ions using methods as outlined in the text. Concentrations used were 1 mM Cd, 3.8 mM Cu and 1.4 mM Zn. The values plotted were the average results of triplicate tests, with standard deviation as shown by error bars. (A - Cd, B - Cu, C - Zn; ○ - non metal-exposed control cells; Δ - non metal-exposed pGPMT1-containing cells; ● - metal-exposed control cells; ▲ - metal-exposed pGPMT1-containing cells)

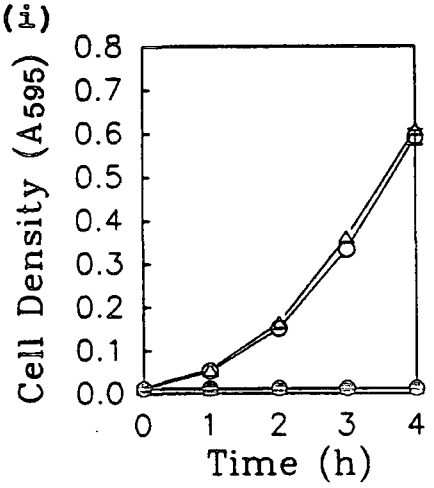
A(i)



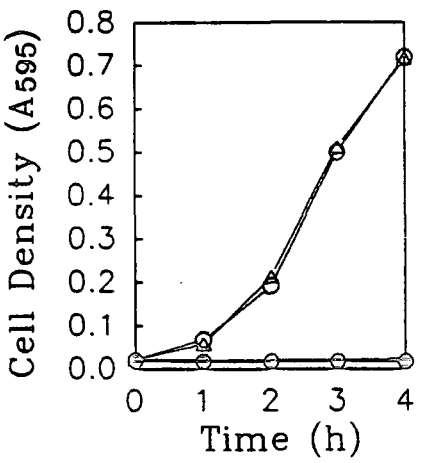
A(ii)



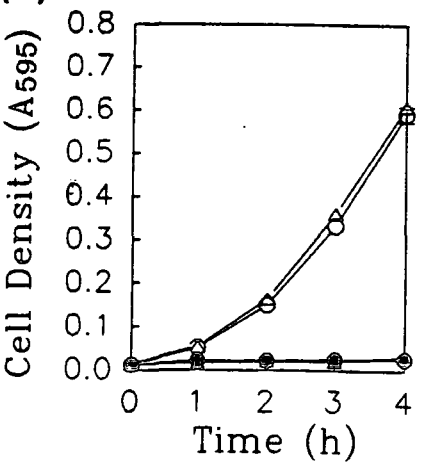
B(i)



B(ii)



C(i)



C(ii)

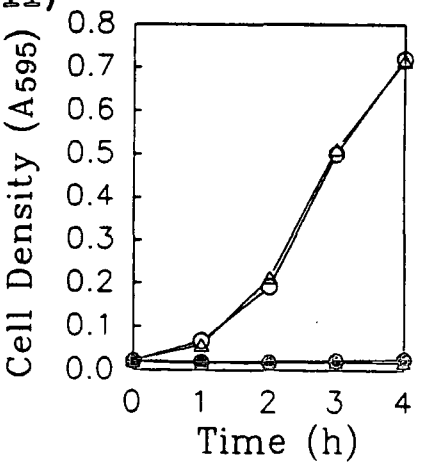
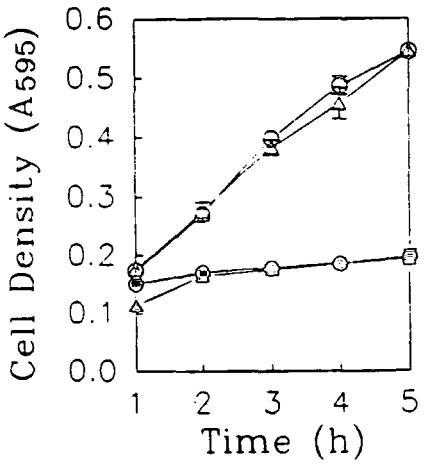
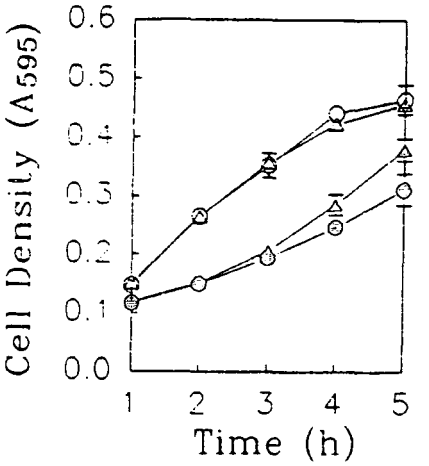


Figure 3.10 Exposure of *E. coli* cells containing either pGPMT1 or pGEX3x (control) to Cd, Cu and Zn (lower concentrations). Cells were exposed to the less inhibitory concentrations of metal ions as defined in section 3.2.3.8.1 (0.6 mM Cd, 2 mM Cu and 1 mM Zn). Symbols are as given in figure 3.9 (A - Cd, B - Cu, C - Zn).

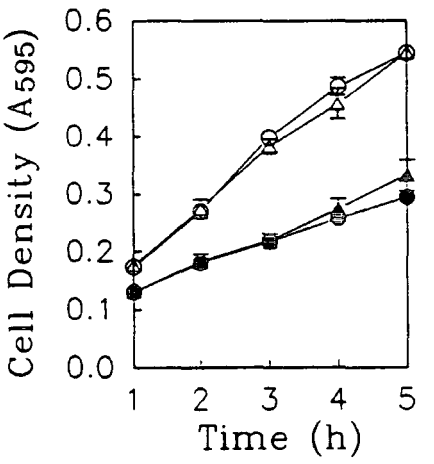
A(i)



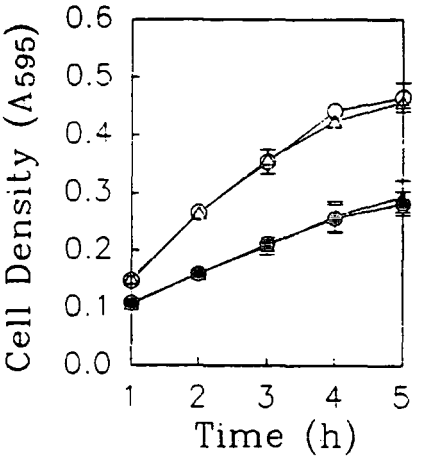
A(ii)



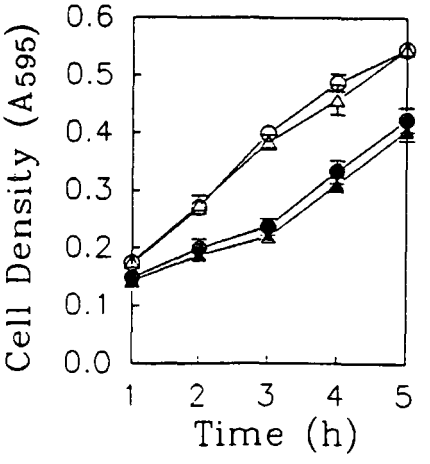
B(i)



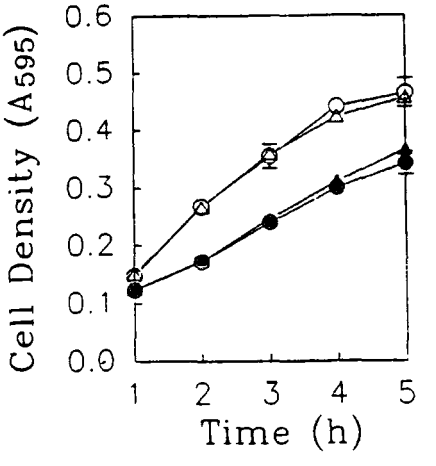
B(ii)



C(i)



C(ii)



complete inhibition of cell growth) and a lower concentration (at which growth was decreased but not completely inhibited). It was considered likely that testing for altered metal tolerance at these concentrations would distinguish any differences between control and test cells.

As the graphs show, no difference in metal tolerance was observed between pGEX3x- and pGPMT1-containing cells under the conditions used. Further discussion of these results, in conjunction with related studies presented in this thesis, is presented in chapter 7.

3.3.6.2 Analysis of metal accumulation by *E. coli* cells expressing *smt*A.

Metal accumulation by cells expressing *smt*A was examined using the protocol given in section 3.2.2.8.3, and the results of these experiments are presented in table 3.3. As for tolerance testing, accumulation was tested at two metal exposures; a higher concentration (H in table 3.3.) which was inhibitory to cell growth, and a lower concentration (L) which was non-inhibitory to cell growth (the precise concentration of each metal is given in section 3.2.3.8.3). No statistically significant increase in Cd accumulation was observed at either concentration of Cd, taking the mean of all 9 experimental samples. Examination of the data at 0.3 mM Cd ("L" in table 3.3A) however, reveals that within all three replicates of the experiment, slightly increased (c.a. 1.5-fold) accumulation was observed for pGPMT1-containing cells compared to control values. Similarly, for Cu and Zn, no consistent effect of expression of *smt*A was observed at high levels of Cu and Zn (2 mM Cu and 1 mM Zn). Exposure of the cells to lower levels (0.5 mM) revealed a slight increase in Cu accumulation by *smt*A-expressing cells (c.a. 1.4-fold increase over control cells), and a more greatly increased accumulation of Zn (3-fold increase over control cells).

This is the first unequivocal evidence that the product of the *smt*A gene is capable of binding metals *in vivo*, and that expression of the gene can affect the phenotype of the host organism with relation to metal accumulation. Further, the results indicate *in vivo* association of Cu ions with the protein, at least in *E. coli* cells. It is of interest in this regard that Olafson et al (1980), demonstrated that exposure of Synechococcal cells to Cu resulted in only a minimal

Table 3.3 Metal accumulation by *E. coli* cells containing either pGPMT1 or pGEX3x (control). Cells were exposed to metal ions and the amount of metal accumulated assayed as described in section 3.2.3. The tables show the result of exposure of cells both to an inhibitory concentration (H), and to a lower, non-inhibitory concentration of each metal (L) as described in section 3.2.3.8.3. The amount of metal accumulated is presented as nmoles per 8 x 10^8 cells, assuming 1 ODU at 595 nm equals 8 x 10^8 cells. Standard deviations from the mean are given in brackets (A - Cd; B - Cu; C - Zn).

(A)

Replicate number	Amount of Cd (nmoles/8 x 10^8 cells) pGEX3x H	pGEX3x L	pGPMT1 H	pGPMT1 L
1	20.82 (0.98)	6.35 (0.50)	24.60 (2.53)	8.50 (0.70)
2	22.48 (1.99)	4.72 (0.35)	17.75 (3.73)	6.08 (0.21)
3	29.26 (0.34)	6.29 (0.11)	28.24 (1.14)	11.57 (0.05)
mean (n=9) (st. dev.)	**24.18 (4.47)**	**5.79 (0.86)**	**23.43 (5.48)**	**8.72 (2.41)**

(B)

Replicate number	Amount of Cu (nmoles/8 x 10^8 cells) pGEX3x H	pGEX3x L	pGPMT1 H	pGPMT1 L
1	18.39 (0.39)	6.53 (0.10)	21.65 (1.98)	9.79 (0.58)
2	24.18 (3.36)	5.64 (0.41)	32.03 (1.51)	7.54 (0.94)
3	7.94 (1.90)	6.41 (0.74)	11.70 (2.17)	9.28 (0.13)
mean (n=9) (st. dev.)	**16.83 (8.23)**	**6.20 (0.60)**	**21.80 (10.16)**	**8.87 (1.18)**

(C)

Replicate number	Amount of Zn (nmoles/8 x 10^8 cells) pGEX3x H	pGEX3x L	pGPMT1 H	pGPMT1 L
1	12.05 (0.20)	2.14 (0.18)	12.79 (0.77)	7.31 (0.19)
2	11.49 (0.56)	1.92 (0.22)	13.14 (1.93)	5.05 (0.09)
3	10.41 (1.08)	1.94 (0.21)	11.96 (1.32)	5.72 (0.13)
mean (n=9) (st. dev.)	**11.32 (0.83)**	**2.00 (0.02)**	**12.63 (0.62)**	**6.05 (1.04)**

increase in synthesis of the protein, and that Cu could only be found in trace amounts when the protein was isolated from cells which had been exposed to Cd or Zn. This led to the suggestion that the Synechococcal MT may not be of importance in the detoxification of Cu. Recent data, showing increases in abundance in *smt*A transcripts in response to Cu (Huckle et al 1992), in conjunction with the data presented here, may indicate a role for SmtA in Cu homoeostasis in Synechococcal cells. Further research will be required to resolve whether or not SmtA has such a role.

It is of interest to note that Zn was accumulated to a greater extent than either Cd or Cu in these experiments, although *in vitro* analysis (section 3.3.5) indicated that the protein had a higher affinity for both Cd and Cu than for Zn. As will be discussed in chapter 7, this anomalous behaviour of the protein *in vivo* may be considered in relation to competition for binding to cellular metal ions by other (endogenous) cellular ligands.

3.3.7 Summary and future work.

3.3.7.1. Implications of results in relation to *smt*A function.

The results of these experiments clearly demonstrate binding of metal ions to the product of *smt*A, with affinity of the order Hg>Cu>Cd>Zn>Co,Cr,Ni. In comparison to equine MT, GST-SmtA exhibited a lower pH of half dissociation of Zn ions, and a slightly higher pH of half dissociation for Cd and Cu ions, indicating that relative to this molecule, SmtA has a high affinity for Zn. Increased accumulation of Cd, Cu and Zn by *E. coli* expressing *smt*A demonstrates that the product of the gene binds these metals *in vivo*, with apparent preferential binding of Zn ions over Cd and Cu.

All of the results obtained in this chapter are consistent with the hypothesis that SmtA is a strong Zn-ligand, indicating that the protein may have an as yet undefined role Zn homoeostasis. A role in tolerance to both Zn and Cd, as implied by Olafson et al (1980) is also consistent with the data presented here. Binding of both Hg and Cu to the protein further suggest a possible involvement in Cu homoeostasis, and Hg detoxification.

The observation that SmtA has a high affinity for Zn relative to equine MT, and is capable of binding Zn in *E. coli* cells, is important in the elucidation of the role of this protein in cellular metabolism.

As suggested in consideration of the possible roles of mammalian MT, evolution of a ligand designed solely for the detoxification of excess Zn is unlikely, given the low toxicity, and (relatively) low abundance of this ion. As seen with mammalian MT, Cd, Zn, Cu and a range of other ions are capable of induction of *smt*A. Although mammalian MT and SmtA are distinctly different in sequence, the data presented in this chapter indicates that the proteins share some similarities in terms of metal ion binding. Thus it is possible that the proteins have evolved (convergently or divergently) to fulfil similar functions. Genetic experiments, such as deletion of the *smt*A gene from Synechococcal cells, will resolve whether or not SmtA has functions not directly relating to metal ion detoxification, which may have implications for MTs in other systems. Experiments with SmtA *in vitro* could also assist in further clarifying the role of the protein in Synechococcal cells. It would be of interest, for example, to demonstrate whether or not SmtA is capable of modulating DNA binding and hence control of gene transcription by Zn-finger type DNA binding proteins, or the activity of metal-requiring enzymes, as has been demonstrated for mammalian MT (section 1.5.5). Such Zn-finger proteins have recently been identified in a cyanobacterium (Ogura et al 1991).

3.3.7.2 Possible biotechnological roles for *smt*A.

The ability of the expressed SmtA protein to cause an increase in Zn accumulation in a heterologous system may be of importance in the possible use of the *smt*A gene in genetically engineering organisms for the accumulation of Zn (possible roles for such molecules are discussed in section 1.7). In none of the studies discussed in section 1.8 were any of the MTs expressed in *E. coli* shown to accumulate Zn, although Cd and Cu accumulation was reported. SmtA, having the lowest pH of half dissociation for Zn of any protein thus far reported, would be likely to bind this ion in many different biological systems, and so would have potential applications where manipulation of Zn metabolism or Zn sequestration is desired.

The finding that SmtA binds Zn with a high affinity suggests the possibility of the use of this protein in the examination of the relationship between structure and metal binding of MT-like molecules. As has been discussed previously (section 1.4), the structural features which determine the affinity and preference of proteins for

specific ligands cannot be easily defined purely in terms of protein sequence. The qualities of SmtA which confer a high affinity for Zn would be worthy of investigation, particularly in view of the divergence of this molecule from other MTs so far characterised. In this respect expression of the protein in *E. coli* and characterisation of the metal-binding sites of the protein using techniques such as NMR and X-ray crystallography, will provide interesting comparisons with other molecules.

CHAPTER 4 EXPRESSION OF A PLANT GENE, *PsMT*$_A$, ENCODING A PROTEIN WITH SIMILARITY TO CLASS I MT IN *E. COLI*.

4.1 Introduction.

The primary structure of the putative product of the pea (*Pisum sativum*) gene, *PsMT*$_A$, has led to suggestions that this protein may have some as yet undefined role in metal ion homoeostasis and/or tolerance. Similar genes and cDNAs have been cloned from several species of higher plant (section 4.1.2), however the proteins encoded by these genes have not been isolated, and therefore remain to be characterised. This research, involving the expression of *PsMT*$_A$ in *E. coli*, was primarily undertaken to provide information regarding the possible functions of the encoded protein.

4.1.1 Metal tolerance in plants.

Reviews examining the broad subject of metal tolerance in plants (Woolhouse 1983; Verkleij and Schat 1990) serve to illustrate the many mechanisms of resistance which have been proposed to occur. As defined by Levitt (1980), these can be described as either avoidance mechanisms (which result in decreased uptake: e.g. alteration of membrane permeability, changes in the metal binding capacity of the cell wall or increased exudation of metal-binding substances) or tolerance mechanisms (by which the plant is able to cope with excessively accumulated metal: e.g. production of intracellular metal-binding molecules, alterations of metal-compartmentalisation, alterations of cellular metabolism or alterations of membrane structure). Distinction should also be drawn between those mechanisms which are common to non metal-selected plants, and alterations in metal resistance which have evolved in response to exposure to metals. The latter may involve modification of normal cellular metal metabolism, or the evolution of specialised mechanisms of metal tolerance within an ecotype of a particular species. Different mechanisms of tolerance to the same metal have been proposed to occur even within the same genus (Duvigneaud and Danaeyer de Smet 1963). Few generalisations can therefore be made concerning the physiological basis for tolerance to any particular ion, as data relating to any particular species cannot necessarily be extrapolated to include other species. Several of the proposed mechanisms which have been described

for various plant species, which serve to illustrate the diversity of metal-tolerance mechanisms in plant cells, are presented below. An exhaustive review of all of the described resistance mechanisms proposed for particular plant-metal associations, however, is not within the scope of this report, and the reader is therefore directed to the aforementioned reviews for a comprehensive treatment of the subject.

Several mechanisms have been proposed for resistance to the toxic effects of Al. Three different patterns of response have been noted (Foy et al 1978). (1) Accumulation in shoot tissues (and possibly additionally in roots). Best characterised in this regard is tea, in which accumulation has been demonstrated to occur in the leaves of resistant species (Matsumoto et al 1976). (2) Accumulation in root without transportation to shoot tissues. This response has been demonstrated in tolerant forms of *Azalea* (Lunt and Kofranek 1970). In the case of both (1) and (2), accumulated metal must be rendered non toxic either by intra- or extra-cellular compartmentalisation or by binding to sequestering ligands. It is postulated that organic acids or phenolic compounds may play a role in intra-cellular sequestration of Al (Sivasubramaniam and Talibudeen 1972). (3) Exclusion of the metal. This has been demonstrated to occur in Al-resistant barley and wheat (Foy 1974). It is proposed that this may be due to decreased membrane permeability or decreased cation-binding capacity of the cell wall (Foy et al 1978). It has also been postulated that exclusion may result from increased Al precipitation at the surface of the root plasma membrane. Dodge and Hiatt (1972) reported that Al-resistant varieties of wheat have a raised pH at root surfaces relative to sensitive varieties, which can result in Al precipitation.

In several species, Mn resistance correlates well with resistance to waterlogging (Robson and Loneragan 1970). Both effects are thought to be brought about by increased diffusion of oxygen from shoot to root tissue (Armstrong 1979). Increased oxygen concentration in roots will result in oxidation of Mn^{2+} to less toxic Mn^{4+}. Plant species show a wide range of resistance to, and accumulation of, Mn (Foy et al 1978). The physiological basis of the observed differences is generally poorly defined, though Momon et al (1980) demonstrated that in *Acanthopanax sciadophylloides*, an accumulator of Mn, the metal was apparently localised in electron-dense particles in cell walls.

Zn resistance has been reported for a great number of species, and several hypotheses have been put forward for the possible mechanisms which may be responsible. These include: (1) immobilisation of Zn in cell walls (Turner and Marshall 1972), (2) compartmentalisation of Zn in soluble complexes (possibly involving organic acids; Mathys 1977), and (3) enzymic adaptation (Cox et al 1976). Grill et al (1987) proposed a role for PCs in Zn tolerance, however there is conflicting evidence regarding this (reviewed in section 5.1.1.). The existing evidence for any proposed Zn resistance mechanism is not conclusive for any system so far described, though the available data (which generally reveal similar Zn accumulation in tolerant and non tolerant plants) is suggestive of internal mechanisms of adaptation of Zn rather than exclusion (Woolhouse 1983).

Detoxification of various metals has been proposed to occur via sequestration by intracellular ligands, in a manner analogous to that proposed for MTs in animal systems. The molecules which have received most attention in this regard, PCs, and their possible role in detoxification of Cd and Cu ions, is discussed in chapter 5.

4.1.2 The genetics of metal tolerance.

Metal resistance may occur via modification of metal detoxification mechanisms which are common to non-selected plants through genetic alteration brought about via selection of a resistant population in a high metal environment. In order to demonstrate that resistance in a particular ecotype is due to genetic alteration, the plant should be put through a seed cycle and the resistance phenotype shown to be heritable (Macnair 1990). Stable resistance in plant cell lines may be demonstrated by retention of resistance through repeated sub-culture in the absence of selection (e.g. Cd resistance in *Datura innoxia* cell lines was demonstrated to be a stable phenotype by growth for over 400 generations in the absence of Cd; Jackson et al 1984). In all selected species of whole plant tested, resistance has been demonstrated to be a highly heritable characteristic (Macnair 1990). In most instances where plants having the resistance phenotype have been analysed by crossing with non-resistant plants, the characteristic has been found to be at least partially dominant (Urquhart 1979; Macnair 1977).

Analysis of segregation of crossed plants has often revealed a lack of clear cut segregation patterns, consistent with the hypothesis that

the resistance phenotype is due to a high number of genes (Antonovics et al 1971), however in the case of Cu resistance in *M. guttatus*, which is perhaps the best characterised system, the phenotype appears to be due to a single major gene (Macnair 1983). Emergence of the resistance phenotype is often rapid. This implies a relatively low degree of complexity of the mechanisms involved, giving a further indication that a single dominant resistance gene may often be responsible for the observed phenotype (Ernst 1976).

Plants selected for tolerance to one metal do not generally exhibit increased tolerance to other metals, suggesting highly specific genetic modification (Gregory and Bradshaw 1965). Co-tolerance has been reported in several instances (Cox and Hutchinson 1980; Symeonidis et al 1985), although the observed increase in tolerance to the metals to which the plants were not originally selected was low. As yet, there are no reports of the isolation of any of the genes involved in metal tolerance.

4.1.3 MT-like genes and proteins in plants.

There has been considerable speculation as to whether plants produce MT, or whether the functions of MT in animal cells are performed by PC molecules in plant cells (Grill et al 1987). MTs have not been isolated from vegetative plant tissue, however the E_c protein isolated from wheat-germ (section 4.1.3.1) is termed a class II MT. Early reports of the isolation of MT from metal-exposed vegetative plant tissue are now considered likely to have been impure isolates of PC complexes. Recently, several groups have reported the isolation of plant genes, the putative products of which show significant similarity to class I MTs (section 4.1.3.2).

4.1.3.1 The E_c protein from wheat-germ.

The E_c protein isolated from wheat-germ is the only MT-like protein to have been isolated from higher plant tissue. Initial isolation of the protein (Hanley-Bowdoin and Lane 1983) was as a result of examination of cysteine incorporation into proteins expressed early in germination of wheat-germ. Computer analysis of the sequence revealed no significant homology to other proteins, however subsequent visual observation (Lane et al 1987) identified several sequences bearing homology to the class I MT from crab, and arrangement of cysteine

residues resembling MT. Isolation of the protein in association with Zn (Lane et al 1987) resulted in the classification of the protein as a class II MT (Kägi and Shäffer 1988). The ratio of Zn to protein was estimated to be approximately 5 : 1.

It was noted that although the E_C protein could be readily isolated from dry wheat-germ, the amount of protein present drops rapidly following germination (Hanley-Bowdoin and Lane 1983). By analogy with animal systems, where in rats, for example, the amount of MT present in the livers of neonatal rats is 20-fold greater than in adults (Kern et al 1981; see chapter 1), Lane et al (1987) postulate that the E_C protein may have a role in the deposition of Zn, the requirement for which may alter during proliferative compared to differentiating stages of development.

4.1.3.2 MT-like genes from plants.

Genes and cDNAs capable of encoding proteins with sequence similarity to class I MTs have recently been isolated from several species of higher plant. These include: *Mimulus guttatus* (de Miranda et al 1990), *Pisum sativum* (Evans et al 1990), *Zea mays* (de Framond 1991); soybean (Kawashima et al 1991) and barley (Okumura et al 1991). Additionally, several as yet unpublished cDNAs and genes have been isolated. These include cDNAs from *Arabidopsis thaliana* and alfalfa, and a gene from *Nicotiana tabacum* (A.M. Tommey unpublished results). All of these cDNAs and genes exhibit significant sequence similarity to each other. The deduced amino acid sequences of the putative protein products of these genes are extremely well-conserved, especially with regard to the cysteine residues which occur in the N- and C-terminal portions of the predicted sequences. For each of the sequences reported, excepting the soybean sequence, the cysteine residues can be aligned perfectly. The soybean sequence has two additional cysteine residues at the N-terminus. The aligned predicted protein sequences of the published DNA sequences are presented in figure 4.1.

All of these MT-like sequences were isolated as a result of screening cDNA libraries. In the case of the genes from *P. sativum* (discussed in detail below), and *Zea mays*, cDNA clones encoding MT-like proteins were isolated as a result of differential screening of cDNA libraries for organ-abundant clones. In each case the MT-like cDNAs were isolated as a result of being highly expressed in root

Figure 4.1 Amino acid alignment of the predicted protein sequences of the MT-like genes and cDNAs isolated from plants. The sequences are presented with breaks to allow maximal alignment of conserved residues. (1) *Pisum sativum*, (2) *Zea mays*, (3) *Mimulus gutattus*, (4) barley, (5) soybean. Cysteine residues are shown in bold. (References are as given in the text).

(1) M.S...GCGCGSSCNCGDSCKCNKRSSGLSYSEMETTETVIL..GVG

(2) M.S....CSCGSSCGCGSSCKCGKKYPDLEETSTAAQPTVVL..GVA

(3) MSS...GCSCGSGCKCGDNCSCSM.....YPDMETNTTVTMIEGVA

(4) M.S....CSCGSSCGCGSNCNCGKMYPDLEEKSGATMQVTVIVLGVG

(5) MSCCGGNCGCGSSCKCGNGCGGCKMYPDLSYTESTTTET..LVMGVA

(1) PAK...IQFEGAEMSAASEDG.GCKCGDNCTCDPCNCK

(2) PEKKAAPEFVEAAAESGEAAH.GCSCGSGCKCDPCNC.

(3) PLK...MYSEGSEKSFGAEGGNGCKCGSNCKCDPCNC.

(4) SAKVQF....EEAAEFGEAAH.GCSCGANCKCNPCNC.

(5) PVKAQF...EGAEMGVPAEN.DGCKCGPNCSCNPCTCK

relative to other tissues. *M. gutattus* cDNA clones, which were isolated from root tissue on the basis of repression by Cu-shock, were subsequently shown to be highly abundant in root tissue relative to leaf tissue.

Okumura et al (1991) isolated the barley gene, *ids1*, as a result of screening for Fe-deficiency-specific cDNAs. These authors speculate that the protein may have a role relating to Fe metabolism, potentially at the level of regulation of the genes involved with synthesis of Fe-binding substances such as mugineic acid (Shojima et al 1990) or that the MT-like protein may have a direct role in the transport of iron (Mori and Nishizawa 1987). No evidence was presented for these roles other than an increase in abundance of these cDNAs in response to Fe-deficiency. Recently reported data has shown a link between Fe metabolism and MT gene expression in *S. cerevisiae* (D.H. Hamer pers. commun.). Cells selected for increased Cu-requirement were found to be mutated in a membrane-associated Fe reductase system (FRE1). This system was shown to be active in Cu uptake and reduction (CuII to CuI). It was subsequently shown that in normal *S. cerevisiae* cells FRE1 is expressed in low Cu concentrations to improve Cu uptake, and is repressed in high Cu concentrations. It is conceivable, though as yet untested, that a similar Fe/Cu reductase is active in the roots of plants grown in Fe-deficient conditions. Evidence that plants grown under low Fe conditions accumulate excess Cu is consistent with such a hypothesis (Robinson et al unpublished).

The cDNA clone containing an MT-like sequence from soybean (*Glycine max* L), reported by Kawashima et al (1991) was isolated as a result of heterologous screening of a cDNA library using a synthetic oligonucleotide probe corresponding to the N-terminal part of mammalian MT. Analysis of expression of the gene by northern hybridisation of the cDNA to mRNA from soybean revealed slightly higher levels of expression of the corresponding gene in leaves relative to root. Exposure of plants to 6 μM Cu resulted in a slight decrease in expression of the gene in root tissue, which is consistent with the response to Cu-shock observed in *M. guttatus*. It is unclear whether this response is specific to the MT-like gene, or is a general effect of reduced transcription in response to Cu toxicity.

4.1.3.3 *PsMT*$_A$ gene and predicted protein structure.

The full sequence of the *PsMT*$_A$ gene, and predicted protein sequence, is presented in figure 4.2. As indicated above, the gene was isolated by screening a genomic library using a cDNA clone which had been isolated on the basis of high abundance in pea root. Comparison of the sequences of cDNA and genomic clones revealed the presence of a large intron in the coding sequence. This feature is conserved in the genomic clone from *Zea mays*. Southern analysis of several enzymic digests of *P. sativum* DNA revealed the existence of a small multi-gene family comprising up to 5 members. Several of these have recently been cloned (J. Bryden, unpublished results).

The predicted 75 amino acid protein sequence has two regions which are rich in cysteine residues (designated domains 1 [residues 4 - 18] and 2 [61 - 74] in figure 4.2). These regions have extensive homology to the Cu-MT from *N. crassa*. The central portion has no homology to MT, and contains several aromatic and hydrophobic residues. This region distinguishes the putative plant MT from all other MT-like proteins so far studied. None of the putative products of the plant MT-like genes described above have yet been isolated, and therefore no information is available on the structure of this class of protein.

4.1.3.4 *PsMT*$_A$ gene expression.

Northern analysis of expression of the *PsMT*$_A$ gene shows that expression is most abundant in root relative to other tissues. Etiolated leaf tissue exhibited increased abundance relative to leaves grown under normal light. In root tissue, *PsMT*$_A$ transcripts could not be detected in RNA isolated from embryonic radicle, but could be detected in pea roots 14 days after germination (Robinson et al 1992). The effects of metal administration on expression of the gene has not been examined, and it therefore remains to be shown whether or not this gene exhibits altered transcription in response to metals, as has been demonstrated for the genes from *M. gutattus*, soybean and barley, as discussed previously. Analysis of the 5' region of the gene reveals the presence of two short sequences having homology to upstream elements found in metal-responsive genes in other organisms. The sequence 5'TGCACACC3', flanked by imperfect repeats is located between -241 and -248 upstream of the start site of translation. This resembles the sequence 5'TGCRCNCX3' (where R represents G or A, X represents G or C, and N can be any base) which has been identified as

Figure 4.2 The sequence of the *PsMT*$_A$ coding region and surrounding DNA. The sequence of the insert of a genomic fragment of lambda clone PR179, which comprises the putative PsMT$_A$ protein coding sequence plus 805 bases of 5' and 493 bases of 3' sequence is presented. This clone was isolated by screening a pea genomic library using a cDNA clone which had been isolated by differential screening for tissue-specific sequences. Details are as given in section 4.1.2. (courtesy of I.M. Evans and L.N. Gatehouse).

.....5'AACCA
AGTTTCTTGTTCTTCAAACATTTTCTAATTCTTCAGCTTAGAGTTTCCTTTCATAATTAATGTAAGAATTGACCCTAGTAAGAAAGTCACTCATACCCTT
TCCTTCTTCTAGCCCTAAATTTCCGTGAAACATATAGTCTTCCCTTAACCCTTTTTGGAACATCCAACATTGCAGCTTATCATATATTGATCCTACTTTG
GTGAAGCGTTATATGTACTCCCGGAGGATTTCCATTTTCATCTATTGGATTCCTATGAAGACAACTATAGTCTTGGATTTCAATTTTTTAATTAATCAAA
AATAAATTATCCCGTGACATTATTAAATACCATTTTGAAATAAAAAGTTTCTAAAAAAAAAGTCCTTCGTCATTATCCATCATTTTTATTAAAGTATATTT
TATTATTTATTGAAACAAGTAATCATCCATTAGCGGAGAAATAGAGGAGTGAAGCATTTAACTTTTCCAAACGAAAGCGACGTAATCAACCTACATTTGA
CTTAGATTGGATTAAGCATGCAACAAATTAAAATTTAATCGCCATTGCAATTTGCACACCACAATAAGACGTGTGATGAAAACGATGATATCTACGTGGA
AATAATCCAAGGGTGGCCTTGTGGACCCATGCAACACAGGATGACAACACGTGGACGGTCAAGATTTCACCAATTATTCTCTCCCACCTTATAAATTGGG
GCACGCAACATCATTAAAAGACATCAATTGTAGTGAAGATAACAGCAACCAAGCAATTAATATCAATTGTTGTTTGCAAAAAATCTTAGGTTCTGAAAAT

ATG TCT GGA TGT GGT TGT GGA AGC AGT TGC AAC TGT GGT GAT AGC TGC AAGTAAGGATCCACCACCTTAATTCTTTGTTGTTTT
Met Ser Gly Cys Gly Cys Gly Ser Ser Cys Asn Cys Gly Asp Ser Cys Ly

TCTGTATAATTTTTTCATTACAATTATTTGTATGTCTATTTTTAATCATATAGATGATTCTTTGGAGATTTTTTTAAATAATTTGTTTAGTTTTATCGCA

TCGAATAATATATGATCTGAGCATGAGAAAAATAAATTTAATATAGACGGATTGTTTTTTATAAATGAATTAGGCTGAATCTAAATTCTAAGACTATGAA

TATGGTTCATAATTCTATGTTAAATCATTTTGTGTAGTGAAATTGGGCAATTTTATGTGTAAACGCATAATTTTGAGGTTTAAAATAAGGATCGTGCTGT

CGCGATAGTTTAAGTGTCGATTGTAGTCGCGTAAAGGCTTTTCTGATTTCGGTTGGTTTAAGTGTTGATTGCAATCGTGTAAAGAGTTTTCTAATTTCGG

TTGGTTTAGGTGCGATTGCAGTCGGGTAAAGATTTTCGTGATTGTCGTCGTTGCGGTGTGAATTAATCACAATTTCTTCTTTATCATAAAAACGTTGAAT

AACATATCGATATCGATTTGAAAACCTTTTTCGTGTAACGGTCTTTCGAAAACCTTTATTTTGACAACCAAGTTTATAATTGATTTGTTTTGCTTGACAG

A TGC AAC AAG AGG TCT AGT GGA TTG AGC TAC TCC GAA ATG GAA ACC ACC GAA ACC GTG ATT CTT GGC GTC GGT
s Cys Asn Lys Arg Ser Ser Gly Leu Ser Tyr Ser Glu Met Glu Thr Thr Glu Thr Val Ile Leu Gly Val Gly

CCG GCG AAG ATC CAG TTT GAA GGT GCT GAA ATG AGT GCT GCT TCT GAG GAT GGT GGC TGC AAG TGT GGT GAT AAC
Pro Ala Lys Ile Gln Phe Glu Gly Ala Glu Met Ser Ala Ala Ser Glu Asp Gly Gly Cys Lys Cys Gly Asp Asn

TGC ACT TGT GAC CCT TGC AAC TGC AAA TGAAGTGTAACATATAAAAGCTTGAAGCAGAGATATTGAAACCATTATGTTTAATTGTGTGTAT
Cys Thr Cys Asp Pro Cys Asn Cys Lys

GAGTACACATGTGTTTGGTTTTTTAGAATAATATAATTAATGTGGTCATGGATCTCTCTTCAGCAGATTGAAATATTTGATTTTGTTGAAAGGTTATGAG
AATTGTGGTTGGTTTGTGTTTGGTTTTCTTGTGTAAAGTGTAGCTAAAAGCTTGAATCATAAATCTCTGCATATGATAAATGGAATACATTATTGTGTGT
TATTTGAATGAAAGGATTGTTGGTACAACATATTATGATGTATATTATTAGAGTCGATGATCCAAAGTCTATTTTTTGTGTGAATGAAGATCTAAAAGGT
AAAAAGTATCTACATTATTTTCTCTCAATTGGCTATAGTGTTTTTTTTAAAGAAGAAAATATGAATTTCTCATTTCATAATCATTCTTTTACCACATGAT
TAGTGACAAGACTATGTAGAGATATGGCCA 3'

the core metal-regulatory element found in vertebrate MT genes (section 6.1.1.1; Palmiter 1987). A second sequence, 5'ATTAAGCATGCAACAATT3', is located between -272 and -290. This sequence shows significant homology (underlined) to a part of the 5' region of the *N. crassa* MT. Part of this sequence is repeated from bases -163 to -171. The significance of these sequences is as yet unknown.

Examination of Cu uptake by the roots of pea plants in response to Fe availability has been investigated (N.J. Robinson pers. commun.). Preliminary data has shown a direct correlation between Fe deficiency and increased Cu uptake. Subsequent analysis of the growth conditions of the plants from which $PsMT_A$ was isolated has demonstrated that these plants were also grown under conditions of low Fe availability. These results are consistent with a the hypothesis that $PsMT_A$ gene expression may be linked to Cu uptake in response to Fe deficiency, via an Fe transport mechanism. Further experiments will be required to support this hypothesis.

4.1.4 Aims.

This research was primarily undertaken in order to attempt to clarify the possible roles of the putative product of the *$PsMT_A$* gene in *P. sativum*. Determination of the ability of the protein to bind various metal ions would indicate whether or not the protein may have a role in metal ion homoeostasis. The *$PsMT_A$* gene was therefore cloned and expressed in *E. coli* in order to monitor metal ion binding *in vivo* by the expressed protein. Additionally, possible biotechnological applications of the *$PsMT_A$* gene for engineering of organisms toward increased metal tolerance and/or accumulation are assessed.

4.2 Materials and methods.

4.2.1 Experimental design.

To express the MT-like gene from *P. sativum* in *E. coli* it was necessary to generate fragments comprising the uninterrupted $PsMT_A$ coding sequence. Due to the difficulty in excising the large intron from the genomic DNA fragment, and in the absence of full-length cDNAs, it was considered that the best option for generation of such a fragment was via PCR amplification from cDNA. This also allowed the inclusion of restriction sites for simple manipulation of the PCR fragment.

4.2.2 PCR-mediated cloning of the $PsMT_A$ coding region.

4.2.2.1 cDNA synthesis.

Pea cDNA was used in the PCR in order to generate a DNA fragment which comprised the uninterrupted $PsMT_A$ coding sequence. cDNA was synthesised from pea tissue using the following method:

1) Total RNA was isolated from root tissue essentially as described in Hall et al (1978), and quantified by measurement of A_{260}.

2) PolyA^+ RNA was purified from total RNA by affinity chromatography using oligodT cellulose columns (Aviv and Leder 1972), precipitated using 2.5 volumes of 100 % ethanol and resuspended in water.

3) Construction of double-stranded cDNA was essentially as described by Sambrook et al (1989). This was used as a template in the reaction as described in section 4.2.2.2.

4.2.2.2 Amplification of the $PsMT_A$ coding sequence.

Two synthetic DNA primers, $^{5'}$GGCGAATTCGTCTGGATGTGGTTGTGG$^{3'}$ and $^{5'}$GGCGAATTCATTTGCAGTTGCAAGGGTC$^{3'}$, were designed to anneal to the 5' and 3' ends of the $PsMT_A$ coding sequence respectively, such that the 3' ends were proximal. Included in the sequence of the primers are EcoRI restriction sites which allow ligation of the amplified fragment into the *E. coli* expression vector pGEX3x, such that the sequence is in frame for expression as a carboxyterminal extension to glutathione-S-transferase (see section 3.2.1). PCR reaction conditions are specified in the legend to figure 4.3.

Other methods used in this chapter, involving cloning the amplification product, the expression of $PsMT_A$ in *E. coli*, and

analysis of the phenotypic effects of expression of the protein, have been described previously (see chapters 2 and 3). Minor alterations to protocols and specific conditions for reactions are described in the text and in figure legends.

4.3 Results and discussion

4.3.1 Amplification and cloning of the *PsMT*$_A$ coding region

Figure 4.3 shows the products of amplification reactions using the primers described in section 4.2, with templates and parameters as described in the figure legend. A genomic *PsMT*$_A$ clone (PR179) was included as a control for the reaction (the sequence of this clone, and details of its isolation are given in section 4.1). Band sizes of 870 bp for amplification from the genomic clone, and of 223 bp for amplification from cDNA were obtained, which is in agreement with predicted sizes.

The 223 bp reaction product amplified from pea root cDNA was excised from the gel, restricted with EcoRI, and ligated into EcoRI-restricted pGEX3x. Several clones were chosen on the basis of hybridisation to ^{32}P-labelled *PsMT*$_A$ probe (figure 4.4). The orientation of several of these clones was analysed by PCR. Reactions were performed using sequencing primers which anneal to the vector, along with the PCR primers described above. Two plasmids were selected on this basis, and the orientation and correct DNA sequence of each was confirmed by direct DNA sequencing. These were named pGPMT3 (which contains the sequence in the correct orientation for expression) and pGPMT3- (in which the sequence is inserted in the incorrect orientation). The DNA sequence obtained from plasmid pGPMT3, and the amino acid sequence resulting from translation of the insert as a fusion protein to GST is shown in figure 4.5. This is in precise agreement with the predicted sequence (figure 4.2).

4.3.2 Protein isolation and SDS-PAGE analysis of GST-fusion proteins.

Figure 4.6 shows that a protein of the predicted size was produced when cells containing either pGPMT3 or pGPMT3- were induced using IPTG. The GST-PsMT$_A$ fusion protein could be readily purified from cultures exposed to Zn or Cd, however, as with the GST-SmtA fusion protein described in chapter 3, exposure of cells to Cu resulted in the detection of multiple bands by SDS-PAGE following purification by glutathione-Sepharose chromatography.

Further *in vitro* analysis of the heterologous protein, as described in chapter 3 for SmtA, was performed by co-workers and is presented as appendix 1. These experiments demonstrated that PsMT$_A$ protein was

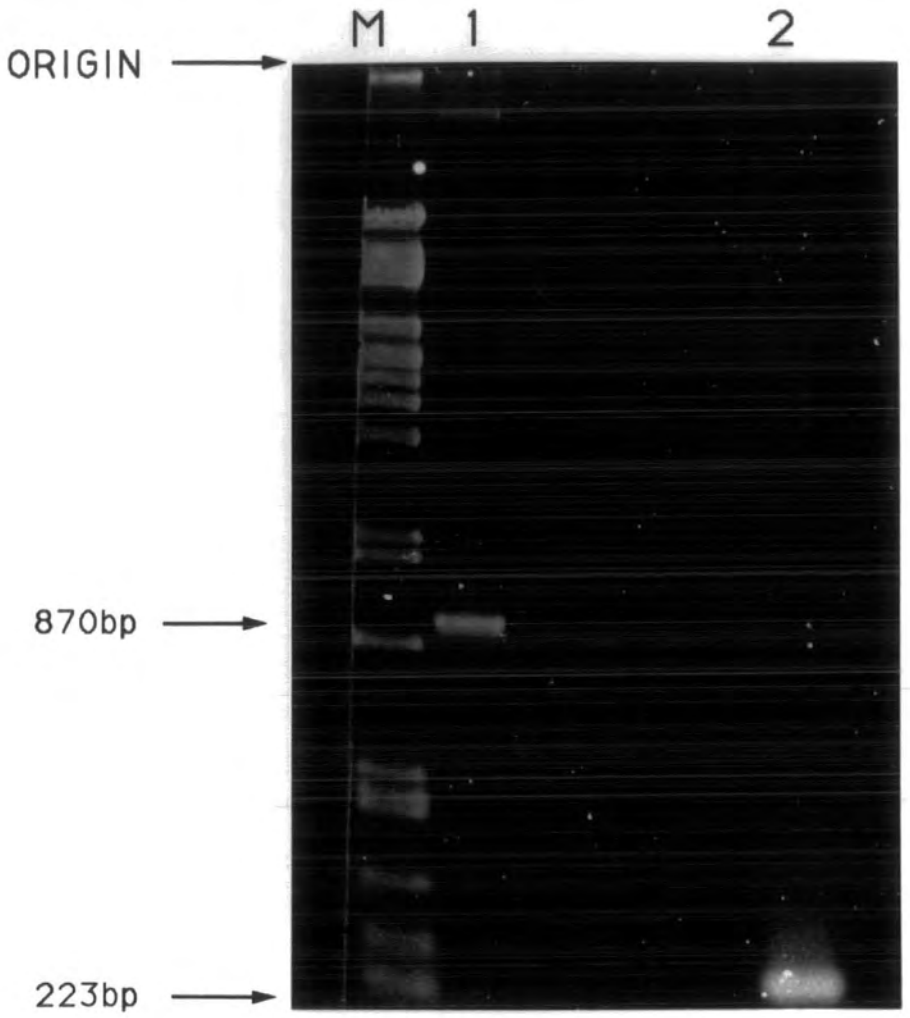
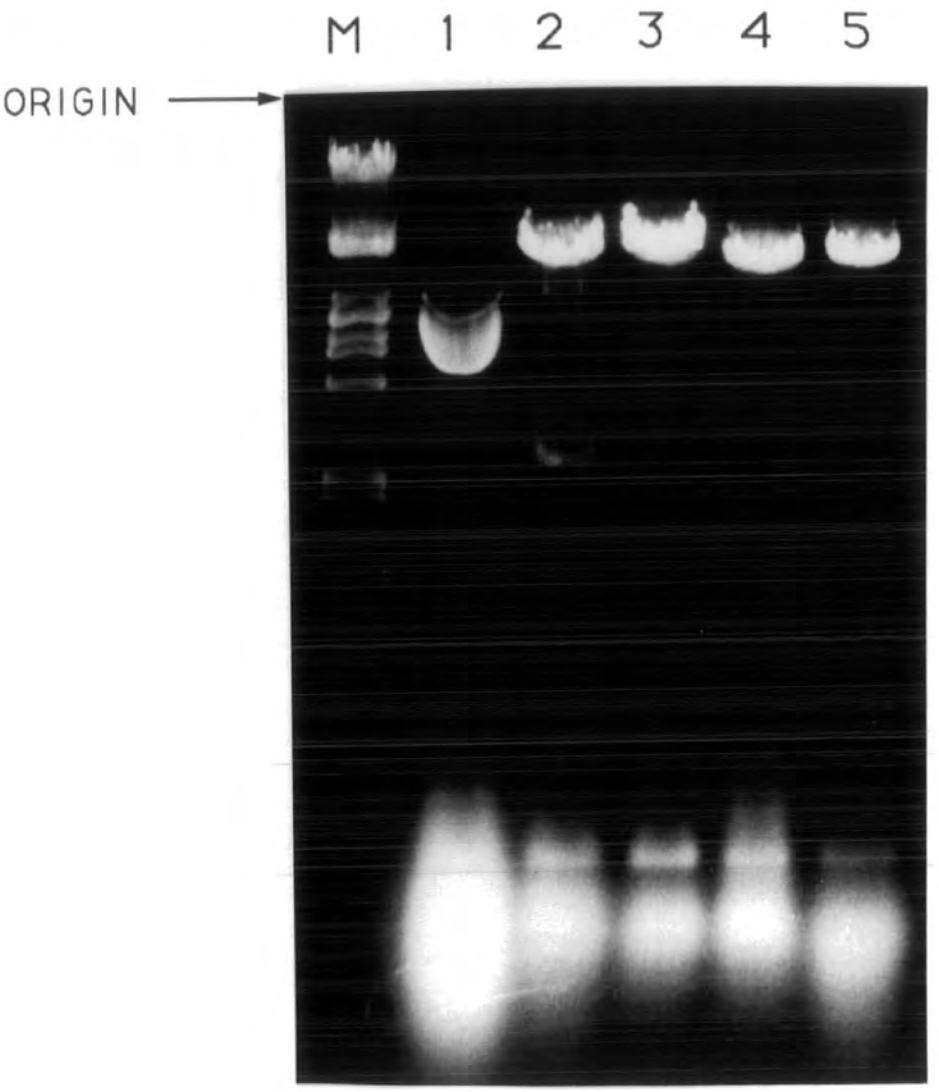

M
1
2
ORIGIN
870bp
223bp

Figure 4.3 *PsMT*$_A$ PCR amplification products. Poly(A$^+$) RNA was isolated from the roots of pea plants (*P. sativum* L cv Feltham First) and used for the synthesis of cDNA (section 4.2.2). 15 ng of cDNA was then used as template in 33 cycles of the PCR with primers as described in the text, and using the following reaction conditions: 92 °C for 1.5 min, 50 °C for 2 min, 72 °C for 2 min. Additionally, as a control, the genomic clone PR179, as illustrated in figure 4.2, was used as template in an identical reaction. Ten µl of each reaction were then loaded onto a 2 % agarose gel. (Lane M - PstI-restricted lambda DNA; lane 1 control reaction using 10 ng of *PsMT*$_A$ genomic clone PR179; lane 2, Ten µl of the PCR from cDNA).

Figure 4.4 Plasmids containing the *PsMT*$_A$ coding region amplified from cDNA. The 223 bp reaction product resulting from amplification of the *PsMT*$_A$ coding region from cDNA was cloned into EcoRI-restricted pGEX3x. (Lane M, PstI-restricted lambda DNA; lanes 2 to 5, pGEX3x/PsMT$_A$ clones re-cut using EcoRI).


M
1
2
3
4
5
ORIGIN

A (i)

5' GGCGAATTCGTCTGGATGTGGTTGTGG 3'

(ii)

3' CTGGGAACGTTGACGTTTACTTAAGCGG 5'

B

L *I* *E* *G* R G I P G N S S G C G C G S S C
CTGATCGAAGGTCGTGGGATCCCCGGG*AATTCG*TCTGGATGTGGTTGTGGAAGCAGTTGC

N C G D S C K C N K R S S G L S Y S E M
AACTGTGGTGATAGCTGCAAATGCAACAAGAGGTCTAGTGGATTGAGCTACTCCGAAATG

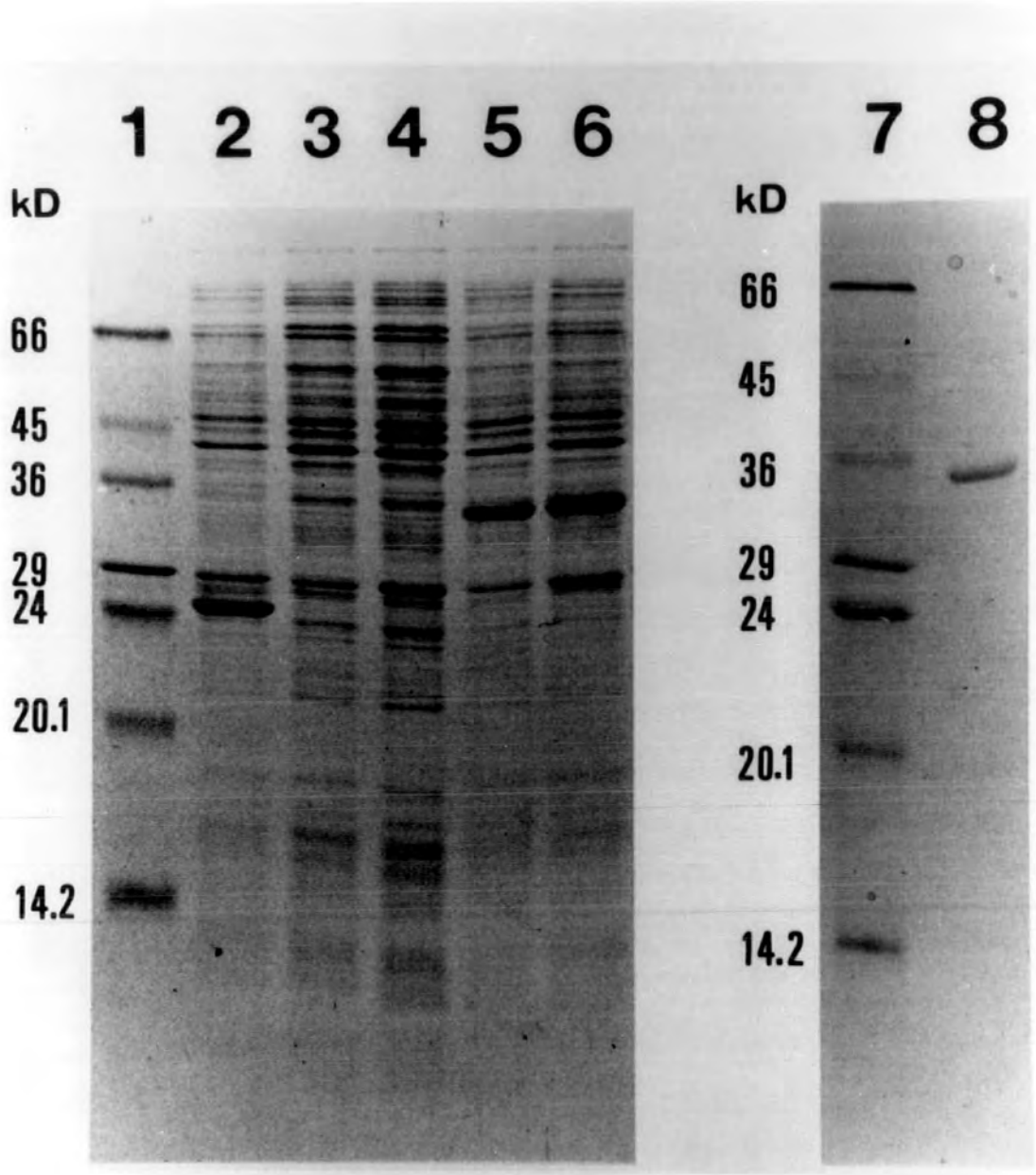
E T T E T V I L G V G P A K I Q F E G A
GAAACCACCGAAACCGTGATTCTTGGCGTCGGTCCGGCGAAGATCCAGTTTGAAGGTGCT

E M S A A S E D G G C K C G D N C T C D
GAAATGAGTGCTGCTTCTGAGGATGGTGGCTGCAAGTGTGGTGATAACTGCACTTGTGAC

P C N C K
CCTTGCAACTGCAAAT*GAATTC*ATCGTG

Figure 4.5 The PCR primers and amplified sequence of *PsMT*$_A$. Primers A (i) and (ii) were used to amplify the *PsMT*$_A$ coding region from cDNA as described in the text. The sequence is shown in the clone pGPMT3. pGEX3x vector sequences are shown in normal type, the *PsMT*$_A$ coding sequence in bold type, and the EcoRI sites used to clone the insert in italics. The factor Xa cleavage site of the resulting fusion protein is shown in italics.

Figure 4.6 SDS-PAGE analysis of the proteins produced from clones pGPMT3 and pGPMT3-. Proteins were extracted and prepared as described in the text. (Lane 2, crude lysate from IPTG-induced cells containing pGEX3x; lanes 3 and 4, crude lysate from IPTG-induced cells containing pGPMT3- in the presence (lane 4) or absence of 500 μM Zn; lanes 5 and 6, crude lysate from IPTG-induced cells containing pGPMT3 grown in the presence (lane 6) or absence (lane 5) of 500 μM Zn; lane 8, glutathione-Sepharose-purified protein from IPTG-cells containing pGPMT3 grown in the presence of 500 μM Zn).


1 2 3 4 5 6
kD
66
45
36
29
24
20.1
14.2
7 8
kD
66
45
36
29
24
20.1
14.2

successfully produced from this clone as a fusion to GST, and that the $PsMT_A$ portion of the protein was responsible for coordination of metal ions. Values for pH of half dissociation of metal ions from GST-$PsMT_A$ were determined as 5.25, 3.95 and 1.45 for Zn, Cd and Cu respectively.

4.3.3 Examination of the phenotypic effects of *$PsMT_A$* expression in *E. coli*.

The results of experiments performed to examine the effect of expression of *$PsMT_A$* on metal tolerance are summarised in figures 4.7 and 4.8. The concentrations used in these experiments were as described previously for examination of cells expressing *smt*A. The results show no consistent, statistically significant alteration of metal tolerance brought about by *$PsMT_A$* expression.

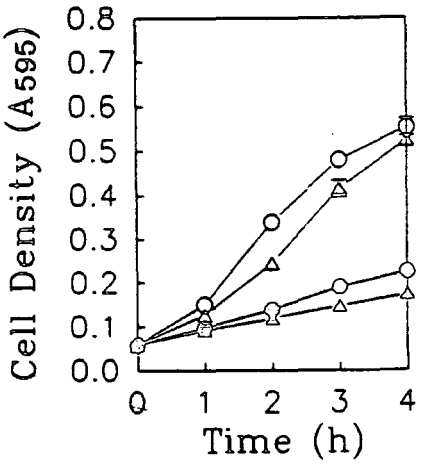
The results of analysis of metal accumulation by *$PsMT_A$*-expressing cells are shown in table 4.1 (A - C). The most marked difference between cells containing pGPMT3 and control cells is the 7.6-fold increase recorded for Cu accumulation. A lesser increase (c.a. 1.8-fold) was observed for cells exposed to the lower concentration of Cu. No statistically significant increase in Cd or Zn at the higher metal exposure was observed, however at lower exposure levels Cd was accumulated 2-fold, and Zn 1.48-fold relative to control cells.

Thus, as observed with *smt*A, induction of *$PsMT_A$* in *E. coli* did not result in any alteration in tolerance to the metal ions tested but did result in a considerable increase in metal (preferentially Cu) accumulation. The possible reasons for the observed result are discussed in chapter 7.

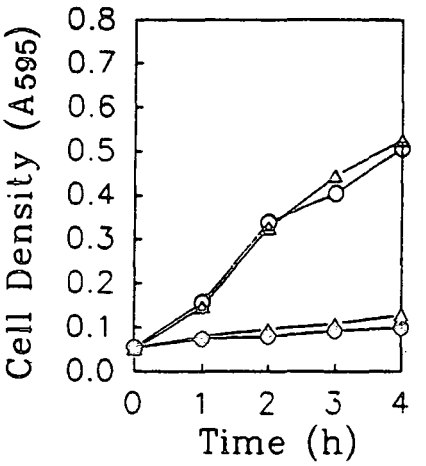
The pattern of increase in accumulation for Cd, Cu and Zn are in marked contrast to those obtained using similar methods to examine metal accumulation by cells expressing *smt*A, and indicate that the putative product of $PsMT_A$ preferentially binds Cu, and to a lesser extent Cd and Zn *in vivo*. It is of interest in regard to the large increase in Cu accumulation due to *$PsMT_A$* expression, that comparison of the amino acid sequence of the *$PsMT_A$* product with other MTs reveals that the protein has greatest over-all sequence similarity to the Cu-thionein of *N. crassa*. (with breaks in the sequence to allow the best possible match between the proteins, a percentage identity of 80 % is observed in comparison of these proteins). Additionally, the *$PsMT_A$*

Figure 4.7 Exposure of *E. coli* cells containing either pGPMT3 or pGEX3x (control) to Cd, Cu and Zn (higher concentrations). Methods, and metal concentrations used, were as described previously for clone pGPMT1 (chapter 3). (A - Cd, B - Cu, C - Zn. Symbols : O - non metal-exposed control cells; Δ - non metal-exposed cells containing pGPMT3; O - metal-exposed control cells; △ - metal-exposed cells containing pGPMT3).

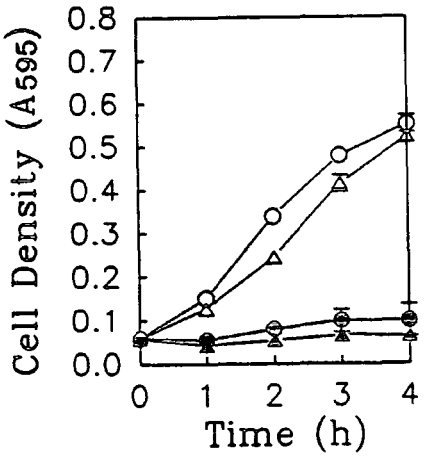
A(i)



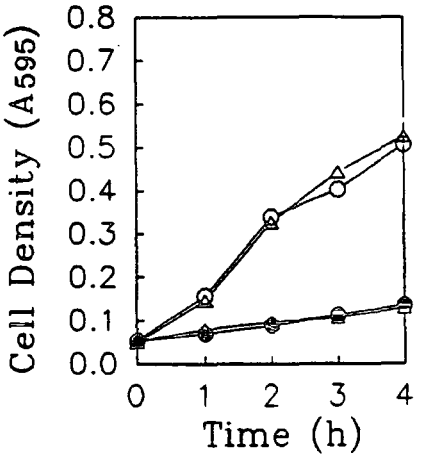
A(ii)



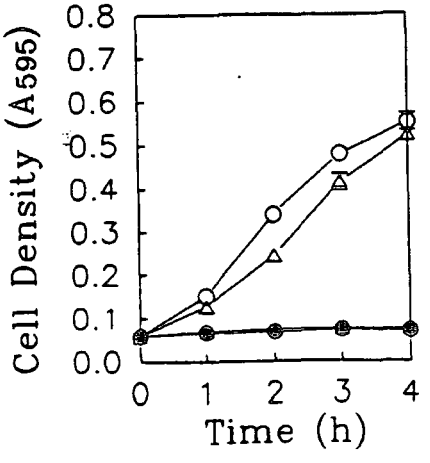
B(i)



B(ii)



C(i)



C(ii)

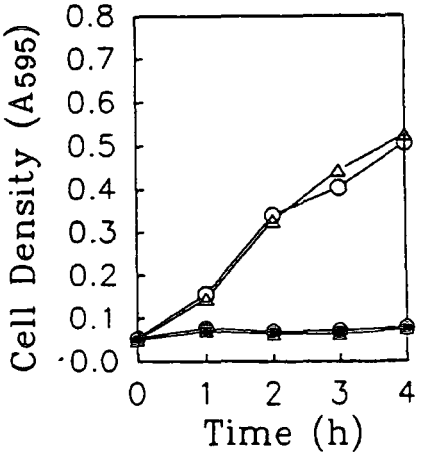
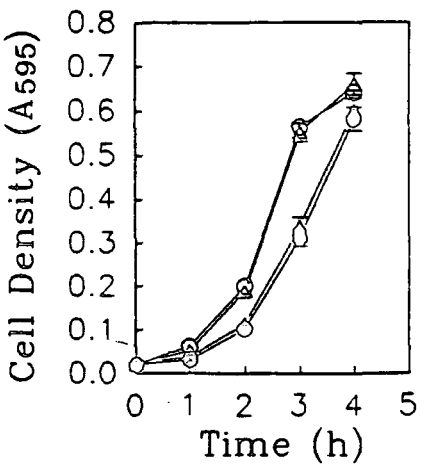
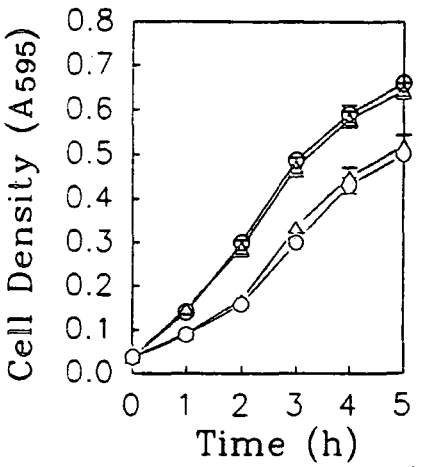


Figure 4.8 Exposure of *E. coli* cells containing either pGPMT3 or pGEX3x (control) to Cd, Cu and Zn (lower concentrations). Methods used were as described previously. (A - Cd, B - Cu, C - Zn. Symbols are as described for figure 4.7).

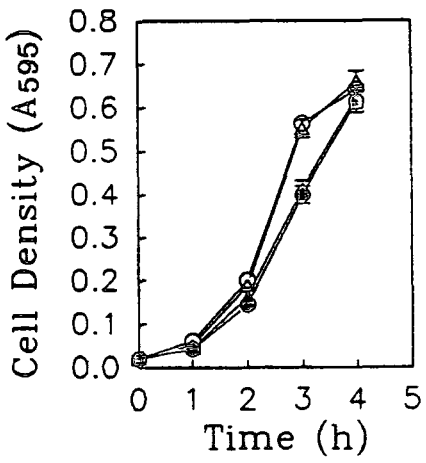
A(i)



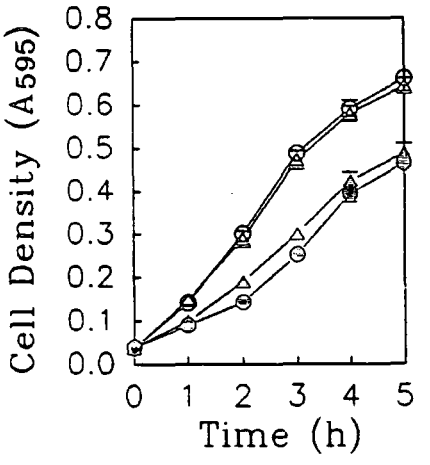
A(ii)



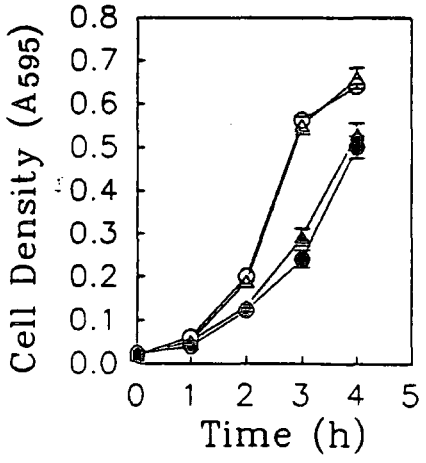
B(i)



B(ii)



C(i)



C(ii)

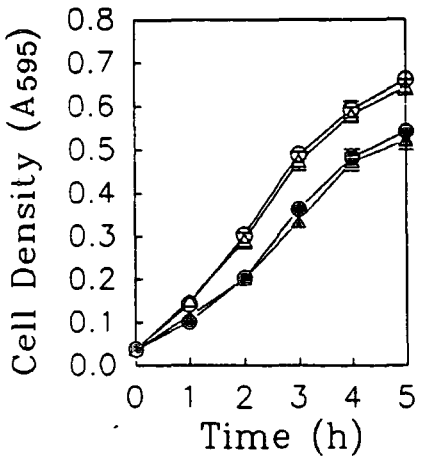


Table 4.1 Metal accumulation by *E. coli* cells containing either pGPMT3 or pGEX3x (control). Metal accumulation was measured using two concentrations of each metal, as described previously (chapter 3). (L) - low metal concentrations (0.3 mM Cd, 0.5 mM Cu or 0.5 mM Zn); (H) - high metal concentrations (0.6 mM Cd, 1 mM Zn, 2 mM Cu). Parameters used to calculate accumulation of metal ions are as described in section 3.2.3.8.3 for cells expressing *smt*A. (A - Cd, B - Cu, C - Zn).

(A)

Replicate number	Amount of Cd (nmoles/8 x 10^8 cells) pGEX3x H	pGEX3x L	pGPMT3 H	pGPMT3 L
1	20.82 (0.98)	6.35 (0.50)	22.03 (2.80)	9.92 (0.50
2	22.48 (1.99)	4.72 (0.35)	29.11 (0.51)	10.15 (0.16)
3	29.26 (0.34)	6.29 (0.11)	25.53 (0.98)	14.16 (0.71)
mean (n=9) (st. dev.)	**24.18 (4.47)**	**5.79 (0.86)**	**25.56 (3.54)**	**11.41 (2.11)**

(B)

Replicate number	Amount of Cu (nmoles/8 x 10^8 cells) pGEX3x H	pGEX3x L	pGPMT3 H	pGPMT3 L
1	18.39 (0.39)	6.53 (0.10)	134.08 (1.00)	11.11 (0.74)
2	24.18 (3.36)	5.64 (0.41)	121.06 (7.01)	8.75 (0.45)
3	7.94 (1.90)	6.41 (0.74)	129.70 (2.14)	12.62 (0.83)
mean (n=9) (st. dev.)	**16.83 (8.23)**	**6.20 (0.60)**	**128.28 (6.65)**	**10.83 (1.80)**

(C)

Replicate number	Amount of Zn (nmoles/8 x 10^8 cells) pGEX3x H	pGEX3x L	pGPMT3 H	pGPMT3 L
1	12.05 (0.20)	2.14 (0.18)	11.66 (0.50)	3.24 (0.04)
2	11.49 (0.56)	1.92 (0.22)	12.18 (2.02)	2.40 (0.04)
3	10.41 (1.08)	1.94 (0.21)	12.04 (0.38)	3.21 (0.33)
mean (n=9) (st. dev.)	**11.32 (0.83)**	**2.00 (0.02)**	**11.96 (0.26)**	**2.95 (0.48)**

upstream sequences which show homology to sequences upstream of the *N. crassa* MT gene (see section 3.1) may be of significance.

4.3.4 Implications of results with respect to functions of $PsMT_A$.

The finding that the protein encoded by $PsMT_A$ is capable of binding metal ions in *E. coli* has implications for the possible roles for the protein in plant cells.

As discussed in section 5.1.1, PCs have been shown to be the major metal ligands produced in many Cu- and Cd-stressed plant cells. These molecules have been isolated from Cd-exposed *P. sativum* (Robinson et al 1992) and *N.tabacum* (Reese and Wagner 1987), and from Cu-exposed *M. guttatus* (Salt et al 1989). Each of these species have also been shown to contain MT-like genes (section 4.1). The inability, thus far, to isolate the putative products of these genes from metal-stressed plant cells, suggests that the product of $PsMT_A$ may not have a primary role in the detoxification of excess intracellular metal ions. The results of this study, however, indicate that the protein will bind metal ions when expressed in *E. coli*. Though this may not be immediately comparable to metal binding *in planta*, the results do suggest that the protein has a relatively high affinity for metal ions, in particular Cu, and so could potentially be involved in metal ion homoeostasis. As no ligands have been characterised with regard to Cu uptake and storage in plants, it may be speculated that this protein has a role relating to these functions. Robinson et al (1992) have examined non-metal stressed *P. sativum* plants in an effort to isolate constitutive metal ligands. Preliminary data has indicated the presence of low molecular weight Cu and Zn ligands. Further characterisation of such ligands in plant cells may reveal the presence of the products of plant MT-like proteins, and allow insight into their functions in non-metal stressed tissue. Analysis of expression of $PsMT_A$ in *P. sativum* has suggested constitutive expression of the gene in this tissue. As discussed in section 4.1.3.4, however, it is possible that high "constitutive" expression of $PsMT_A$ may, in fact, represent a response to elevated Cu, as a result of increased Cu uptake by plants grown under Fe-deficient conditions. This would suggest a role for the protein in sequestration of excess intracellular Cu. Preferential Cu accumulation by *E. coli* cells expressing $PsMT_A$ is consistent with this hypothesis. As yet, although many authors have examined metal-stressed

plant tissue for metal-binding molecules, no ligand corresponding to the product of the MT-like genes described above have been isolated from any species. The possible reasons for this are discussed below.

Amino acid analysis of factor Xa-cleaved GST-$PsMT_A$ fusion protein indicated that some proteolytic cleavage of the protein had occurred *in vitro* during purification (appendix 1). The site at which proteolysis had occurred (the valine residue at position 39) is situated within the hydrophobic region which intervenes between the two MT-like putative metal-binding domains, indicating that the cysteine-rich regions may have been protected from proteolysis by virtue of binding metal ions, as has previously been noted for other MT molecules (Nielson and Winge 1983). Subsequent work by Kille et al (1991) who also expressed *$PsMT_A$* in *E. coli*, showed a similar pattern of proteolysis during purification (or *in vivo* prior to purification) of the protein. In addition to the authentic $PsMT_A$ protein sequence, four other sequences corresponding to cleavage sites within the central portion of the protein were obtained by Edman degradation. Interestingly, digestion of the various metal-binding $PsMT_A$ fragments with protease K *in vitro*, followed by gel permeation purification of the digestion products and amino acid analysis, resulted in only two sequences being detected. These corresponded to the cysteine rich domains of the protein. Elution of these fragments as one metal-containing peak from Sephadex G-50 and G-75 gel permeation columns led these authors to postulate that the two cysteine-rich domains had remained held together by virtue of metal coordination, and had thereby resisted proteolysis, while the hydrophobic non-metal-binding loop between the two domains had been completely digested by protease K. de Miranda et al (1990) noted the presence of a Cu-ligand which had some similarity to the amino acid composition of the product of the MT-like gene (but minus the hydrophobic/aromatic central portion of the protein) as a component of extracts from Cu-exposed *M. guttatus* cells (Salt et al 1989). This indicates that the protein may be post-transationally modified in plant cells via excision of the central portion of the protein. The evidence from examination of the protein following expression in *E. coli* presented above provides a model by which such processing may occur, via proteolysis of the protein following its association with metal ions. This may explain difficulty in purifying the putative product of MT-like genes from plant cells,

as the small protein dimer (ca 30 amino acids) would prove difficult to purify from plant cells in the presence of abundant PC molecules which are induced in metal-stressed tissue.

The results of this study suggest that the product of *PsMT*$_A$ is a metal-ligand with preference for Cu binding in *E. coli*, though the protein does bind Cd, and to a lesser extent Zn. Further studies will be required to demonstrate whether or not the protein has a role in metal ion homoeostasis in plant cells. In this regard, it would be of interest to further examine the potential roles of *PsMT*$_A$ via complementation of functions of metal-binding proteins in other organisms. It could be presumed that *PsMT*$_A$ would complement the metal-detoxification functions of CUP1 in *S. cerevisiae*, assuming the protein was stable in this system. Another potential role for the protein, storage and intracellular transport of Cu, could be examined if *PsMT*$_A$ were expressed in *cut*E-deficient *E. coli*.

4.3.5 Biotechnological applications of *PsMT*$_A$.

The expression of various MTs in *E. coli* has led to differing effects on metal accumulation, as discussed previously (section 1.8). The Cu-thionein of *N.crassa* resulted in accumulation of both Cu (3.5-fold over control) and Cd (5-fold over control; Romeyer et al 1990), whereas expression of rainbow trout MT resulted in increased accumulation of only Cd (Kille et al 1990). The PsMT$_A$ protein is the only ligand so far expressed in *E. coli* cells which gives a greater increase in Cu accumulation than in Cd accumulation. The *PsMT*$_A$ gene may therefore have potential applications, as discussed in section 1.7, relating to genetically engineering organism for increased accumulation of Cu.

Increased tolerance to Cu was not observed when *PsMT*$_A$ was expressed in *E. coli*. Possible reasons for this observation are discussed in chapter 7. This does not preclude the possibility that expression of *PsMT*$_A$ in other organisms, and under the control of different promoters, may have an effect on metal tolerance.

The PsMT$_A$ protein, though having certain sequence similarities to other MTs (especially those of *N. crassa* and *A. bisporus*) also has features which distinguish it from other metal ligands. Insight into the metal binding characteristics of the protein will provide useful information concerning the design of metal binding sites, since metal

stoichiometries (appendix 1; Kille et al 1991) are distinct from other MTs. The isolation of large amounts of pure protein by over-production in *E. coli* using clone pGPMT3 will provide material for analysis of the metal binding site by ^{113}Cd NMR.

CHAPTER 5 EXPRESSION OF αPC_3 IN *E. COLI*.

5.1 Introduction.

Phytochelatin (PC) molecules, as discussed below, are thought to have a role in detoxification of excess intracellular metal ions in some plants. Genes encoding enzymes involved in the biosynthesis of PCs have not yet been cloned, and hence gene transfer experiments to assist in determining the possible roles of these molecules cannot yet be performed. As an alternative to this, an analogue of a PC molecule (PC_3) was expressed in *E. coli* via the manufacture of a synthetic gene.

5.1.1. Phytochelatins (PCs).

As indicated in section 1.6, PCs were first isolated from *S. pombe* (Murasugi et al 1981), and subsequently from higher plants (Bernhard and Kägi 1985; Grill et al 1985; Robinson et al 1985). They have been identified in a wide variety of plant species (reviewed by Rauser 1990), the unicellular flagellate *Euglena gracilis* (Shaw et al 1989) and eukaryotic algae (Gekeler et al 1988).

5.1.1.1 Structure and metal binding.

PCs have the general structure $(\gamma Glu\text{-}Cys)_nGly$, where n can range from 2-11. The repeating γGlu-Cys unit represents glutamate and cysteine residues which are linked via the γ-carboxyl group of glutamate. This repeating unit is terminated with an α-linked glycine residue (in plants of the order Fabales glycine is replaced by beta-alanine; Grill et al 1986). In this respect these molecules resemble glutathione (γGlu-Cys-Gly), which is a precursor in their synthesis (section 5.1.1.2).

PC molecules have been isolated from plant cells in association with Cd and Cu, although Zn was a minor constituent of a complex isolated from tomato root, which was subsequently shown to have a PC component (Lue-Kim and Rauser 1986). Zn was also a minor component of the Cd-PC complex isolated from Cd-exposed *E. gracilis* (Shaw et al 1989). Wagner (1984) demonstrated Hg and Cu (but not Zn) binding to PC *in vitro*, as monitored by changes in absorbance at 250 nm brought about by addition of these metals to a solution containing Cd-PC. Thumann et al (1991)

demonstrated that Zn-PC complexes could be formed in vitro by the addition of Zn to apo-PC.

Reese and Winge (1988) reported that PC-Cd complexes isolated from Cd-exposed *S. pombe* cells are isolated as a cluster composed of several PC molecules. Two distinct forms are present, one of which contains acid-labile sulphide (S^{2-}). These authors reported that the presence of S^{2-} resulted in an increase in the affinity of the complexes for Cd, as demonstrated by a decrease in the pH required to displace Cd from complexes reconstituted with S^{2-}, relative to complexes without S^{2-}. The presence of S^{2-} in reconstituted complexes formed *in vitro* resulted in an alteration in the UV absorption characteristics of the complexes which was consistent with increasing size of Cd-S^{2-} crystallites (Reese and Winge 1988). It is postulated from the results of these experiments that the Cd-S^{2-}-peptide complexes consist of a Cd-S^{2-} crystallite core, surrounded by peptide molecules. Reese et al (1988) demonstrated that the S^{2-}-containing complexes isolated from *S. pombe* had increased capacity for Cd, having a higher stoichiometric ratio of Cd : peptide relative to complexes which did not contain S^{2-}. S^{2-} has also been shown to be present in the Cd-complexes isolated from *Datura innoxia* (Robinson et al 1990a), *E. gracilis* (Shaw et al 1989) and *C. glabrata* (Dameron and Winge 1989). There are no reports of the presence of S^{2-} in Cu complexes from any source. A comparison of the published data for the pH of half dissociation of metal from various PC complexes shows, however, that the affinity of PCs for Cu is much higher than for Cd (cited in Robinson 1990). It has been shown that *D. innoxia* cell cultures selected for resistance to Cd produce more of the longer chain PCs than do non selected cultures (Delhaize et al 1989). The finding that longer chain PCs have a higher affinity for Cd and Cu than do shorter forms (Hayashi et al 1988; Mehra and Winge 1988) indicates that the length of the molecule may be of importance in metal sequestration leading to metal resistance in metal-resistant cells.

5.1.1.2 Biosynthesis of PCs.

The observation that PC synthesis is accompanied by a concomitant depletion in cellular pools of glutathione (Scheller et al 1987) indicated that glutathione is a precursor in the synthesis of PC molecules. Inhibition of the production of PCs in cells exposed to

BSO, a potent inhibitor of γ-glutamyl-cysteine synthetase, provided further evidence for a biosynthetic pathway which involved glutathione consumption (Reese and Wagner 1987). Additionally, mutants of *S. pombe* which were unable to synthesise cadystin (PC) were also unable to synthesise glutathione (Mutoh and Hayashi 1988). Pulse-chase experiments using ^{35}S-labelled cysteine have indicated that longer chain forms of PC are synthesised from shorter chain forms. Gradual migration of ^{35}S-labelled cysteine was observed from glutathione to elongating species of PC molecule (Robinson et al 1988).

Grill et al (1989) reported the partial purification of an enzyme from *Silene cucubalus* which acts as a PC synthase, catalysing the formation of $(\gamma Glu\text{-}Cys)_{n+1}Gly$ from $(\gamma Glu\text{-}Cys)_nGly$ via transfer of the (γGlu-Cys) from glutathione, thereby acting as a γEC dipeptidyl transpeptidase. In a cell-free reaction system the enzyme was activated by Cd, and the reaction was stopped by sequestration of the activating metal ion by the newly formed PC molecules. Hayashi et al (1990) reported the existence of two pathways for cadystin synthesis in *S. pombe*. As well as the reaction described above, they report that synthesis proceeds via the addition of γGlu-Cys units from glutathione to elongating chains of $(\gamma Glu\text{-}Cys)_n$ units, to which glycine is added as the final step of the reaction. These authors found no evidence for enzyme activation by Cd. Alternative mechanisms by which PC formation could be stimulated in the presence of metal ions, is by product removal, via complex formation, or by substrate availability if Cd-associated substrates are required by the enzyme.

5.1.1.3 The role of PC in metal detoxification.

The synthesis of PC molecules in response to elevated concentrations of metal ions, and their association with the inducing metal ion, strongly suggests that they play a role in metal ion detoxification via sequestration. PCs isolated from metal-stressed *D. innoxia* cell cultures which had been selected for tolerance to Cd were found to be associated with up to 80 % of the cellular Cd (Jackson et al 1987). The observation that mutants of *S. pombe* which are unable to synthesise these molecules are hypersensitive to Cd toxicity (Mutoh and Hayashi 1988), and that tobacco cells exposed to BSO are Cd-hypersensitive (Reese and Wagner 1987), also indicates a role in the detoxification of metal ions.

It has been shown that a Cu-ligand (subsequently shown to be PC) was induced in both Cu-tolerant and non-tolerant ecotypes of *M. guttatus* in response to metal administration (Robinson and Thurman 1986). Similarly, Delhaize et al (1989) reported that a *D. innoxia* cell line which had been selected for Cd tolerance synthesised PCs at a similar rate to a non Cd-selected line when exposed to Cd. It was noted, however that in the Cd-tolerant line, Cd-PC complexes were formed more rapidly, indicating that factors other than PC synthesis contributed to tolerance. It has been shown that PC-Cd complexes in tobacco leaves are transported to the vacuole after synthesis (Vögeli-Lange and Wagner 1990). Evidence from Oritz et al (1991), indicates the presence of a specific transporter of these complexes into the vacuole in *S. pombe* cells. Transformation of *S. pombe* cells with multiple copies of the gene encoding this protein resulted in increased tolerance to, and accumulation of, Cd. Thus, it is likely that although synthesis of PCs is essential for detoxification of free Cu and Cd ions, other factors such as sequestration of the complexes in the vacuole have a role in the cellular response to supra-optimal concentrations of these metal ions. It was observed that decreased peptide formation in tobacco cells exposed to BSO, while increasing Cd-toxicity, did not affect Zn uptake or toxicity (Reese and Wagner 1987). Thus it is likely that PCs do not have a role in Zn detoxification.

5.1.1.4 Possible alternative functions of PCs.

Several alternative functions, not directly relating to metal ion detoxification, have been proposed for PCs. This is suggested by the observation that the enzymes involved in their synthesis are constitutively present (Steffens et al 1986). Thumann et al (1991) reactivated Cu- and Zn-requiring enzymes using *in vitro*-reconstituted Cu-PC and Zn-PC complexes, and suggested a role in the storage and mobilisation of these ions *in vivo*. The presence of inorganic sulphide in one of the two types of Cd-PC complexes isolated has led to the suggestion that PCs may have a role in sulphur metabolism (Steffens et al 1986). Structural similarity to glutathione has also led to suggestions that PCs may have analagous functions to those proposed for glutathione (e.g. detoxification of hydrogen peroxide). Rieger et al (1990) demonstrated that *Vicia fabia* root cells could be protected

from the effects of both triethylenemelamine and maleic hydrazide (which cause chromatid aberrations in dividing cells) by pretreatment with $NiCl_2$. BSO treatment of the roots prior to treatment with $NiCl_2$ nullified the effect, leading to the hypothesis that PC molecules have a role in protection of the plant from these compounds. As is the case for MTs, however, the evidence for a non-detoxification role is indirect. Further research is required to demonstrate whether or not these molecules have a constitutive function in non metal-stressed tissue.

5.1.2 Metal binding studies on novel metal-ligands.

Several studies have examined the metal binding characteristics of molecules related to MT and PC *in vitro*. Such studies have depended on standard methods such as increases in absorbance at 250 nm and 265 nm to monitor thiol binding to Cd and Cu respectively. In some cases more detailed spectral analysis, such as CD have been undertaken to provide comparison of the nature of the binding site with relation to natural molecules.

Okada et al (1989) and Nishiyama et al (1990), chemically synthesised various peptide fragments relating to fragments of *N. crassa* and *A. bisporus* MT molecules respectively. Analysis of the metal binding characteristics of these fragments gave similar results for each protein. It was shown that amounts of the different protein fragments which provided similar quantities of thiols, exhibited different capacities for Cu. The capacity of the larger MT peptides was greater than that of smaller fragments. Cd binding affinity was more dependent on the structure of the molecules than was Cu binding. It was found that at least two closely spaced cysteine residues (especially in the arrangement cys-X-cys) were important in maximising Cd binding affinity. As with Cu, comparison of binding capacity using equimolar amounts of thiols revealed that smaller MT fragments generally exhibited decreased capacity for Cd relative to larger fragments. One 5-residue fragment, however, with the sequence Gly-Cys-Ser-Cys-Ser, exhibited equal capacity for Cd sequestration to the entire *N. crassa* protein. Longer peptides containing the same sequence, and with additional cysteine residues, had lesser Cd capacity, indicating that cysteine availability and spacing are the most important factors in Cd coordination.

Ohta et al (1983) found similar results with fragments corresponding to portions of human MT-II. By monitoring changes in UV absorbance of Cd-peptide complexes, they were able to conclude that Cd binding was optimised by sequences having the general structure Cys-Cys-X-Cys, which are prevalent in the alpha domain of MT. Yoshida et al (1979) synthesised portions of mouse MT-I. Surprisingly, they report that one peptide, having the sequence Gly-Cys-Ser-Lys-Cys-Ala-Gln-Gly-Cys-Val, which corresponds to part of the alpha fragment, had greater affinity for Zn than for Cd. This is the only report of a thiol-rich molecule which has a higher affinity for Zn than for Cd.

Matsumoto et al (1990) synthesised peptides with the structure $(\gamma Glu\text{-}Cys)_nGly$, with n values 2-5. In this report it was shown that whereas Cu(I) binding was not greatly affected by the length of the peptides, affinity and capacity for Cd was greatly increased with increasing peptide length. A mixture of $(\gamma Glu\text{-}Cys)_5Gly$ with Cd and Cu in equal concentrations resulted in complete saturation of the peptides with Cu, with no Cd being found in peptide complexes following gel filtration chromatography of the mixture. Winge et al (1989) synthesised both α- and γ-linked PC analogues in order to examine the ability of these molecules to coat Cd-S crystallites *in vitro*, as has been observed *in vivo* in *S. pombe* cells. It was found that the type of linkage was apparently not important in this regard, and that the crystallites formed by both types of molecule were similar in size. A peptide of the sequence Lys-Cys-Thr-Cys-Ala was also tested for the ability to coat Cd-S crystallites, and was found to produce similar complexes to the other 2 peptides, suggesting that the availability of cysteine residues was the only important feature regarding the ability of such molecules to coat Cd-S crystallites *in vitro*.

5.1.3 Studies on structural alteration of MT molecules.

Romeyer et al (1990) used synthetic DNA oligonucleotides to clone genes encoding variants of the *N. crassa* MT in *E. coli*. Five such genes were successfully expressed, encoding proteins in which the each cysteine residues of the natural MT were replaced by either Asp, Asn, His, Lys or Tyr. Only the protein containing His residues showed any metal binding activity *in vivo*. This protein caused increased Cu and Cd accumulation by *E. coli*, but at a reduced level to that exhibited by expression of the natural cysteine-containing protein. Cu and Cd

were selectively accumulated from a range of 16 different metals by the histidine derivative.

Oikawa et al (1991) chemically synthesised a derivative of *N. crassa* MT in which all the cysteine residues were replaced with selenocysteine. This protein was capable of binding only 3 Cu ions, due to the change in the size of the binding pocket. CD spectroscopy suggested asymmetric Cu coordination.

Two recent studies examined the effect of alterations to mammalian MT. Chernaik and Huang (1991) expressed several variants of the Chinese hamster MT-II in CUP1-deleted *S. cerevisiae*. Specific alterations were made to the MT gene by *in vitro* mutagenesis, to replace specific cysteine residues with serine. This was done in order to examine which cysteine residues were of most importance in retaining the metal binding characteristics of the MT molecule. Using the criterion of the ability of the molecule to restore metal tolerance to the *S. cerevisiae* strain as a marker of the metal binding characteristics of the protein, they demonstrated that bridging ligands (as assigned by Schultze et al 1988), were of most importance in retaining the metal binding properties of MT. Similarly, Rhee et al (1990) used metal resistance as a biological marker in an examination of the effect on metal coordination of the expansion of the hinge region joining the two MT domains. In natural mammalian MT molecules this region consists of three residues (Lys-Lys-Ser). These authors expanded this region via insertional mutagenesis of the MT gene, to give variously sized hinge regions of between 5 and 19 amino acids. The level of resistance to metal ions decreased with increasing size of the hinge region, though it was not demonstrated whether this was due to decreased metal binding to the intact molecule, or to decreased protein stability.

5.1.4 Aims.

This chapter details the expression of an analogue of the metal chelating molecule PC_3 in *E. coli*, and an examination of its metal binding properties by examination of its physiological effects *in vivo*. A synthetic gene encoding the protein analogue of PC_3, $(\alpha Glu\text{-}Cys)_3Gly$ was made for this purpose and cloned for expression in *E. coli*. The aim of these experiments was to determine the effect of expression of this protein, and by inference from these results, gain

information on the possible roles of PCs. The potential for the design of novel proteins for enhancement of metal tolerance and accumulation is also addressed.

5.2 Methods.

5.2.1 Experimental design.

Oligonucleotides $^{5'}$CTAGACTAGGAGGAATTCTATGGAGTGTGAATGCGAATGTGGTAG$^{3'}$ and $^{5'}$GATCCTACCCACATTCGCATTCACACTCCATAGAATTCCTCCTAGT$^{3'}$ were synthesised and annealed to form a DNA fragment capable of encoding an α-analogue of PC. Figure 5.1 describes the sequence and features of the annealed DNA strands which encode the phytochelatin analogue αPC_3 [or $(\alpha Glu\text{-}Cys)_3Gly$]. The annealed strands were designed to be inserted into the expression vectors pUC18 and pUC19. If the whole insert is cloned into the XbaI/BamHI sites of these vectors, it is incorrectly orientated for expression in pUC18, and correctly orientated in pUC19. The insert may be restricted with EcoRI for insertion into EcoRI/BamHI restricted pUC18 to give a carboxyterminal fusion to the first six amino acids of the *lacZ* fragment of beta-galactosidase. Additionally, since the insert includes a ribosome-binding-site (Shine/Dalgarno region) which is independent of vector sequences, a number of other expression vectors could potentially be employed.

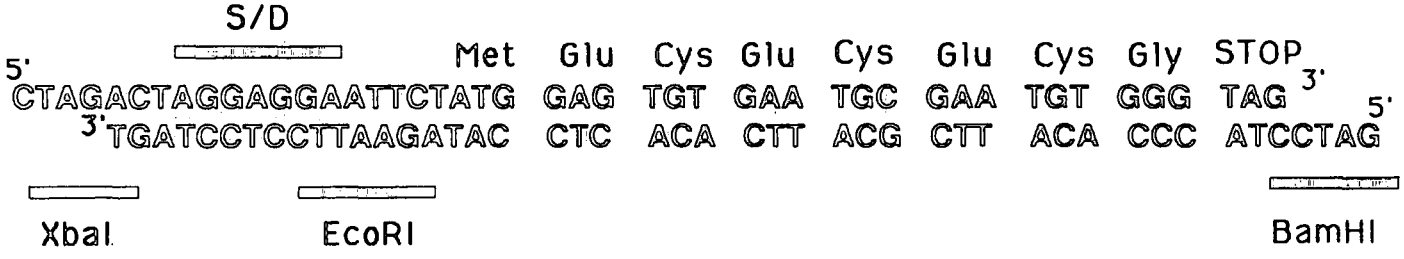
The sequence 5'AGGAGGA 3' was designed to maximise initiation of protein synthesis from the start codon of the synthetic gene. The sequence is derived from a consensus of *E. coli* ribosome binding sites (Stormo et al 1982), and is placed at a distance from the ATG codon which, by analogy to natural *E. coli* translational signals, should allow efficient protein initiation (the optimal distance is generally seven, plus or minus two nucleotides; cited in Kozak 1983). Jay et al 1981, derived a similar sequence (5'AAGGAGG 3') which they chemically synthesised and incorporated into an *E. coli* expression vector. This vector was found to efficiently initiate the translation of several cloned mammalian proteins in *E. coli*. Full details of the various constructs created in order to facilitate the expression of an α-analogue in *E. coli* are described below and in section 5.3.

5.2.2 Purification, kinasing and annealing of DNA oligonucleotides.

The DNA strands used to form the synthetic gene were synthesised as outlined in section 2. To allow further purification of the oligonucleotides used to form the constructs described in this section, DNA synthesis was performed by the "trityl on" method, which omits the final detritylation step. A dimethoxytrityl group is therefore retained on the 5' end of the DNA strand, allowing

Figure 5.1 The sequence and structural features of the annealed DNA strands used to form a synthetic gene encoding αPC_3.

The DNA oligonucleotides used in the formation of the synthetic gene are presented as an annealed fragment. (S/D - Shine/Dalgarno region for ribosome binding; XbaI - overhang allowing insertion into an XbaI site; BamHI - overhang allowing insertion into a BamHI site; EcoRI - EcoRI restriction site which allows cleavage of the construct for cloning into EcoRI/BamHI-restricted pUC18 to give a carboxyterminal fusion to LacZ).


S/D
5'
Met Glu Cys Glu Cys Glu Cys Gly STOP
CTAGACTAGGAGGAATTCTATG GAG TGT GAA TGC GAA TGT GGG TAG 3'
3' TGATCCTCCTTAAGATAC CTC ACA CTT ACG CTT ACA CCC ATCCTAG 5'
XbaI
EcoRI
BamHI

purification of the full length product from shorter length incomplete oligonucleotides using a reverse phase column (Oligo Purification Column; Applied Biosystems product No.400771). The newly synthesised oligonucleotides were bound to the column in ammonium hydroxide solution, washed, and then detritylated using TFA. The resulting detritylated species were eluted from the column using 20 % ammonium hydroxide, and collected by vacuum centrifugation.

After resuspension of the oligonucleotides in TE buffer (pH 8.0), the DNA was quantified by measurement of A_{260}. An aliquot (1 µg) of each oligonucleotide was checked by visualisation on a 20 % acrylamide gel, and 5 µg of each DNA strand was then added to a reaction containing 1X LKB, 10 mM ATP and 5 units of T4 polynucleotide kinase. After incubation at 37 °C for 1 h, the reaction tube was placed in a waterbath and heated to 90 °C. This was then left for 5 h to return to room temperature. The sequence and features of the annealed oligonucleotides as described in section 5.3.1.

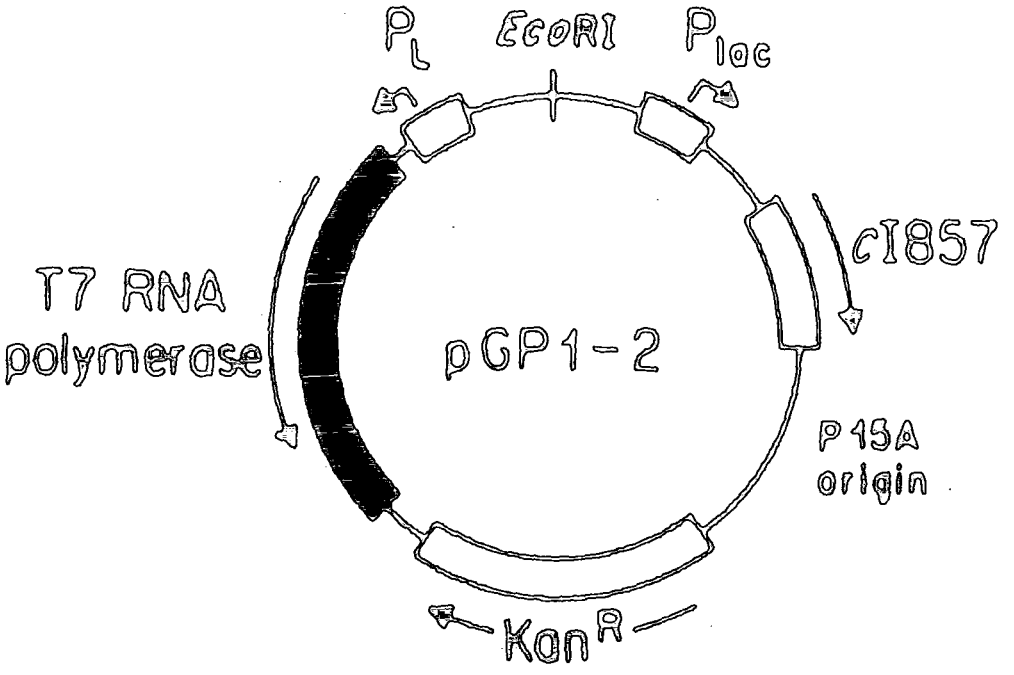
5.2.3 Cloning of annealed synthetic oligonucleotides.

Cloning of the annealed oligonucleotide strands was carried out according to standard protocols (Sambrook et al 1989). The insert was cloned as an XbaI/BamHI fragment into pUC18, pUC19 and pGEM4z (pGPSG1, 2 and 4 respectively). The fragment was subsequently sub-cloned from pGPSG1 by restriction with EcoRI and BamHI, the 33 bp fragment isolated from a 20 % polyacrylamide gel and inserted into EcoRI/BamHI-restricted pUC18 (plasmid pGPSG3).

5.2.4 The use of plasmid pGP1.2 to allow heat-inducible transcription of the synthetic gene.

Plasmid pGP1.2 (Tabor and Richardson 1985; figure 5.2) constitutively expresses a heat-sensitive form of the lambda cI repressor protein (mutant cI857; Hecht et al 1983). This mutant form of the protein binds to the lambda P_L promoter at 30 °C, shutting off expression of genes downstream of the promoter. At higher temperatures the protein becomes gradually denatured, and hence does not bind effectively to the promoter sequence. In plasmid pGP1.2, the lambda P_L promoter is placed upstream of, thereby controlling the expression of, the T7 RNA polymerase gene. *E. coli* cells are capable of harbouring this plasmid along with a pBR322-derived plasmid such as pGEM4z. Thus, DNA cloned

Figure 5.2 Features of plasmid pGP1.2. The 7.2 Kb plasmid pGP1.2 allows heat-induction of genes which are under the control of T7 promoters, via heat-induction of T7 RNA polymerase. Full details of the structural features shown in the diagram, and of the mechanism by which the construct can be used to control the expression of cloned genes, are given in section 5.2.4.


P_L
EcoRI
P_{lac}
cI857
T7 RNA
polymerase
pGP1-2
P15A
origin
Kan^R

downstream of the T7 promoter in pGEM4z can be made heat-inducible by co-transformation with plasmid pGP1.2, via heat-induction of T7 polymerase. Cells containing pGP1.2 were made competent using standard procedures, for subsequent transformation with either pGEM4z or pGPSG4.

5.2.5 IPTG-induction and northern analysis of cells containing pGPSG2 and pGPSG3.

To enable the detection of transcripts which included the synthetic gene coding sequence, cells containing the constructs described above were grown overnight in LB (+50 µg/ml Ap). Log. phase cultures were set up from these, and the cells grown to A_{595} = 0.5. At this point IPTG (1 mM) was added to the cultures, and incubation continued for a further 20 min. An aliquot of the cells (1 ml of culture) was harvested by microcentrifugation, and RNA extracted using the method described in chapter 2. RNA species were prepared and separated on a 1.4 % formaldehyde-agarose gel and blotted onto a nylon membrane, as described previously.

5.2.6 Heat-induction, RNA extraction and transcript detection in cells containing plasmids pGP1.2 and pGPSG4.

To detect transcripts of the synthetic gene under control of the T7 promoter in pGPSG4, cells containing both this plasmid and pGP1.2 were grown overnight at 29 °C in LB (+ 50 µg/ml Ap and 25 µg/ml Km). Eight 2 ml cultures were set up from this, and growth continued under the same conditions until a cell density (A_{595}) of 0.5 was reached. At this point, the cultures were heat-induced at a range of temperatures between 29 and 42 °C for 10 min (see legend to figure 5.4). The cultures were then returned to 29 °C for a further period of 30 min. 1 ml aliquots of these cells were harvested by microcentrifugation, and RNA extracted as described in chapter 2.

5.2.7 Labelling of *E. coli* proteins with ^{35}S-cysteine.

E. coli cells containing plasmids pGP1.2 and either pGPSG4 or pGEM4z (control) were exposed to ^{35}S-cysteine to attempt to label the product of the synthetic gene to allow detection either by fluorometry of polyacrylamide gels or by scintillation analysis of fractions from a gel permeation HPLC column (SW3000 column; section 5.2.11). In either

case the method used to expose cells to ^{35}S-cysteine was identical except that, in the case of gel permeation HPLC analysis, Cd was added at step 5:

1) Cells were grown for 16 h at 29 °C in 5 ml of M9 minimal medium supplemented with 50 µg/ml Ap and 25 µg/ml Km, 20 µM thiamine, plus each of the following L-amino acids at a concentration of 40 µM : alanine, arginine, asparginine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine.

2) Cells were diluted 1/10 (0.5 ml in 5 ml) into fresh M9 medium as above but containing each of the amino acids listed above except cysteine, and grown until the optical density of the culture (A_{595}) was 0.4.

3) The cultures were incubated at 42 °C for 10 min to induce transcription of the T7 polymerase gene from the lambda P_L promoter.

4) After a further incubation period of 20 min at 29 °C, for experiments where transcription by *E. coli* RNA polymerase was inhibited, rifampicin was added at a final concentration of 200 µg/ml.

5) ^{35}S cysteine was immediately added at a final concentration of 2 µCi/ml, (plus Cd to a final concentration of 500 µM for gel permeation HPLC) and the cells incubated for a further period of 3 h at 29 °C.

Extracts were then prepared using the method outlined in section 5.2.8 (for gel permeation HPLC analysis) or by that described in section 5.2.9 (for SDS-PAGE analysis).

5.2.8 Exposure of *E. coli* cells to Cd for gel permeation HPLC analysis.

Cells containing pGP1.2 plus pGPSG4 were exposed to Cd to allow detection of Cd-ligands expressed in the cells using the following method:

1) Cultures (5 ml) were grown for 16 h in M9 medium containing 20 µM thiamine plus each of the amino acids as listed in section 5.2.6 at a concentration of 40 µM.

2) Log phase cultures were initiated from these by 1/10 dilution in the same medium, and grown to A_{595} = 0.4.

3) The cultures were incubated at 42 °C for 10 min, Cd added to a final concentration of 500 µM, and the cultures returned to 30 °C for a further 3 h.
4) Proteins were extracted as described in section 5.2.8.

5.2.9 Extraction of total *E. coli* protein for analysis of extracts by gel permeation (SW3000) HPLC chromatography.

Cell extracts for separation on SW3000 (TSK, Japan) columns were prepared as follows:
1) Cells from 5 ml of culture were harvested by centrifugation, and were resuspended in 250 µl of extraction buffer (10 mM tris-Cl, 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol; pH 7.4).
2) The resupended cells were sonicated on ice for 2 min at full power using 4 x 30 sec bursts each separated by 30 sec.
3) Cell debris was removed by microcentrifugation at high speed for 10 min.
An aliquot of the supernatant was then applied to the column without further treatment.

5.2.10 Preparation of ^{35}S-labelled *E. coli* proteins for PAGE.

^{35}S-labelled proteins were prepared from ^{35}S-cysteine-exposed cells according to the method used by Berka et al (1988). Cell pellets from 200 µl of culture were resuspended in 25 µl of lysis buffer (25 mM tris-Cl pH 8.0, 10 mM EDTA, 50 mM glucose, and 1 mg/ml lysosyme). After incubation on ice for 15 min, the lysed cells were mixed for 30 min with 25 µl of a solution consisting of 25 mM dithiothreitol, 100 mM NaCl, 200 mM MgCl, and 0.8 % (v/v) Triton X-100. The crude protein preparation was subjected to carboxymethylation by the addition of 5 µl of sodium iodoacetate [625 mg/ml in 100 mM tris-Cl (pH 8.6)], and incubation in the dark at 25 °C for 3 h. Samples were prepared for PAGE as described previously (section 2.3.3.14).

5.2.11 Gel permeation HPLC chromatography.

Gel permeation HPLC was performed using an SW3000 column equilibrated in 150 mM NaCl to counteract hydrophobic interactions between the sample and separating resin. A Gilson model 302 pump was used to maintain buffer flow through the column. Samples of 100 µl were

injected. Column flow rate was 1 ml/min. Fractions sizes collected are described in the results section for each experiment.

5.2.12 Preparation of tricine gels.

Tricine gels were prepared essentially as described by Shägger and von Jagow (1987) Stock solutions and amounts used were as follows:

1) Stacking gel (6 %):

48 % acrylamide/bis-acrylamide	- 0.35 ml
Gel buffer (3 M tris-Cl, 0.3 % SDS pH 8.45)	- 0.77 ml
Water	- 1.9 ml
Ammonium persulphate (3 %)	- 2.5 µl
TEMED	- 6.3 µl

2) Running gel (12 %):

48 % acrylamide/bis-acrylamide	- 9.7 ml
Gel buffer (as above)	- 13.3 ml
Glycerol	- 5.3 ml
Water	- 8.0 ml
Ammonium persulphate (3 %)	- 130 µl
TEMED	- 30 µl

Acrylamide stock	-	48 g acrylamide, 1.5 g bisacrylamide in 100 ml of water.
Anode buffer	-	0.2 M tris-Cl (pH 8.9)
Cathode buffer	-	0.1 M tris-Cl, 0.1 M tricine, 0.1 % SDS (pH 8.25)

Electrophoresis was performed using the Bio-rad Protean II system as described in chapter 2.

5.2.13 Fluorometic analysis of polyacrylamide gels.

Fluorometry of polyacrylamide gels was performed according to the following procedure (adapted from Hames and Rickwood 1988).

1) The gel was soaked in DMSO for 1 h.

2) DMSO was removed and replaced with an equal volume of PPO for 3 h.

3) The gel was washed in water for 20 min, wrapped in cellophane film, and clamped on a frame to avoid distortion during drying.

4) The gel was dried overnight by placing it six inches below the bulb of an anglepoise lamp.

After drying, the gel was exposed to X-ray film. Films were developed at the appropriate time using standard methods.

5.2.14 Reduction of protein samples using sodium borohydride.

Vigorous reduction of ^{35}S-labelled protein samples was performed using the following method:

1) A 20 mg/ml solution of $NaBH_4$ (freshly made in 1 N NaOH) was added to protein sample to give a final concentration of 2 mg/ml $NaBH_4$.

2) After 30 min incubation at room temperature, the reaction was stopped, and the solution neutralised by the addition of 1/10 volume of 3.6 N HCl.

3) The sample was diluted to a volume of 2.5 ml for separation using a PD-10 column.

5.2.15 G-25 (PD-10) chromatography of samples following borohydride reduction.

Following borohydride reduction, samples were applied to a PD-10 column. Columns were equilibrated in PBS (pH 7.3) containing 25 mM DTT and 10 mM BSA. Samples were applied in a volume of 2.5 ml, and 0.5 ml samples collected for analysis by liquid scintillation counting.

5.2.16 Liquid scintillation analysis.

Samples were prepared for liquid scintillation analysis by mixing 100 µl of sample with 2.4 ml of scintillant (Ecoscint A). Scintillation analysis was performed using a Packard 2000CA TRI-CARB liquid scintillation counter, using standard operating procedures.

5.2.17 Protein precipitation following borohydride reduction.

Fractions corresponding to the void volume and to the total volume of the PD-10 column were pooled and precipitated using 4 volumes of acetone overnight at -80 °C. Protein was then resuspended in 40 µl of water and prepared for analysis on a 12.5 % polyacrylamide gel as described above.

5.2.18 Determination of inhibitory metal ion concentrations.

The inhibitory concentrations of Cd, Cu and Zn were established for *E. coli* cells containing pGP1.2 and pGEM4z as described earlier for pGEX3x-containing cells. The method used monitor metal toxicity was as

outlined below (section 5.2.19) for metal tolerance tests. Metal tolerance was examined using two concentrations of each metal, selected using the same criteria described in chapter 3 (section 3.2.3.8.1). Thus, the higher concentrations chosen were: 0.8 mM Cd, 1.8 mM Zn and 3.2 mM Cu, and the less inhibitory concentrations used were: 0.6 mM Cd, 1 mM Zn and 2 mM Cu.

5.2.19 Metal tolerance testing of synthetic gene-containing clones.

E. coli containing plasmids pGP1.2 and pGPSG4 were tested for tolerance to Cd, Cu and Zn, using the concentrations given above. The following protocol was used:

1) *E. coli* containing plasmid pGP1.2 and either pGPSG4 (synthetic gene) or pGEM4z (control) were grown overnight at 30 °C in LB medium containing Ap (50 µg/ml) and Km (25 µg/ml).

2) Cells were diluted 1/10 in fresh medium, and growth monitored until cells reached mid-logarithmic phase. Cell density was then adjusted by addition of LB to give synchronously growing cultures of equal density.

3) Cultures (two sets in triplicate for each test) were diluted 1/10 in fresh medium, and grown at 32 °C for 1 h.

4) Metal was added to one set of cultures at the concentrations indicated above, and incubation continued at 32 °C. Cell density was measured at appropriate intervals during growth.

In several experiments, dependent on the rate of growth, incubation was continued for extended periods. Experiments were continued until the non metal-exposed cells reached stationary phase.

5.2.20 Metal accumulation tests using synthetic gene-containing cells.

Metal accumulation was tested using the following protocol:

1) *E. coli* cells containing pGP1.2 and either pGEM4z or pGPSG4 were grown for 16 h under standard conditions at 28 °C.

2) Cultures inoculated from these were grown to log-phase, and diluted to standardise growth.

3) Log-phase cells at an optical density of 0.3, were heat-shocked at 42 °C for 10 min, and re-incubated at 30 °C for a further 3.5 h.

4) At the end of the incubation period, the optical density of the cultures was measured, and equal numbers of cells removed from each culture.

5) The cells removed from these cultures were collected by centrifugation, washed twice in sterile growth medium, and resuspended in 70 % nitric acid.

6) After incubation for 16 h, the cell digests were subjected to AAS as described previously.

5.3 Results and discussion.

5.3.1 Cloning of the synthetic gene.

5.3.1.1 The use of expression vectors pUC18 and pUC19.

After synthesis, the DNA strands were annealed and kinased prior to attempted cloning in *E. coli* expression vectors pUC18 and pUC19. These vectors allow cloning of fragments into a multiple cloning site within the *lacZ* gene, giving blue/white colour selection of transformed *E. coli* cells (constitutively in *E. coli* strain DH5α, or by IPTG-induction in strain JM101). Cloning of the synthetic construct in the incorrect orientation for expression in pUC18 was accomplished on the first round of screening (42 of 67 white colonies re-screened by *in situ* analysis contained insert DNA; the sequence of one of these was checked by direct sequencing, and named pGPSG1). Conversely, of 175 white colonies analysed during several rounds of screening using identical methods with vector pUC19, no positive colonies were isolated. This indicated the possibility that the product of the gene may have affected cell viability, and screening was continued by colony lifts of non-induced JM101 cells. Several thousand transformed cells were screened by this method, yielding 4 positive clones by *in situ* analysis. Similarly, attempts to isolate clones in which the EcoRI/BamHI fragment of the synthetic insert (excised from pGPSG1) was cloned between the EcoRI and BamHI sites of pUC18 required many rounds of screening of uninduced cells in order to obtain 5 positive clones.

Figure 5.3 (A-E) shows sequences of several of the pUC18 and pUC19 constructs which were isolated during attempts to clone the synthetic gene in the correct orientation for expression. Three pUC19 clones were isolated in which the insert or vector sequence had undergone alterations. In clone (A), a portion of the synthetic gene sequence is duplicated, and in both of the replicated sequences the TAG stop codon is altered to TCG. In clone (B), the S/D region of the gene is altered, and in clone (C), although the gene is cloned intact, DNA of the vector sequence is rearranged. Two pUC18 clones (D) and (E) are also shown in figure 5.3. One of these, clone (D) contains an additional sequence following the synthetic gene insert, whereas in clone (E) the insert sequence is repeated three times, allowing the potential for the mRNA produced from this plasmid to form secondary structure. Although the effects of these alterations, if any, on production of the protein product of the synthetic gene are unknown,

Figure 5.3 Mutated DNA sequences of several pUC19 and pUC18 plasmids in which the synthetic DNA construct was cloned in the correct orientation for expression.

Plasmids were directly sequenced without sub-cloning using the M13 primer binding sites of the pUC vectors. In each case, the sense strand of the gene is written in the correct orientation. Alterations which were found in the gene and surrounding sequences are indicated as described below. (M13RP1 - Annealing site for M13 reverse primer; HIII - HindIII restriction site; MCS - multiple cloning site of pUC vector)

Description of altered sequences:

(A) pUC19 - the sequence of part of the synthetic insert is repeated, and in both repeats of the sequence there is an A to C transversion (shown in bold type) in the stop codon of the gene.

(B) pUC19 - the synthetic gene sequence shows an A to G transition in the Shine/Dalgarno region (shown in bold type).

(C) pUC19 - in this clone the sequence of the synthetic insert was found to be successfully cloned unaltered between the XbaI and BamHI sites. Further analysis of the DNA surrounding the insertion, however, revealed that rearrangement of sequences between the start of the polylinker and the M13 primer binding site had occurred. The portion of sequence underlined represents inserted DNA of unknown origin.

(D) pUC18 - the gene sequence is cloned in frame with the LacZ protein, however additional DNA is inserted 3' to the insert (underlined).

(E) pUC18 - The insert DNA has formed a concatamer of three repeats which has subsequently been cloned into the BamHI/EcoRI-restricted vector. The repeated sequence is indicated in the diagram.

(A)

```
    XbaI ------------Gene sequence (I)---------   -- Repeated
 5' TCTAGACTAGGAGGAATTCTATGGAGTGTGAATGCGAATGTGGGTCGGAATTCTATGG

    sequence------------- BamHI
    AGTGTGAATGCGAATGTGGGTCGGATCC 3'
```

(B)

```
   XbaI --------------Gene sequence----------- BamHI
5' TCTAGACTAGGAGGGATTCTATGGAGTGTGAATGCGAATGTGGGTAGGATCC 3'
```

(C)

```
    ---M13RP1 ------          __HIII                              HIII
 5' AACAGCTATGACCATGATTACGCCAAGCTCTAATACGACTCACTATAGGGAGACAAGC

           MCS            XbaI --------Gene sequence----------
    TTGCATGCCTGCAGGTCGACTCTAGACTAGGAGGAATTCTATGGAGTGTGAATGCGAA

    --------BamHI
    TGTGGGTAGGATCCCGGGG 3'
```

(D)

```
    ---M13RP1------       EcoRI ------Gene sequence--------
 5' AACAGCTATGACCATGATTACGAATTCTATGGAAGTGTGAATGCGAATGTGGGTA

    BamHI
    GGATCCCGTAACGAGCTCGAATTCGAGCTC 3'
```

(E)

```
     EcoRI---Gene sequence (I)------- BamHI----Sequence (II)--
 3' GAATTCTATGGAGTGTGAATGCGAATGTGGGTAGGATCCTACCCACATTCGCATTCAC

    -------- EcoRI--------Sequence (III)----- BamHI
    ACTCCATAGAATTCTATGGAGTGTGAATGCGAATGTGGGTAGGATCCA
```

and despite the fact that other plasmids were isolated and sequenced in which the insert sequence was correct and which produced mRNA containing the synthetic gene coding sequence (plasmids pGPSG2 and pGPSG3; figure 5.4), these plasmids were not used in further experiments due to the possibility that they were abnormal in some other respect. Difficulty in isolating significant amounts of these plasmids using standard methods increased doubts about the validity of their use in further experimentation.

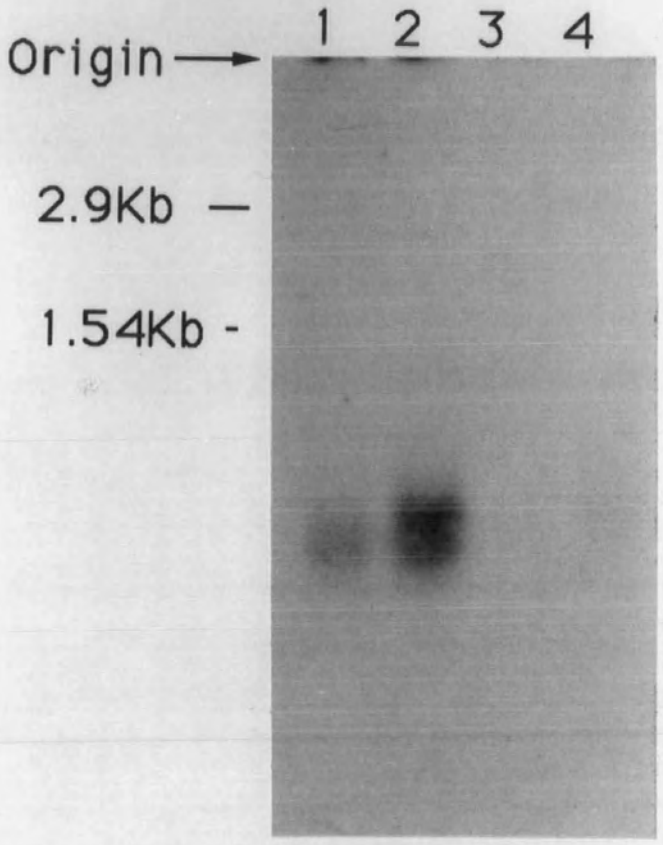
5.3.1.2 Cloning the synthetic gene under control of the T7 promoter.

The results described above indicated that the product of the synthetic construct may be detrimental to cell viability. Although the *lacZ* gene is in the repressed state in the absence of lactose or IPTG, the "leaky" nature of expression from the *lacZ* promoter means that a low level of constitutive expression is observed (e.g. see lane 1, figure 5.4). The selection of mutant sequences in plasmids selected in the uninduced state suggested that a more tightly controlled expression system would be beneficial for cloning the synthetic gene. The expression vector pGEM4z was selected on this basis. This vector, and its use with plasmid pGP1.2 is described in section 5.2.3. Insertion of DNA into the multiple cloning site of pGEM4z allows blue/white colour screening of recombinant cells by interruption of the *lacZ* gene, however, DNA inserted in the correct orientation for expression from the T7 promoter is in the incorrect orientation for expression from the *lacZ* promoter. The synthetic gene was ligated into XbaI/BamHI-restricted pGEM4z, which places the gene under control of the T7 promoter. The ligation was transformed into JM101 cells, and several white colonies chosen and re-screened by *in situ* analysis. All of the colonies screened gave a positive signal. The plasmid from one of these clones was subjected to direct DNA sequencing, and the inserted sequence found to be correct. This plasmid was retained for further work, and named pGPSG4.

Since T7 RNA polymerase promoter recognition sequences share no homology with those for *E. coli* RNA polymerases, there is very little leaky expression of sequences cloned downstream of the T7 promoter in pGEM4z (Davanloo et al 1986). To gain expression of the synthetic gene from the T7 promoter in plasmid pGPSG4, it was necessary to supply the *E. coli* cell with plasmid pGP1.2 (figure 5.1; section 5.2.). Competent

Figure 5.4 Northern blot showing IPTG-induction of the synthetic gene from plasmids pGPSG2 and pGPSG3.

Cells were exposed to IPTG (section 5.2.4), and RNA was extracted, blotted and probed as described in chapter 2. Lane 1, pGPSG2 (non-induced); lane 2, pGPSG2 (induced); lane 3, pGPSG3 (non-induced); lane 4, pGPSG3 (induced). The probe used was the missense stand of the synthetic gene, end-labelled using γ-^{32}PdATP.


1 2 3 4
Origin
2.9Kb
1.54Kb

cells containing pGP1.2 were transformed with plasmid pGPSG4, and also with pGEM4z for use as a control in subsequent experiments. Transformants were selected for Km (pGP1.2) and Ap (pGEM4z) resistance. Colonies which were Km and Ap resistant were incapable of growth above 34 °C, and were maintained at 28 °C. Loss of viability of induced cells is attributed to the high level of mRNA induction from T7 promoters, which may cause a depletion of the cellular pool of ribonucleoside triphosphates (Tabor and Richardson 1985).

Insertion of the synthetic gene into a completely non-induced vector ensured that cloning could be performed with minimal risk of rearrangement. It was necessary after cloning to determine whether the protein could be effectively heat-induced in order to allow its biological effects in terms of *in vivo* binding of metals to be assessed. Analysis was performed firstly at the level of transcription, followed by attempts to detect the protein directly via ^{35}S-labelling of induced cellular proteins.

5.3.2 Analysis of heat-induced mRNA induction from pGPSG4.

Heat-induction of mRNA encoding the synthetic gene is shown in figure 5.5. No defined RNA bands are visible on this blot due to the fact that the vector contained no transcription termination signals. The blot shows, however, that mRNA encoding the αPC_3 product is induced between 34 and 36 °C.

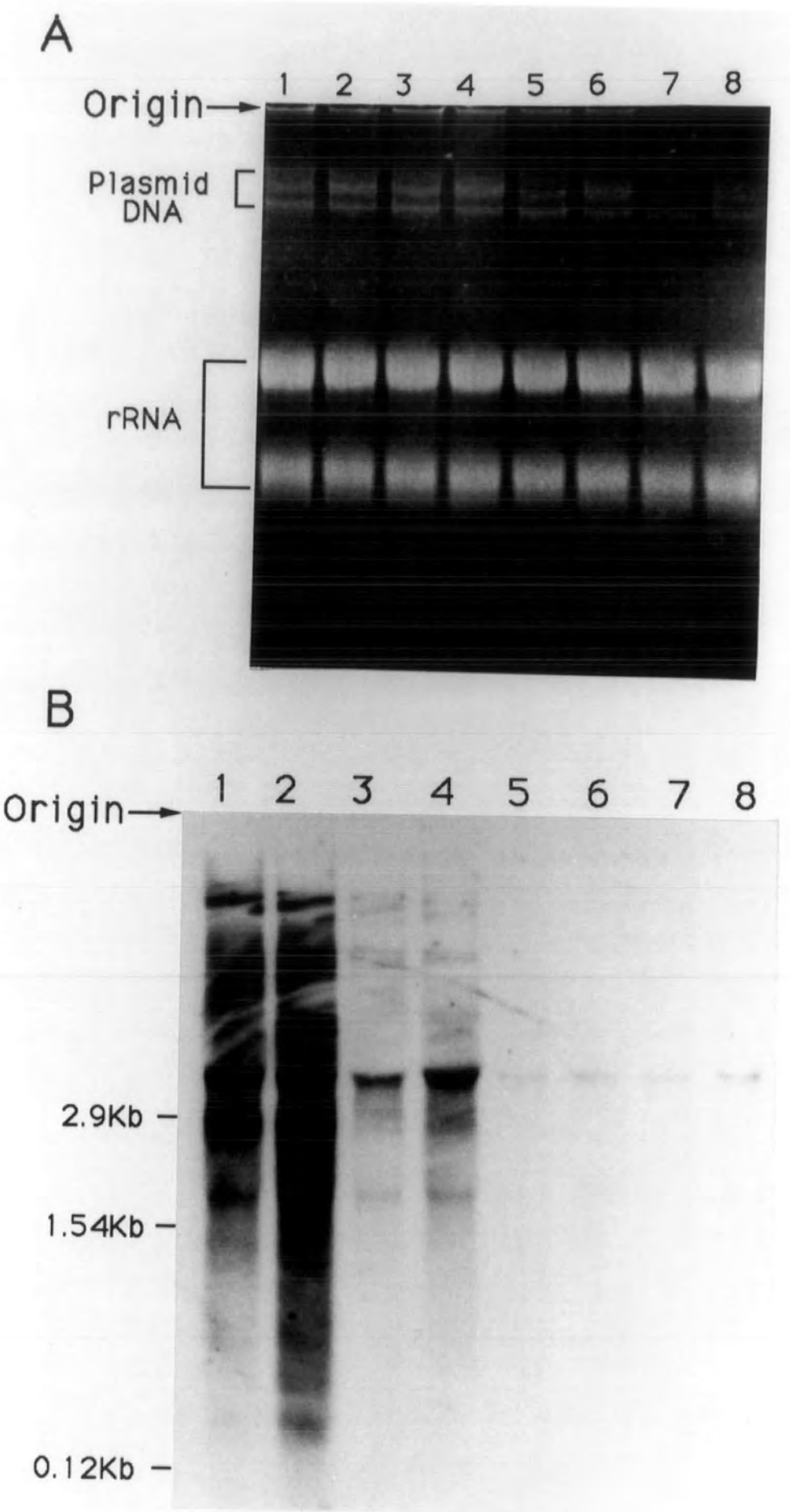
5.3.3 Analysis of *E. coli* extracts for the putative product of the synthetic gene.

Having shown that *E. coli* harbouring pGP1.2 and pGPSG4 produced heat-induced transcripts, it was necessary to determine whether αPC_3 could be detected in induced cells. The method of choice for the separation of small molecules, namely reversed phase HPLC, was not available for these experiments, and so alternative methods were employed. The results of several experiments describing attempted detection of the gene product are given below.

5.3.3.1 Gel permeation HPLC analysis of ^{35}S-labelled and Cd-exposed cell extracts.

The high cysteine content of the putative gene product meant that labelling with ^{35}S-cysteine was the most sensitive method for

Figure 5.5 Northern blot showing heat-induction of the synthetic gene from plasmid pGPSG4. Cells containing plasmids pGP1.2 and pGPSG4 were heat-induced as described in the text. Photograph (A), showing the formaldehyde-agarose gel used for northern blotting demonstrates equal loading of the RNA samples. For both (A) and (B): lane 1, 42 °C; lane 2, 40 °C; lane 3, 38 °C; lane 4, 36 °C; lane 5, 34 °C; lane 6, 32 °C; lane 7, 30 °C; lane 8, 28 °C. The probe used was identical to that described in the legend to figure 5.4.


A
1 2 3 4 5 6 7 8
Origin
Plasmid DNA
rRNA
B
1 2 3 4 5 6 7 8
Origin
2.9Kb
1.54Kb
0.12Kb

detection. Since it was also hoped that the protein would be capable of binding metals intracellularly, cells were exposed to Cd to allow detection of bound metal. The small size of the protein (1 Kd) meant that it was not possible to detect directly the free protein using this method. It is likely however that, due to its high cysteine content, the protein would tend to form aggregates with free cellular cations. This would result in ^{35}S or Cd eluting before the total volume of the column. The results of gel permeation HPLC analysis of extracts from Cd- and ^{35}S-exposed cells are shown in figure 5.6. Using both methods of analysis, a slight, but consistent increase in the amount of ^{35}S [figure 5.6 (A)], or Cd [figure 5.6 (B)] was detected in the void volume of induced compared to non-induced cells.

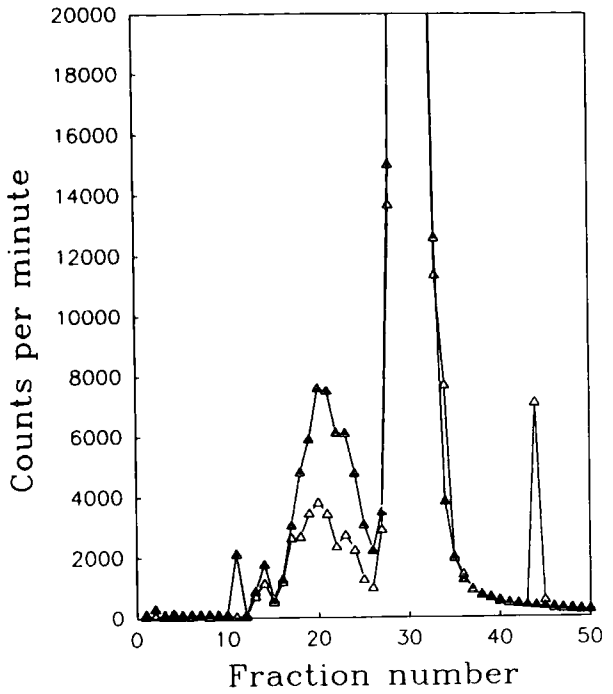
5.3.3.2 Polyacrylamide gel analysis of ^{35}S-labelled *E. coli* proteins.

Shägger and von Jagow (1987) describe a method for the separation of small proteins and peptide fragments, involving the use of polyacrylamide gels in which glycine is replaced with tricine (section 5.2.12). Such gels were used in initial attempts to visualise the product of the synthetic gene (figure 5.7). As can be seen in the tricine gel shown, no concise band is visible in the expected low molecular weight region at which the protein product of the synthetic gene could be expected to run (proteins of ca. 1 KDa would migrate close to the buffer front of such gels). All tricine gels, run either with or without Cd added to the cultures prior to extraction, showed a similar group ^{35}S-labelled bands in extracts from cells containing the synthetic gene. By inhibition of transcription of genes encoding endogenous *E. coli* proteins using rifampicin, it was possible to confirm that the induced bands were due solely to the T7-transcribed synthetic gene (lane 1, figure 5.7). Three replicate samples, showing identical induction patterns were separated using a "normal" (glycine) polyacrylamide gel (figure 5.8). This shows that several major bands (of between approximately 30 and 45 KDa) which are ^{35}S-labelled as a result of induction of the synthetic gene. Three bands corresponding to this region are visible in ^{35}S-labelled extracts from control cells and non-induced synthetic gene-containing cells which were not exposed to rifampicin. The bands in these extracts, however, are noticeably smaller and less intense than those observed in the induced synthetic gene-containing cells.

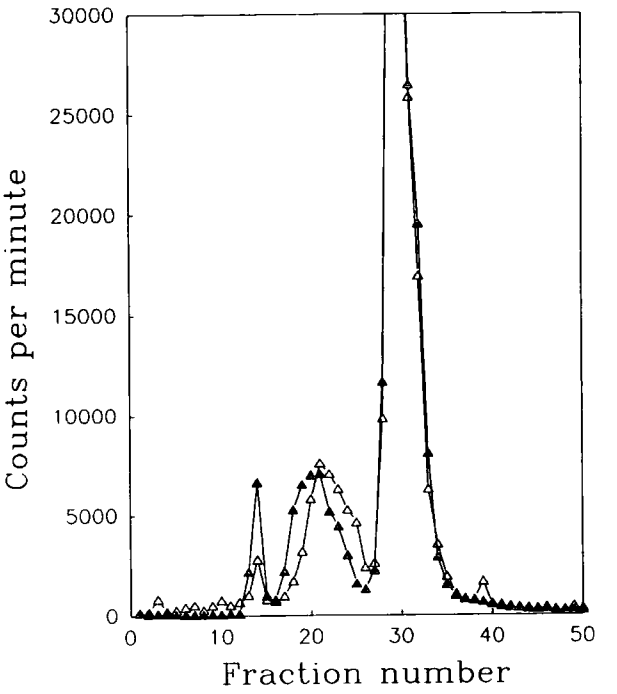
Figure 5.6 Gel permeation HPLC (SW3000) elution profiles of extracts from *E. coli* cells containing plasmids pGPSG4 and pGP1.2.

Graphs A (i) and (ii) show the elution profiles of ^{35}S from SW3000 gel permeation separations of extracts from *E. coli* cells exposed to 800 µM Cd in the presence of ^{35}S-cysteine. Extracts were passed through the column at a flow rate of 0.5 ml/min, and 0.5 ml fractions collected. Graphs B (i) and (ii) represent duplicate experiments in which cells containing plasmids pGP1.2 and PGPSG4 were exposed to 800 µM Cd. Column parameters were as outlined above, except B (i) for which 0.75 ml fractions were collected. Full details of the methods used for ^{35}S-cysteine labelling and Cd-exposure are given in the text. (Symbols: △ - induced cells; Δ - non-induced cells).

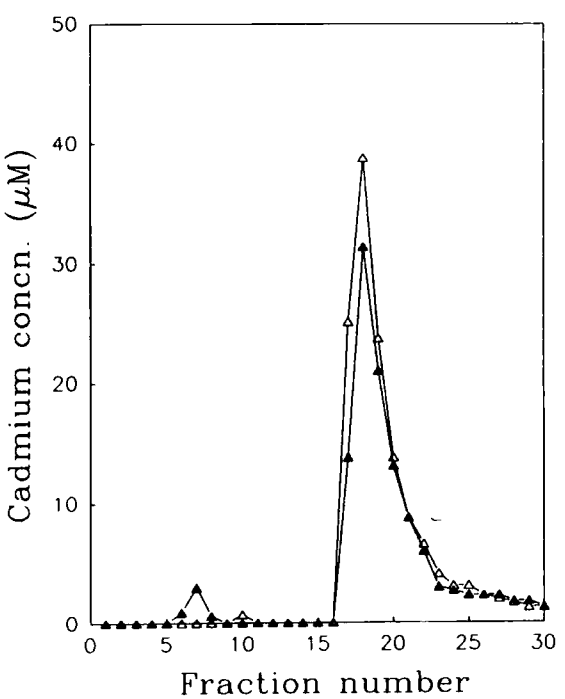
A(i)


Counts per minute
Fraction number
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
20000
0
10
20
30
40
50

A(ii)


Counts per minute
Fraction number
0
5000
10000
15000
20000
25000
30000
0
10
20
30
40
50

B(i)


Cadmium concn. (μM)
Fraction number
0
10
20
30
40
50
0
5
10
15
20
25
30

B(ii)

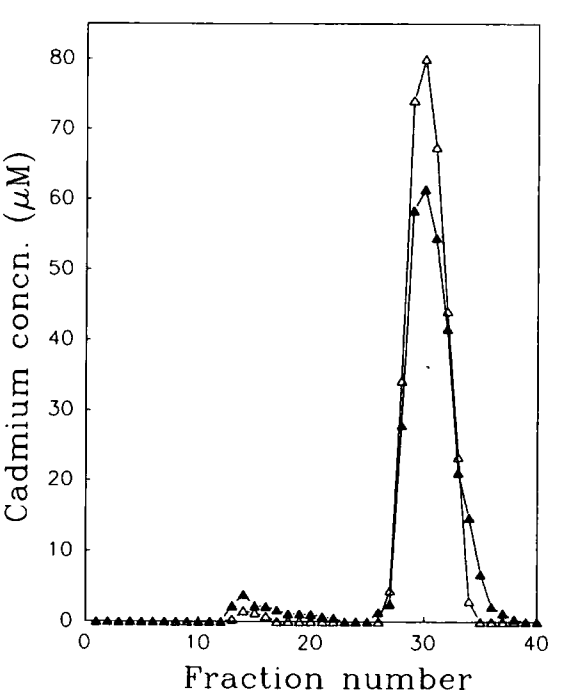

Cadmium concn. (μM)
Fraction number
0
10
20
30
40
50
60
70
80
0
10
20
30
40

Figure 5.7 Autoradiogram of ^{35}S-labelled proteins separated on a 12 % polyacrylamide tricine gel.

In vivo labelling, protein preparation and gel analysis of the cell extracts is described in section 5.2. The gel was exposed to X-ray film for 4 h prior to development. Molecular weight markers were not available and are therefore not included. The intense bands visible in the induced lanes are in the range of 25 - 45 KDa, not in the approximately 1 KDa size range of the putative product of the synthetic gene. Lane 1 - rifampicin-exposed pGPSG4-containing cells, lane 2 - rifampicin exposed control cells, lane 3 - pGPSG4-containing cells not exposed to rifampicin, lane 4 - control cells not exposed to rifampicin.

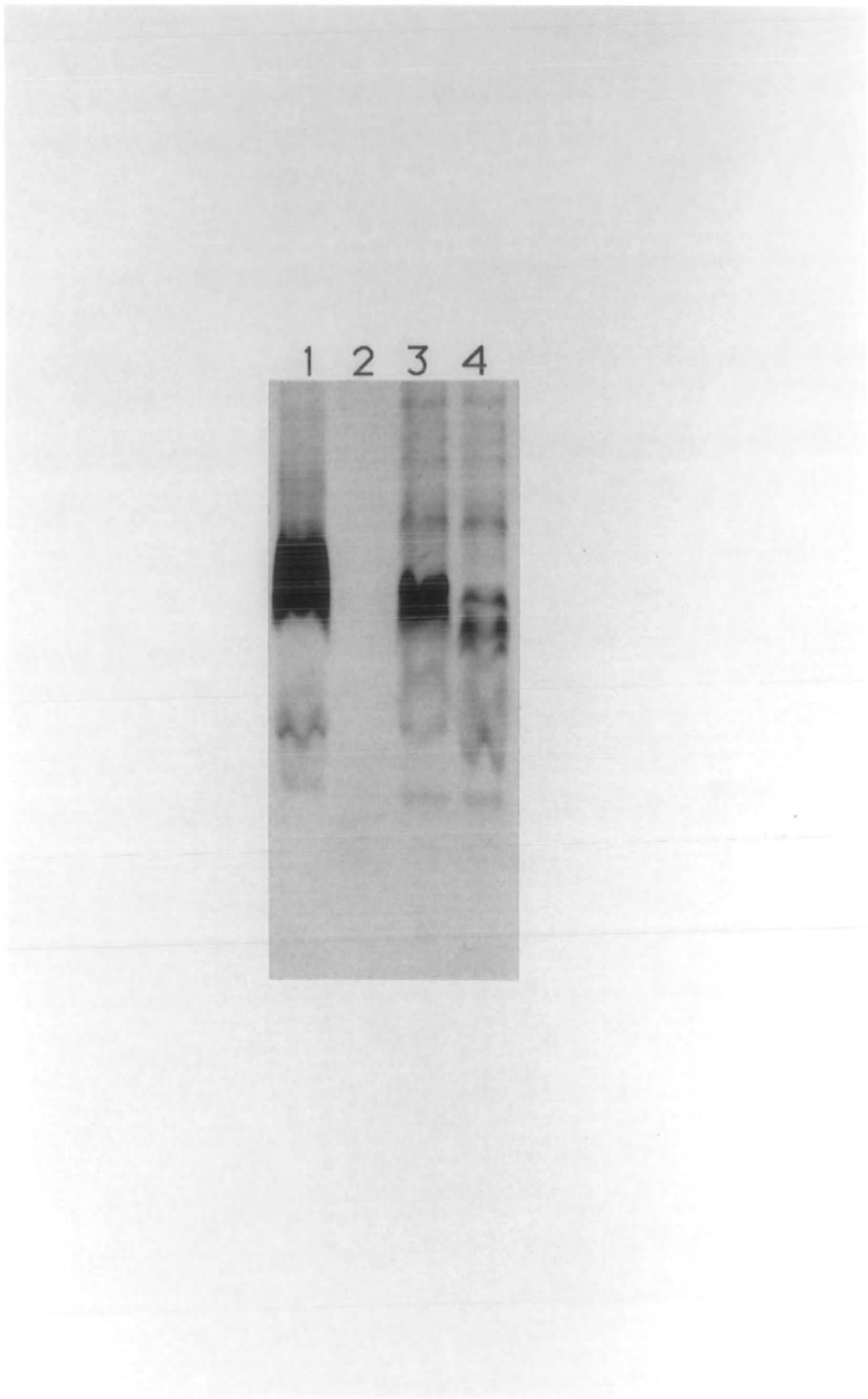
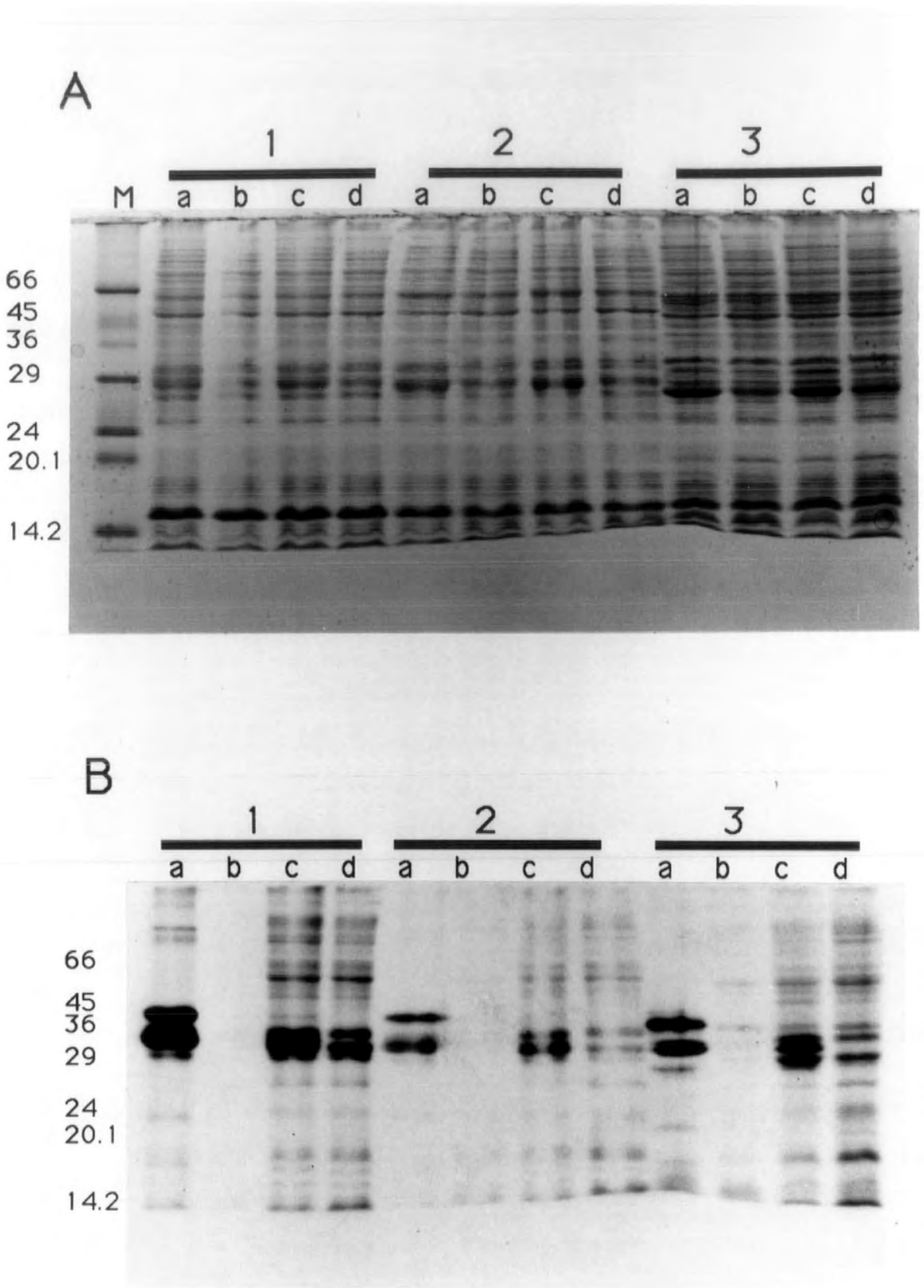

1 2 3 4

Figure 5.8 Gel photograph and autoradiogram of ^{35}S-labelled proteins on a 12.5 % polyacrylamide gel.

Cell extracts were prepared as for the tricine gel described above and separated on a 12.5 % "normal" (glycine) polyacrylamide gel. Lanes (a) - rifampicin-exposed pGPSG4-containing cells, lanes (b) - rifampicin-exposed control cells, lanes (c) - pGPSG4-containing cells not exposed to rifampicin, lanes (d) - control cells not exposed to rifampicin. Sets 1, 2 and 3 represent 3 replicate extracts.


A
1
2
3
M
a
b
c
d
66
45
36
29
24
20.1
14.2
B
1
2
3
a
b
c
d
66
45
36
29
24
20.1
14.2

5.3.3.3 Sodium borohydride reduction and G-25 separation of labelled protein samples.

The results described in section 5.3.3.2 suggested that αPC_3 was being expressed, but that the polypeptide was associating with endogenous *E. coli* proteins *in vivo*. This conclusion is supported by the observation that the endogenous proteins which are present in this molecular weight size range are the most highly labelled by ^{35}S-labelled cysteine, indicating that these are cysteine-rich proteins. Association of these proteins with αPC_3 could therefore occur via disulphide formation. Additionally, the observed shift in the apparent molecular weight of the proteins via induction of the synthetic gene is consistent with the hypothesis that these proteins are associating with αPC_3.

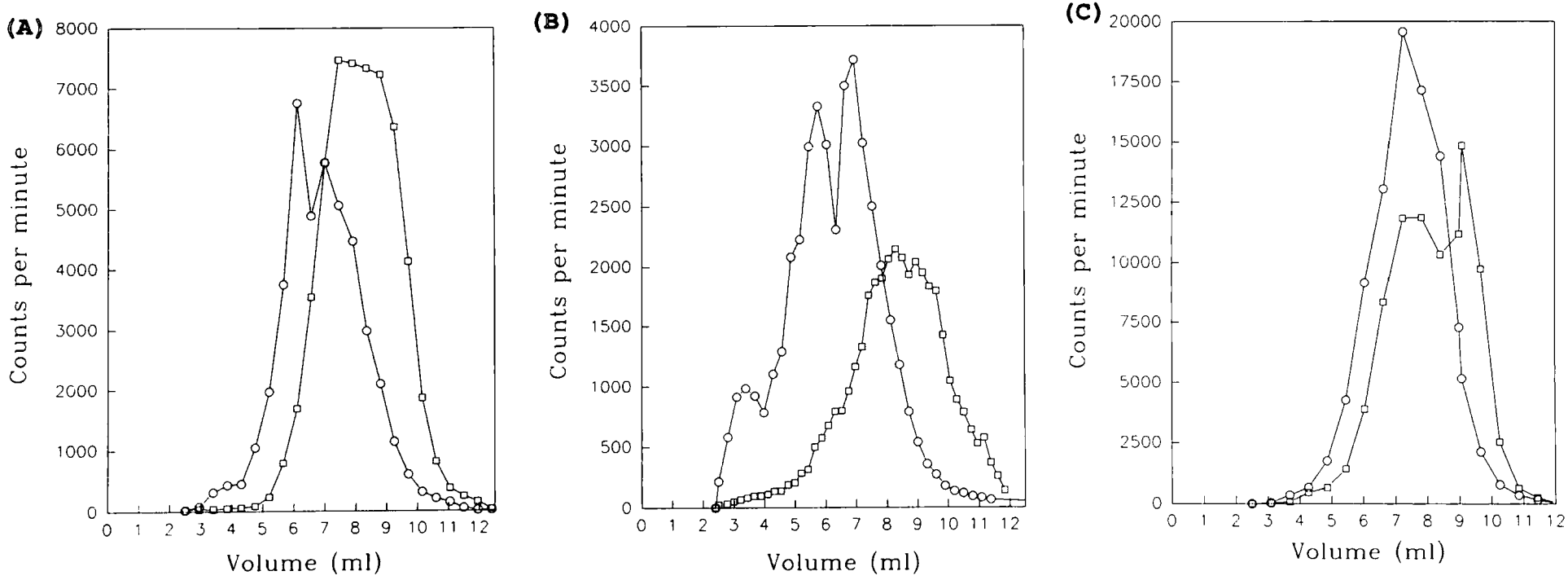
In order to test the hypothesis outlined above, the following procedures were employed to attempt to disrupt the association of the putative synthetic gene product with higher molecular weight proteins and to separate the high molecular weight proteins from low molecular weight species. To reduce disulphides, the protein samples were treated with sodium borohydride (section 5.2.14). Protein samples from induced cells either containing vectors only, or including the synthetic gene insert, were prepared exactly as for polyacrylamide gel analysis. Samples were divided in half, and one half reduced using sodium borohydride. The elution profiles of reduced and non-reduced samples from PD-10 columns are shown in figure 5.9. The major features which differ between ^{35}S elution profile for the protein samples from cells containing pGPSG4 (figure 5.9, A) and protein from control cells (figure 5.9, C) is the peak which is present in the void volume prior to the reduction process, and the subsequent abolishment of this peak post-reduction. This alteration in the distribution of label was observed in all 4 replicates of the same experiment. To clarify the result, free ^{35}S-labelled cysteine and other ^{35}S-labelled low molecular weight thiols were removed from one sample, by desalting of the sample prior to the reduction procedure (figure 5.9, B). In this experiment, a clear shift was observed in the distribution of label following reduction of the sample.

To establish that the change in the observed elution profile was due to the displacement of low molecular weight thiols from void

Figure 5.9 The elution profile of ^{35}S from ^{35}S-labelled cell extracts eluting from G-25 (PD-10) columns either with or without borohydride reduction.

Full experimental details are described in section 5.2.14 and 5.2.15. (A) cell extracts from cells containing pGP1.2 and pGPSG4; (B) cell extracts prepared exactly as for (A), but the with non-incorporated label removed prior to analysis by passage through a PD-10 column; (C) cell extract from *E. coli* containing vectors only (control).

[O - pre-reduction; ❐ - post-reduction].


(A)
Counts per minute
8000
7000
6000
5000
4000
3000
2000
1000
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Volume (ml)
(B)
Counts per minute
4000
3500
3000
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Volume (ml)
(C)
Counts per minute
20000
17500
15000
12500
10000
7500
5000
2500
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Volume (ml)

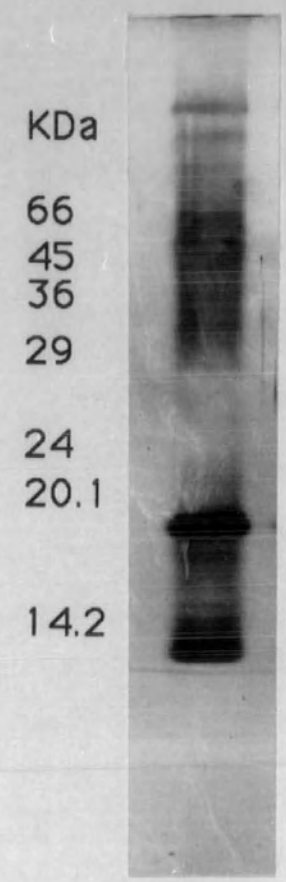
volume proteins, and not to altered protein mobility through the PD-10 column, the void volume of a reduced protein sample was precipitated using 4 volumes of acetone. The precipitated protein, separated using a 12.5 % polyacrylamide gel is shown in figure 5.10. No protein was detected in the fractions following the void volume. Fluorometry of this gel, followed by exposure of the gel to X-ray film for 30 days gave no detectable signal.

These results indicated that the synthetic gene product was being expressed in heat-induced cells containing pGPSG4. The product was apparently expressed at a high level when the cells were induced at 42 °C, and, using the methods of induction outlined here, tended to associate with other thiol-rich proteins in the *E. coli* cell, possibly via disulphide formation between the reactive sulphydryl groups present on the synthetic gene product and those of endogenous cellular proteins. In this regard, the observed association resembles S-thiolation reactions which have been described in eukaryotic cells. These enzymically catalysed associations between small thiol-rich molecules (such as glutathione disulphide) and thiol groups of cellular enzymes may be important in the modification of enzyme activity in response to oxidative stress (Ziegler 1985). Such reactions have not been studied in prokaryotes, but could potentially be responsible for the results observed during these experiments. Other possibilities for the observed phenomena are spontaneous association of the reactive thiols *in vivo*, or during the extraction procedure. This latter possibility is unlikely since extractions were performed in the presence of DTT. These observations also provide a possible explanation of the results observed in attempting to produce clones in pUC vectors. It is possible that the implied toxicity of the synthetic gene was due to disruption of cellular metabolism, via association with other *E. coli* proteins, rather than to effects on metal metabolism.

Association of the synthetic gene product with other cellular proteins does not preclude the possibility of association of a proportion of the αPC_3 with free metal ions if these are present in the cell. Gel permeation HPLC profiles gave some indication of the binding of Cd in extracts from Cd-exposed cells when the synthetic gene was induced at a high level. The following sections describe attempts to determine whether cells expressing the synthetic gene were

Figure 5.10 Polyacrylamide gel analysis of sodium borohydride-reduced protein.

Protein eluting in the first 5 fractions of a sample as shown in figure 5.5 (B) (after reduction), was pooled and precipitated using 4 volumes of acetone. This material was then separated on a 12.5 % acrylamide gel, and the protein visualised by silver staining. After photographing, the gel was subjected to fluorometry as described previously. No bands were visible after 30 days exposure.


KDa
66
45
36
29
24
20.1
14.2

capable of sequestering more metal compared to control cells, and whether expression of the gene at a low level in the presence of metal ions could confer increased metal tolerance.

5.3.4 Phenotypic effects of expression of αPC_3 in *E. coli*.

5.3.4.1 Metal tolerance testing of *E. coli* cells expressing αPC_3.

The results of metal tolerance testing of cells containing pGP1.2 and pGPSG4 or pGEM4z (control) are shown in figures 5.11, 5.12 and 5.13. Cells containing pGPSG4 exhibited a clear, reproducible increase in tolerance toward Cd. As this result appeared inconsistent with the previously described data concerning expression of *smt*A and $PsMT_A$ in *E. coli*, exposure to Cd of cells expressing the synthetic gene was repeated several times. A total of eight replicates showed a clear increase in growth of pGPSG4-containing cells over those containing pGEM4z. No effect on tolerance to Cu or Zn was observed. To confirm that the observed effect was attributable to transcription of the synthetic gene by T7 polymerase, the experiments were repeated using cells without the pGP1.2 plasmid (figure 5.13). In these experiments, growth of cells containing pGPSG4 and pGEM4z were inhibited to the same extent by Cd.

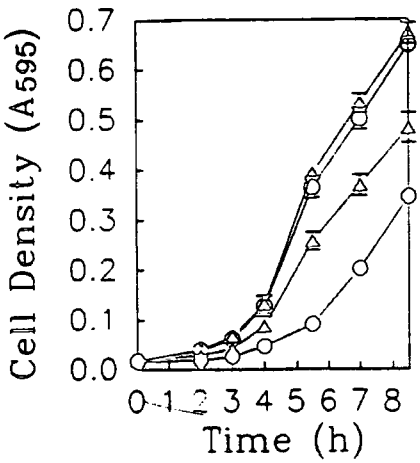
Examination of the literature relating to the expression of metal ligands in *E. coli*, reveals only one instance where increased metal tolerance was observed (Hou et al 1988; section 1.7). It is of interest that in this case, as with tolerance studies with the synthetic gene, the expression of MT was markedly lower than in other studies where accumulation, but not tolerance was observed (e.g. Romeyer et al 1988, Kille et al 1990). This may indicate that a low, rather than a high level of expression is advantageous in increasing metal tolerance in such clones. As is discussed in chapter 7, a low level of expression may result in closer coupling of the amount of free thiol groups with intracellular metal ion concentrations. Coupling of ligand concentration to intracellular metal levels would appear to be critical in intracellular metal tolerance mechanisms (hence the intricate mechanisms which have evolved to control the expression of MTs and other metal sequestering ligands - reviewed in chapter 6). It remains to be demonstrated unequivocally that intracellular metal-sequestering proteins are implicated in responses of prokaryotic cells to metal ions, however the results presented here

Figure 5.11 Graphs showing the growth of *E. coli* containing the synthetic gene construct exposed to Cd, Cu and Zn (lower metal concentrations).

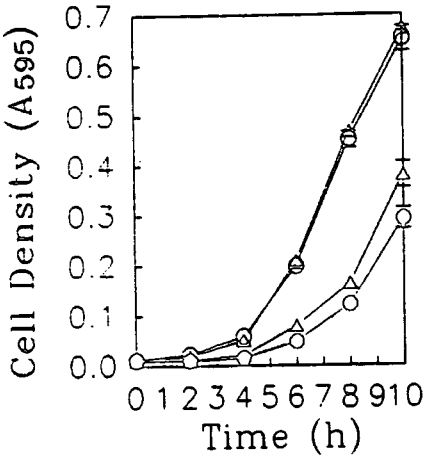
Cultures (in triplicate) were exposed to 0.6 mM Cd, 1 mM Zn and 2 mM Cu, and growth monitored as described in section 5.2.19. The metal concentrations were chosen as described in section 5.2.18. Graphs for two replicates of the experiment using each metal ion are shown. (Graphs (A) - Cd-exposed cells; (B) - Cu-exposed cells; (C) - Zn-exposed cells).

(Symbols: O - cells containing pGP1.2 and pGEM4z not exposed to metals; ○ - cells containing pGP1.2 and pGEM4z exposed to metals; Δ - cells containing pGP1.2 and pGPSG4 not exposed to metals; △ - cells containing pGP1.2 and pGPSG4 exposed to metals).

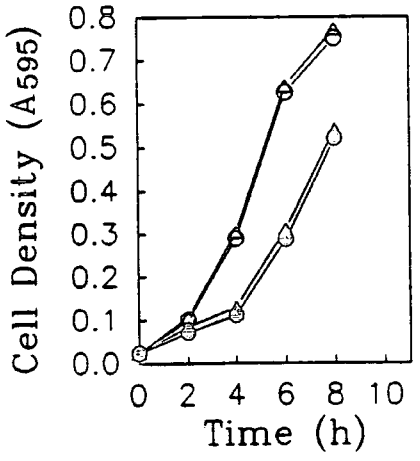
A(i)



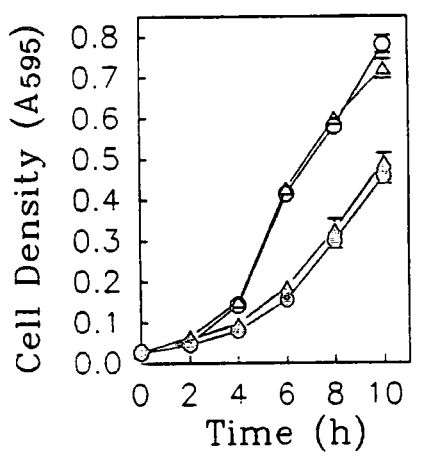
A(ii)



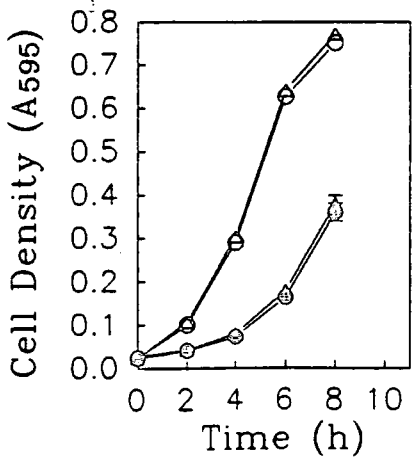
B(i)



B(ii)



C(i)



C(ii)

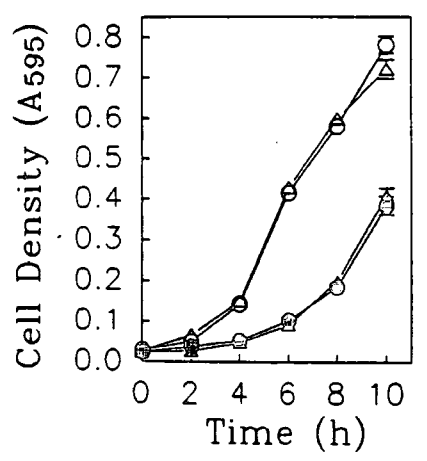
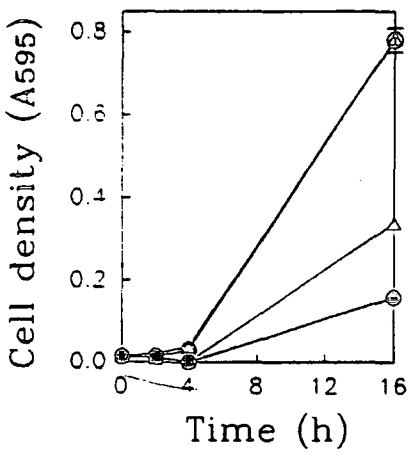


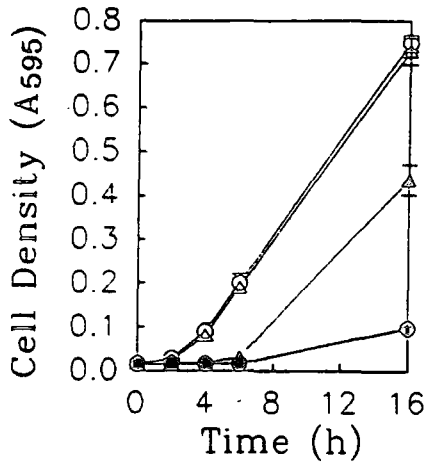
Figure 5.12 Graphs showing the growth *E. coli* cells containing the synthetic gene construct in Cd, Cu and Zn (higher metal concentrations).

Cells containing pGP1.2 with either pGPSG4 or pGEM4z were exposed to the higher concentrations of Cd, Cu and Zn (0.8 mM, 3.4 mM and 1.6 mM respectively), using identical methods to those outlined for lower concentrations. (Graphs A - Cd-exposed cells; graphs B - Cu-exposed cells; graphs C - Zn-exposed cells. The symbols used are as described for figure 5.11).

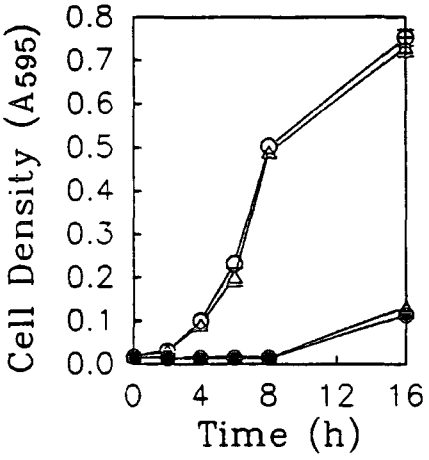
A(i)



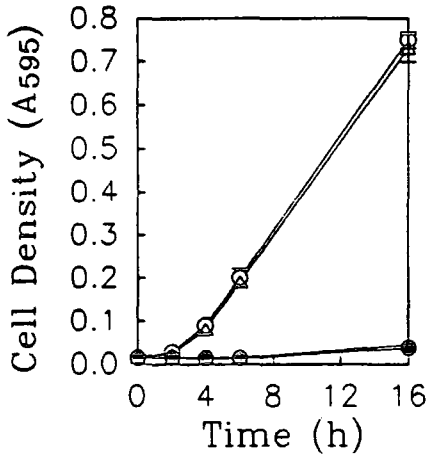
A(ii)



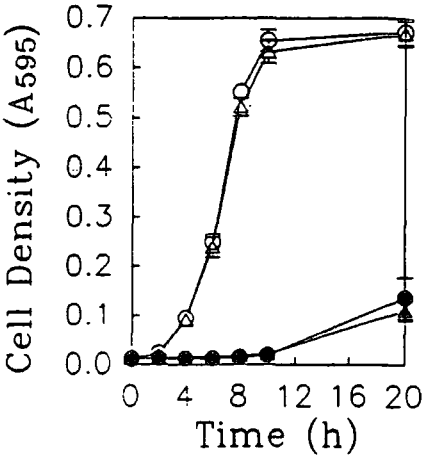
B(i)



B(ii)



C(i)



C(ii)

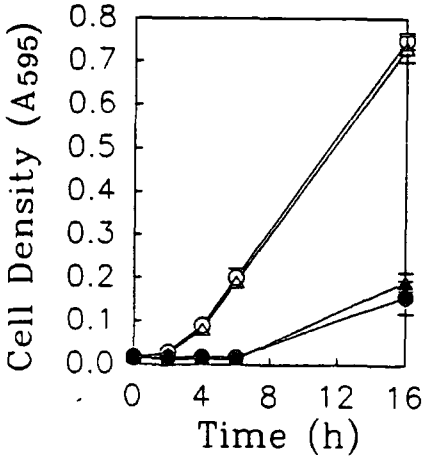
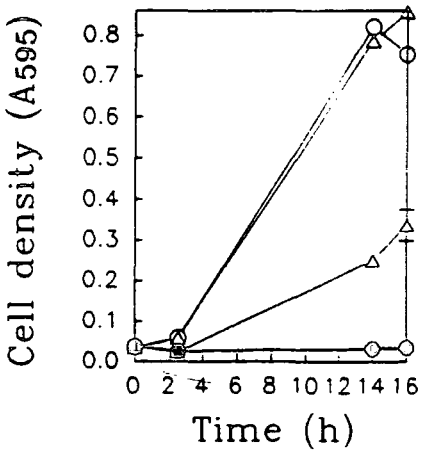
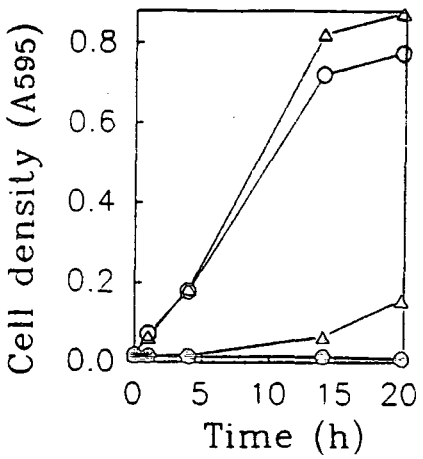


Figure 5.13 Replicates of Cd-exposure of cells containing pGP1.2 and pGPSG4 or pGEM4z, and Cd-exposure of cells containing pGPSG4 or pGEM4z (without plasmid pGP1.2). Graphs (A) (i) to (iv) represent four additional replicates of exposure of cells to 800 μM Cd and were performed as outlined for figure 5.12 in order to confirm the result of the previous experiments. Symbols are as described for figure 5.11. Graphs (B) - cells containing pGPSG4 or pGEM4z, but not the T7 polymerase-donating plasmid pGP1.2 were exposed to 800 μM Cd using identical methods to those outlined for cells containing both plasmids (but without Km in the medium). [Δ - non Cd-exposed cells containing pGPSG4; ○ - non Cd-exposed cells containing pGEM4z; △ - Cd-exposed cells containing pGPSG4; ○ - Cd-exposed cells containing pGEM4z].

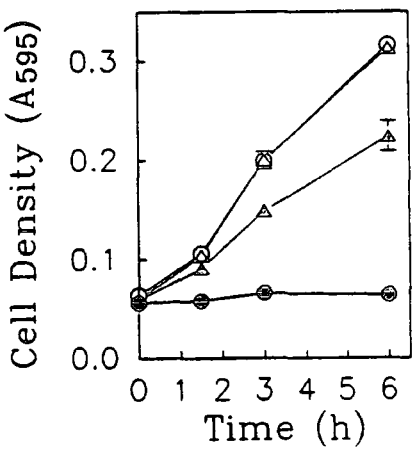
A(i)



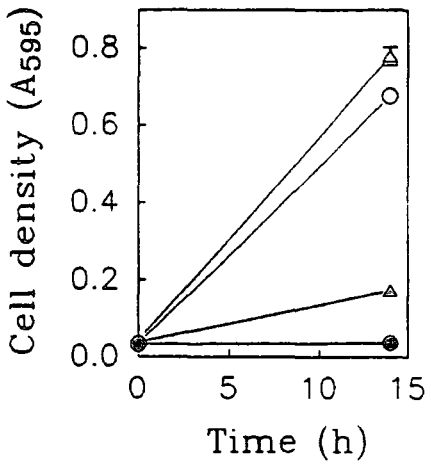
A(ii)



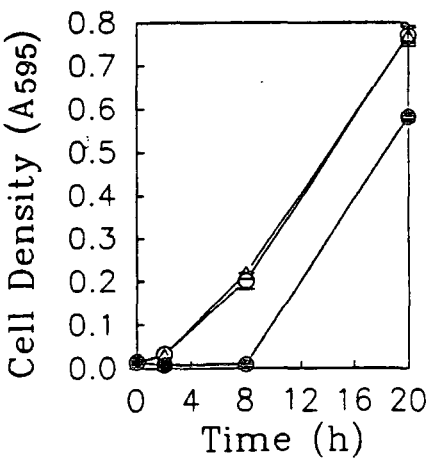
A(iii)



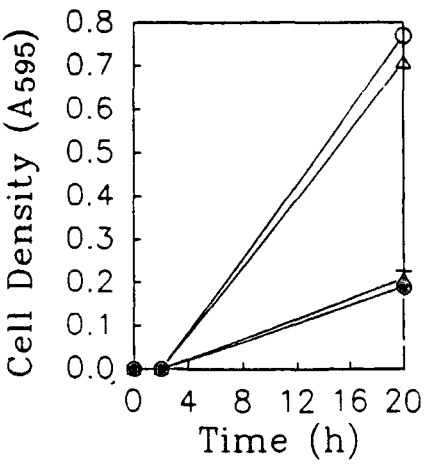
A(iv)



B(i)



B(ii)



demonstrate that controlled expression of such proteins can have an effect on metal tolerance. This is investigated further in chapter 6.

5.3.4.2 Metal accumulation studies on *E. coli* cells expressing αPC_3.

Table 5.1 (A - C) show the results of examination of metal accumulation by *E. coli* cells expressing αPC_3. No significant increase in accumulation was observed for any of the metals when nine replicates of each test were averaged. In the case of Cd, however, for both levels of exposure each separate experiment (each comprising three replicates) a slight but statistically significant increase in Cd accumulation by test relative to control cells was observed. This was also observed for cells exposed to the lower concentration (0.5 mM) of Cu. The variation between different experiments, however, conceals this observed difference when all 9 repeats of the test are considered as one group. This may reflect the difficulty in precise replication of experimental conditions with respect to the temperatures to which cells were exposed. As has been demonstrated in earlier experiments, slight alterations in transcription of the synthetic gene may potentially result in changes in cellular metabolism. It is probable, from examination of the results of Cd tolerance tests, that the product of the synthetic gene would exhibit *in vivo* metal binding. Hypotheses relating to the reasons for the observed phenotypes are discussed in chapter 7. It should be noted, however, that accumulation studies were carried out under different conditions to metal tolerance testing. The gene was induced at a minimal level for tolerance testing, and at a high level for accumulation studies. A high level of expression of the protein may result in increased interaction with other molecules in the cell (as observed previously), resulting in lack of association with metal ions. The possibility exists, however, that the observed change in Cd tolerance is due to unknown factors not relating to metal binding.

5.3.5 Summary and future work.

5.3.5.1 Implications regarding the functions of PCs.

Although the molecules produced via expression of the synthetic gene in *E. coli* differ from PCs in terms of the bond between the glutamate and cysteine residues, they are sufficiently similar in structure to exhibit similar qualities *in vitro* (Winge et al 1989). Thus, the data

Table 5.1 Metal accumulation by cells containing plasmid pGP1.2 and either pGPSG4 or pGEM4z. Methods were as outlined in the text. The concentrations of Cd, Cu and Zn chosen for metal exposure were as defined in section 5.2.20. (Table A - Cd; B - Cu; C - Zn. H - higher metal concentration; L - low metal concentration).

(A)

Replicate number	Amount of Cd (nmoles/8 x 10^8 cells) pGP1.2/pGEM4z H	pGP1.2/pGEM4z L	pGP1.2/pGPSG4 H	pGP1.2/pGPSG4 L
1	13.82 (0.23)	6.78 (0.13)	15.91 (0.30)	7.79 (0.20)
2	14.68 (0.03)	8.83 (0.10)	16.15 (0.54)	12.62 (0.58)
3	12.78 (0.30)	5.82 (0.30)	14.25 (0.60)	6.45 (0.25)
mean (n=9) (st. dev.)	**13.76 (0.97)**	**7.14 (1.34)**	**15.56 (1.21)**	**8.94 (2.68)**

(B)

Replicate number	Amount of Cu (nmoles/8 x 10^8 cells) pGP1.2/pGEM4z H	pGP1.2/pGEM4z L	pGP1.2/pGPSG4 H	pGP1.2/pGPSG4 L
1	18.03 (0.95)	2.68 (0.12)	21.45 (0.68)	3.08 (0.11)
2	9.86 (0.92)	3.18 (0.21)	10.23 (0.92)	5.64 (0.42)
3	16.60 (0.41)	3.70 (0.21)	17.63 (0.62)	4.63 (0.10)
mean (n=9) (st. dev.)	**14.83 (4.36)**	**3.19 (0.47)**	**16.44 (5.70)**	**4.36 (1.13)**

(C)

Replicate number	Amount of Zn (nmoles/8 x 10^8 cells) pGP1.2/pGEM4z H	pGP1.2/pGEM4z L	pGP1.2/pGPSG4 H	pGP1.2/pGPSG4 L
1	6.60 (0.33)	2.42 (0.19)	6.75 (0.10)	2.77 (0.23)
2	6.97 (0.34)	2.67 (0.22)	7.56 (0.13)	2.70 (0.10)
3	6.39 (0.12)	1.35 (0.15)	6.63 (0.28)	1.60 (0.04)
mean (n=9) (st. dev.)	**6.65 (0.29)**	**2.15 (0.63)**	**6.98 (0.50)**	**2.36 (0.58)**

obtained here may be extrapolated to provide indications of the functions of PCs in plant cells. The data suggest that αPC_3 molecules are capable of providing protection against the toxic effects of Cd *in vivo* at least in *E. coli* cells. Although the basis of this protection is unclear, it is likely that the molecules function by internal sequestration of metal ions. This is consistent with the observed effect of PCs in the systems described in section 5.1. It is noted that all of the organisms from which these molecules have been isolated have vacuoles, which have been implicated as having a function in the detoxification of metal ions via compartmentalisation of metal-PC complexes (see section 5.1.1.3). The data for expression of the synthetic gene in *E. coli* indicate that such compartmentalisation of PC complexes may not be critical in allowing detoxification by such molecules. The observation that the expression of heterologous MTs in plant cells (which are unlikely to be transported in a similar fashion to PC complexes) are capable of increasing metal tolerance in plants (Misra and Gedamu 1990), also indicates that intracellular sequestration alone may be sufficient to detoxify metal ions in plants. Similar experiments, involving the expression of genes encoding analogues of PCs in plant cells would be useful in further elucidation of their function.

5.3.5.2 Metal binding to αPC_3; Biotechnological aspects.

The data presented above, revealing a slight increase in Cd and Cu accumulation (within replicated experiments), and a reproducible increase in tolerance to Cd in cells expressing the synthetic gene, may indicate that the product of the synthetic gene has a preference for binding to these ions relative to Zn. This is consistent both with the implied function of PC molecules in detoxification of these ions in plant cells, and with data presented in section 5.1.2 regarding the nature of metal coordination sites. Section 5.1.2 indicates that liganding to Zn, through sulphydryl groups, is more highly dependent on the spacing of cysteine groups, on other amino acid residues, or on protein secondary structure than are sites for Cd or Cu coordination, although the precise nature of such sites is as yet poorly defined. Ligand binding to Cu and Cd is less structure-dependent, and the spacing of cysteine residues of the synthetic gene product, by

comparison with results obtained with other proteins and polypeptide fragments, would be likely to allow coordination of these ions.

The results in this chapter also suggest that it may be possible, given correctly controlled expression of novel ligands, to design and test the effectiveness of such ligands *in vivo* via alterations in metal tolerance. This has been described previously for the examination of analogues of MT, using restoration of Cu-tolerance in CUP1-deleted mutants as a biological marker for the production of effective Cu-ligands (Chernaik and Huang 1991; section 5.1.3). Similar experiments, using variants of PC-like molecules would assist in defining the nature of binding sites for metal ions.

The production of vectors such as that containing the synthetic gene, which establish a novel phenotype (Cd resistance) may also have applications in relation to recombinant DNA technology. Such vectors would provide an alternative to antibiotic selection and thereby allow the generation of novel plasmid constructs.

CHAPTER 6 EXPRESSION OF αPC_3 UNDER CONTROL OF *smt*A 5' SEQUENCES.

6.1 Introduction.

Many metal detoxification systems, in both prokaryotes and eukaryotes have evolved to rapidly respond to increased concentrations of intracellular metal ions. This response is often brought about by a metal-induced increase in transcripts encoding proteins (enzymes and metal-binding proteins) involved in the detoxification response. Metal-induced gene expression has been studied in several systems, the details of which are presented below. This chapter describes the use of the presumed metal-responsive elements of the *smt*A gene to attempt to achieve metal-regulated expression of the synthetic gene encoding αPC_3 [or $(\alpha Glu\text{-}Cys)_3Gly$] in *Synechococcus* PCC7942.

6.1.1 The regulation of MT genes.

As discussed previously, all MT genes so far studied show an increase in transcript abundance in reponse to elevated intracellular concentrations of specific free metal ions. The molecular genetics of metal-regulated expression of vertebrate MT genes, and of the CUP genes of *S. cerevisiae* have been elucidated in some detail, and these are described in the following sections. The *cis*-acting sequences present in animal MT genes which respond to other stimuli, such as glucocortacoid hormones and interferon, will not be discussed.

6.1.1.1 Metal-regulated expression of animal MT genes

Reverse genetic studies, involving the re-introduction of mutated or hybrid control sequences into cells to examine alterations in gene expression, have provided detailed information regarding the metal-regulatory *cis*-acting regions of MT genes. The primate and rodent genes, which have been studied in most detail, are similar in structure. Two *cis*-acting control sequences are positioned 5' to the MT structural genes. A "proximal" element lies between bases -15 to -84, and a "distal" element between -84 to -151 (Carter et al 1984). When introduced separately into cells, the proximal region gives higher induction ratios with low endogenous transcriptional efficiency, whereas the distal region gives higher transcriptional efficiency with a lower induction ratio. Both regions include a core regulatory sequence (5'TGCXCXCG3') which has been identified as the

major metal regulatory element in animal systems, and is present in multiple copies in both orientations in mammalian genes (Foster and Gedamu 1990). By insertion of this sequence 5' to a marker gene (herpes simplex thymidine kinase gene) and analysis of metal-inducibility of the constructs (Searle et al 1985), it was found that this sequence conferred a low level of metal inducibility on the marker gene when present in one copy, and a much higher level of inducibility when present in two or more copies. The effect was not dependent on the precise location of the sequence relative to the gene. This core sequence has been found in the DNA sequences surrounding all animal genes studied, and a variant of this sequence is also present 5' to the plant MT-like gene $PsMT_A$ (see chapter 4).

Initial studies to isolate the *trans*-acting factors responsible for metal-induction of mammalian MT genes have shown metal-dependent binding of liver nuclear proteins to DNA sequences which include copies of the core element (Searle 1990; Andersen et al 1990). Further research should identify the proteins involved in MT activation and their mode of action.

6.1.1.2 Cu-regulation of CUP1 expression in *S. cerevisiae*.

Cu-regulated expression of the *S. cerevisiae* MT gene (CUP1) has been extensively studied at the molecular level. Studies involving mutation of sequences 5' to the MT transcriptional start site (Butt et al 1984; Hamer et al 1985) identified two adjacent regions having imperfect repeats of 32 and 34 bases, centred at positions -123 and -165 relative to the transcriptional start site. Either region was capable of conferring Cu-inducibility, but both were required to give normal transcription and induction ratios.

The trans-acting CUP1 activating factor (CUP2 or ACE1) has been cloned and sequenced by complementation of mutants deficient in CUP1 activation (Szczypka and Theile 1989; Welch et al 1989). The protein was subsequently isolated directly from yeast cells (Buchman et al 1990). It was noted that the amino terminal portion of the protein was characterised by the occurrence of Cys-X-Cys and Cys-X-X-Cys motifs, which allowed interaction of the protein with Cu. Binding of Cu ions to the protein and the effect on binding to *cis* elements of the CUP1 gene has revealed that coordination of Cu is necessary to give binding to CUP1 sequences. Mutagenesis to substitute one of the cysteine

residues with a serine residue resulted in decreased interaction with CUP1 metal responsive elements (Buchman et al 1990). Interaction with CUP1 sequences is thought to occur in a fashion analogous to that demonstrated for Zn-finger-type proteins, the Cu-induced conformational alteration allowing direct interaction with DNA. EXAFS analysis of Cu interaction with CUP2 has demonstrated that Cu binds cooperatively to form a Cu cluster, with Cu-S distances suggestive of trigonal coordination (Nakagawa et al 1991). It is postulated that cooperative Cu-clustering allows the induction of CUP1 at high intracellular Cu concentrations, while a lower, essential level of Cu does not result in activation.

6.1.2 Metal-regulated prokaryotic genes.

Several metal-regulated detoxification systems have been described in prokaryotes, however relatively few have been extensively studied with regard to the mode of gene activation at the molecular level. The proposed mechanisms of metal-activation of the best characterised of these systems are described below.

6.1.2.1 The *mer* operon.

Eight analogous Hg detoxification (*mer*) systems have been isolated and studied. The plasmids on which they are located derive from both Gram positive and Gram negative cells, and in each case the mode of Hg detoxification (described in section 3.1) and gene activation is the same. The *mer*R (regulator) gene, which is responsible for Hg-induction of the operon, reads divergently to the other genes of the detoxification system, and is separated from these genes by the operator/promoter region to which the 144 amino acid MerR protein binds (as a dimer). The operator region to which the MerR protein binds has the sequence 5'TCCGTACATGAGTACGGA3', which exhibits dyadic symmetry of seven amino acids with a spacer region of 4 bases (Frantz and O'Halloran 1990). In the absence of Hg ions the MerR protein binds to the operator region and represses both transcription of the *mer*R gene and of the divergent structural genes encoding the proteins involved in transport and detoxification of Hg (Lund and Brown 1989). Binding of Hg to the MerR-DNA complex results in localised bending of the DNA at this region, which results in the activation of transcription of the operon. The spacing between the -35 and -10

transcriptional control elements (19 bases) is two bases (approximately 70°) out of phase for a typical *E. coli* promoter. This spacing is known to be critical for correct regulation of the *mer* operon (Parkhill and Brown 1990). It is proposed that the distortion produced in the DNA molecule by Hg binding to the MerR protein results in realignment of the transcriptional promoter elements, and hence activation of transcription (Ansari et al 1992).

6.1.2.2 The arsenical resistance operon.

The arsenical resistance operon located on the *E. coli* plasmid R773 encodes an ATP-dependent pump for the extrusion of arsenite, antimonite and arsenate (Chen et al 1986). Three structural genes have been identified. The *ars*A and *ars*B gene products are alone capable of forming a pump for the extrusion of arsenite and antimonite, while the *ars*C product alters the specificity of the pump to include arsenate. The *ars*R gene product, which is required for inducibility of the system has been cloned and sequenced (San Francisco et al 1990). This protein controls transcription of the *ars* operon by repression of transcription in the absence of arsenate ions. Upon induction of the system with arsenate, both the structural genes and the *ars*R protein are transcribed on the same transcription unit. No DNA-binding activity has benn reported for the ArsR protein, and its mode of action therefore remains unknown (Wu and Rosen 1991).

Other prokaryotic metal-inducible systems which have been studied at the molecular level include the plasmid-encoded *cad* operon of *S. aureus* (see section 3.1; Yoon and Silver 1991), the plasmid-encoded Cu resistance genes of the *pco* operon in *E. coli* (Rouch et al 1985) and the chromosomally-encoded *cut* genes, which are involved in Cu uptake and transport in *E. coli* (Rogers et al 1991). The precise regulatory mechanism for these systems remain to be elucidated.

6.1.3 Metal-responsiveness of the *smt*A gene.

As discussed in chapter 3, transcripts of *smt*A increase in abundance in response to elevated concentrations of metal ions. Examination of the 5' region of the gene (see figure 3.1, chapter 1) reveals two features which resemble metal-regulatory elements which have been identified in other bacterial systems. Firstly, the sequence 5'CTGAATCAAGATTCAG3', beginning 73 bases upstream from the ATG start

codon has a region of seven bases of dyadic symmetry, separated by two adenosine nucleotides. Similar dyadic sequences are located upstream to the start of the *cut*E gene in *E. coli*, and upstream of the structural genes of the *mer* operon, suggesting that such sequences may be characteristic of metal-responsive genes.

A second feature of the 5' region of the *smt*A gene is the presence of an open reading frame in the opposite orientation to the *smt*A gene, which encodes a protein with some sequence similarity to the regulatory protein of the *E. coli* arsenical resistance operon, ArsR (San Francisco et al 1990), and to the CadC protein, which is associated with Cd resistance in *S. aureus* (Yoon and Silver 1991). It has recently been shown that this second *smt* gene is transcribed (Huckle et al 1992). The predicted secondary structure of the protein encoded by this region has revealed the presence of potential helix-turn-helix motifs, indicating that the protein may be capable of interaction with DNA, supporting the concept that this is a regulator of *smt*A transcription (A.M. Morby pers. commun.).

6.1.4 Aims.

The research described in chapter 5 indicated that the synthetic gene was effective in establishing expression of αPC_3 in *E. coli*, and that the expressed protein was capable of protection against the toxic effects of Cd. The results indicated, however, that over-expression of the protein may be detrimental to cell survival. The aim of the research described in this chapter was to establish whether controlling transcription of the gene via coupling to the presumed metal-responsive elements of *smt*A would allow metal-induction of the gene in *Synechococcus* PCC7942 (PIM8), and result in any modification in Cd tolerance in cells expressing αPC_3. Metal-induction of the synthetic gene product should lessen the potentially toxic effect of producing a high cellular concentration of reactive thiols in the absence of free metal ions. Cd-tolerance as a result of expression of the gene would provide evidence that metal-sequestering ligands can enhance metal tolerance in these organisms.

It was hypothesised, although untested, that sequences adjacent to *smt*A would contain *cis*-elements responsible for metal-induced expression of the *smt*A coding sequence, and hence could be utilised to gain metal-regulation of other genes cloned adjacent to such

sequences. An investigation of the effect on transcription of the synthetic gene cloned adjacent to *smt* sequences therefore provided a test of this hypothesis.

Coupling of the 5' region of *smtA* to the synthetic gene was presumed to be likely to give metal-activated transcription of the gene in Synechococcal cells, in which all *trans*-acting transcription factors will be present. Additionally, the presence of a gene encoding a putative regulatory protein in the 5' region (see figure 3.1) could potentially allow metal-regulated transcription of the gene in other organisms.

6.2 Materials and methods.

6.2.1 Experimental design.

6.2.1.1 Design of a PCR primer for the amplification of *smt* sequences.

The PCR was used to couple a DNA sequence encoding a PC analogue (chapter 5) to presumed metal-regulatory sequences from *smt*A. Figure 6.1 shows the sequence and features of a PCR primer which was designed to allow amplification of the sequence directly 5' to the *smt*A coding region. Amplification using this primer will result in addition of the synthetic gene coding sequence to the amplified fragment. The 3' portion of the oligonucleotide primer is designed to anneal to the 13 bases directly adjacent to the *smt*A coding region and to the initiator codon and first codon of the *smt*A coding region. The primer is complementary to the sense strand and so anneals such that polymerisation will proceed into the 5' region of *smt*A. The annealing portion of the primer is followed by a sequence encoding $(Glu\text{-}Cys)_3Gly$, as described in the previous chapter. A BglII restriction site was included after this sequence to simplify genetic manipulation following amplification.

It has been noted that the nucleotides directly following the start codon are often conserved in prokaryotic genes, particularly with regard to an adenine base in the +4 position in *E. coli*. It is postulated that these bases can exert an effect on translation (Stormo et al 1982; Scherer et al 1980). For this reason the threonine codon ACC was retained in the synthetic gene sequence.

6.2.1.2 Amplification strategy.

Figure 6.2 outlines the strategy employed for use of the primer described above in amplification of the *smt*A 5' region. The template used in the reactions outlined was plasmid pJHNR4.9 (figure 3.1). This plasmid contains a 1.9 Kb genomic fragment from *Synechococcus* PCC7942 which includes the *smt*A gene plus 620 bp 5' and 1.1 Kb 3' to the *smt*A coding sequence. This plasmid was included as template in a reaction with the PCR primer described above, along with the M13 forward sequencing primer. Reaction parameters are described in the legend to figure 6.2.

Full details of the constructs created using the products of the reactions described are given in section 6.3. Two constructs were made using the *E. coli*/*Synechococcus* PCC7942 shuttle vector pUC105 (section

Figure 6.1 Diagram showing the PCR oligonucleotide primer used to couple the *smtA* 5' region to a DNA sequence encoding the phytochelatin analogue αPC_3.

The oligonucleotide shown was designed to hybridise to thirteen bases directly adjacent to the *smtA* coding region, and to the six bases encoding the first two amino acids of *smtA*. This primer region is followed by the synthetic gene coding sequence, followed by a stop codon and a BglII restriction site to allow subsequent manipulation of the amplification product.

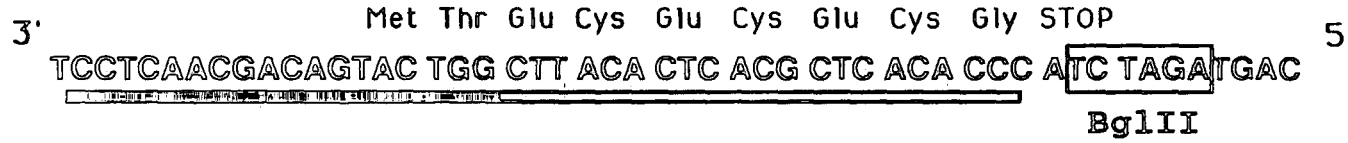
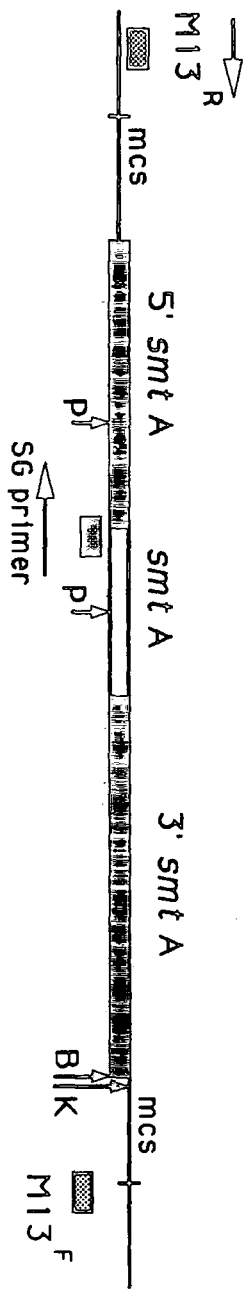

3'
Met Thr Glu Cys Glu Cys Glu Cys Gly STOP
5'
TCCTCAACGACAGTAC TGG CTT ACA CTC ACG CTC ACA CCC ATC TAGATGAC
BglII

Figure 6.2 Diagram showing the amplification strategy for production of a DNA sequence encoding the synthetic gene under the transcriptional control of putative *smtA* 5' regulatory sequences. The oligonucleotide primer shown in figure 6.1 was used in the PCR reaction along with one of the M13 sequencing primers, using 200 ng of plasmid pJHNR4.9 as template as illustrated. PCR conditions for the reaction were ten cycles as follows : 96 °C for 1.5 min. (denaturing); 55 °C for 1.5 min. (annealing); 72 °C for 4 min. (extension). Thin lines - vector sequence of plasmid (pGEM4z); thick lines - insert sequence (genomic fragment of *smt* region); SG primer - oligonucleotide described in figure 6.1; $M13^F$, $M13^R$ - M13 sequencing primer binding sites of vector; mcs - vector multiple cloning site; B - BamHI restriction site; K - KpnI restriction site.


M13 R
mcs
5' smt A
P
SG primer
P
smt A
3' smt A
BlK
mcs
M13 F

6.2.2.2), which replicates in both organisms, therefore allowing the expression of genes in Synechococcal cells.

6.2.2 Materials.

6.2.2.1 *Synechococcus* PCC7942 (variant PIM8).

The cyanobacterium *Synechococcus* PCC7942, also referred to as *Anacystis nidulans* R2, was used throughout these experiments. A variant of this strain, PIM8 (van der Plas et al 1990), was used as the endogenous 7.5 Kb plasmid normally present in Synechococcal cells has been cured from this strain. Transformation of this organisms with a vector containing sequences derived from the 7.5 Kb plasmid (such as plasmid pUC105 - see below) is therefore less likely to result in rearrangement via homologous recombination than transformation into a non-cured strain. Also, variant PIM8 is a methionine auxotroph (due to disruption of the *met*F gene), and is therefore amenable to biological containment.

6.2.2.2 Shuttle vector pUC105.

The 12.8 Kb shuttle vector pUC105 (Kuhlemeir *et al* 1981), which includes an origin of replication for both *E. coli* and *Synechococcus* PCC7942 is described in figure 6.3.

6.2.2.3 Plasmid pHP45Ω.

Full details of plasmid pHP45Ω (Prentki and Krisch 1984) are provided in figure 6.4 and the accompanying figure legend.

6.2.3 Methods.

6.2.3.1 Maintenance of Synechococcal cultures.

Synechococcal cultures were maintained in Allen's medium (Allen 1952), either on 1.5 % agar (slopes or plates) or in liquid cultures under constant light conditions (100 µmol photon $m^{-2}s^{-1}$) at 32 °C. Since strain PIM8 was used, the medium was supplemented with 30 µg/ml methionine. Generally, cultures were maintained in 50 ml flasks without aeration. For experiments requiring larger volumes of cells (500 ml - 1 l), filter-sterilised air was bubbled through the culture. Strains were also maintained in long-term storage by freezing 1 ml aliquots of culture in liquid nitrogen, both with and without added DMSO (10 µl per 1 ml of culture).

Figure 6.3 Restriction map of pUC105. The thin lines of the plasmid diagram represent sequence derived from the Synechococcal plasmid pUC1 (Van den Hondel et al 1980), thick lines represent sequence derived from pACYC184 (Chang and Cohen 1978). pUC105 was created as a hybrid of these two plasmids by Kuhlemeier et al (1981). The BamHI and BglII sites are unique sites, and were used for cloning in the experiments described in this chapter.

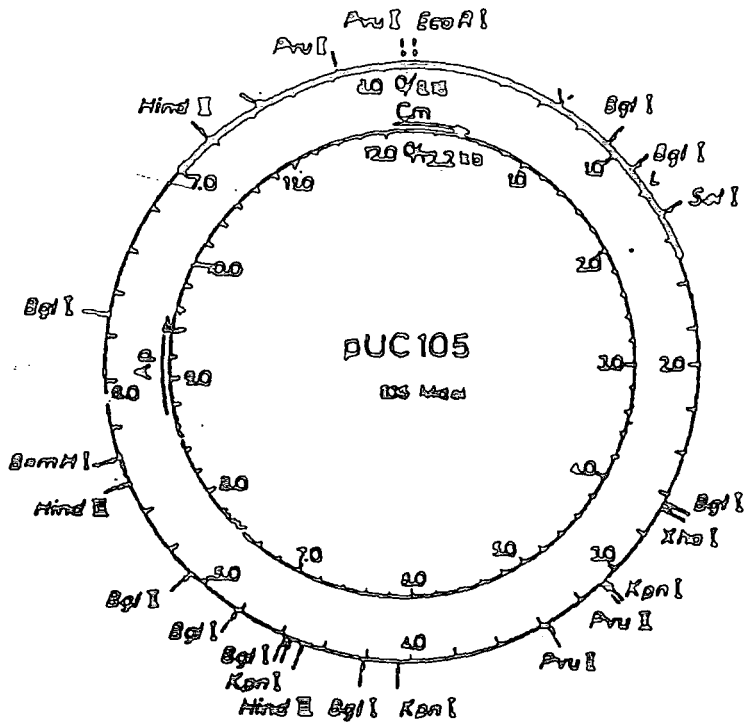
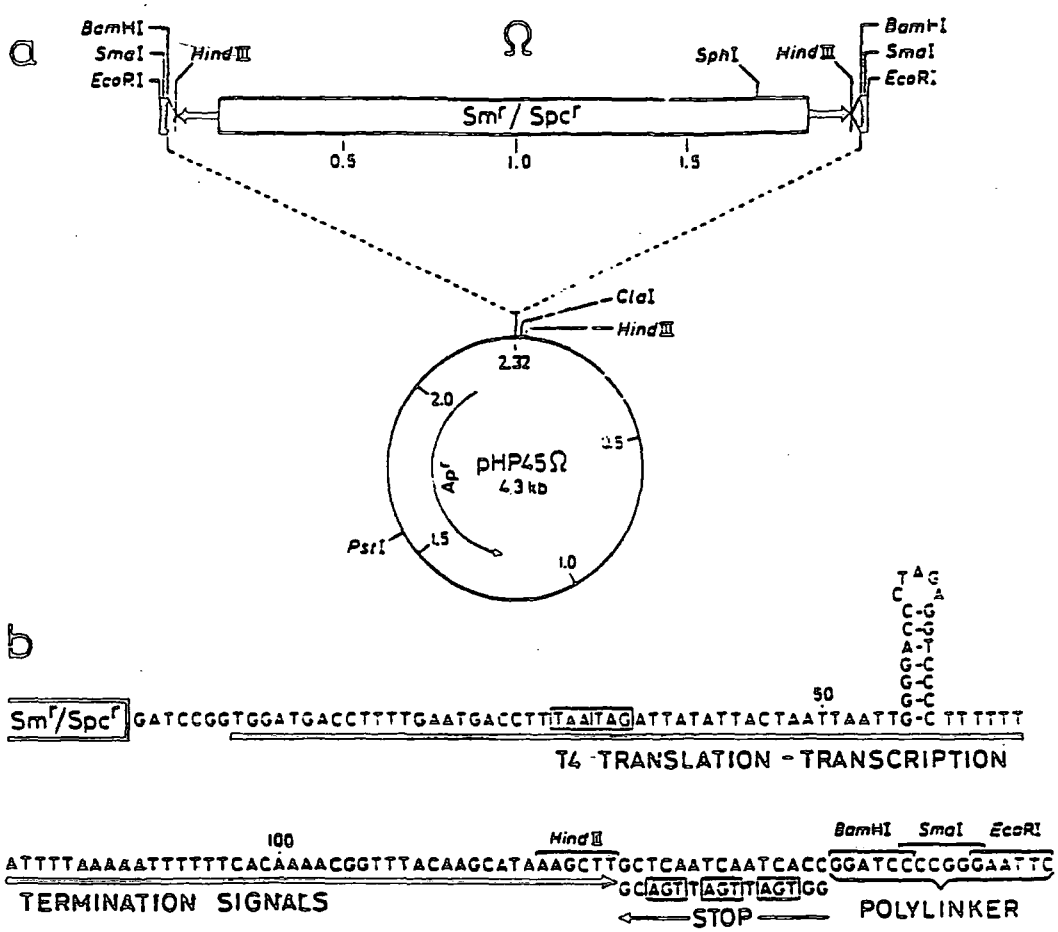

pUC 105
Cm
Ap
Ori
Pvu I
EcoR I
Hind III
Bgl I
Sal I
BamH I
Xho I
Kpn I
Pvu II

Figure 6.4 Plasmid pHP45Ω and the Ω fragment. Plasmid pHP45Ω (A), and the essential features of the Ω fragment (B) are shown. The 2 Kb Ω fragment includes the gene for streptomycin resistance, and carries the transcription termination signals from gene 32 of bacteriophage T4 in both orientations (from Prentki and Krisch 1984).


a
BamHI
SmaI
EcoRI
HindIII
Ω
SphI
HindIII
BamHI
SmaI
EcoRI
Smr / Spcr
0.5
1.0
1.5
ClaI
HindIII
2.32
2.0
0.5
Apr
pHP45Ω
4.3 kb
PstI
1.5
1.0
b
Smr/Spcr
GATCCGGTGGATGACCTTTTGAATGACCTTTAAATAGATTATATTACTAATTAATTG-C TTTTTT
50
T4 -TRANSLATION - TRANSCRIPTION
100
ATTTTAAAAATTTTTTCACAAAACGGTTTACAAGCATAAAGCTTGCTCAATCAATCACCGGATCCCCGGGAATTC
HindIII
BamHI SmaI EcoRI
GCAGTTAGTTAGTGG
TERMINATION SIGNALS
STOP
POLYLINKER

6.2.3.2. Estimation of cell numbers.

Where it was necessary to determine Synechococcal cell numbers, two methods were employed. Direct counts were done using a haemocytometer, using standard procedures. Where comparative data were required (e.g. when monitoring cell growth), it was possible to estimate cell numbers by measurement of absorbance at 540 nm. This was performed using the micro-titre plate reader as described for *E. coli* cells. Using this measure, 1 absorbance unit at 540 nm is approximately equivalent to 4 x 10^7 Synechococcal cells.

6.2.3.3 Isolation of DNA from Synechococcal cells.

1. Cells were harvested by centrifugation at 3000 rpm using Beckman benchtop centrifuge, the supernatant discarded, and the cells resuspended in an equal volume of extraction buffer.
2. Resuspended cells were added drop-wise to liquid nitrogen, and ground to a fine powder using a mortar and pestle.
3. 200 µl aliquots of ground cells were placed in an Eppendorf tube containing 2 µl of 2-mercaptoethanol, and 200 µl of DNA extraction buffer (1.4 M NaCl, 100 mM tris, 20 mM Na_2EDTA; pH 8.0) was added. 400 µl of phenol/chloroform was then added, and the tube contents thoroughly mixed. The mixture was allowed to return to room temperature, and was then microcentrifuged at top speed for 5 min.
4. The supernatant was removed to a fresh tube, and re-extracted once more using phenol/chloroform, followed by several extractions using chloroform alone, until no protein contamination was visible at the aqueous/organic interface.
5. After the final extraction with chloroform the supernatant was made up to 320 µl and 80 µl of 5 M ammonium acetate added. 1 ml of 100 % ethanol was then added to this, and the nucleic acids allowed to precipitate overnight at -20 °C.
6. Total nucleic acid was recovered by microcentrifugation for 10 min at high speed, briefly washed using 70 % ethanol, and dried under vacuum. This material was resupended in 40 µl of sterile water, and quantified by measurement of absorbance at 260 nm.

6.2.3.4 Exposure of cells to Cd for northern analysis of metal-induced gene expression.

1) Cells (1 litre) were grown to mid log phase in Allen's medium.

2) Aliquots (50 ml) of culture were removed to 50 ml Falcon tubes, and 5 μM Cd added at various times over a 1 hr period to give exposure times of 2, 5, 10, 15, 30 and 60 min.

3) Cells were collected by centrifugation at 3000 rpm (MSE Centuar centrifuge), and frozen in liquid nitrogen prior to RNA extraction performed as outlined below.

6.2.3.5 Isolation of total RNA from Synechococcal cells.

(From Dzelzkalns et al 1988)

1. Cells were harvested and ground to powder as for extraction of DNA.
2. Ground cells (200 μl) were added to an equal volume of pre-warmed (37 °C) lysis buffer, and incubated at 37 °C for 30 min.
3. Lysed cells were extracted twice using phenol/chloroform, and then several times using chloroform until no contaminating proteins were visible at the aqueous/organic interface.
4. The supernatant was then removed to a fresh Eppendorf tube, and nucleic acids precipitated in the same manor as for DNA extraction.
5. The isolated nucleic acid was quantified both by absorbance at 260 nm, and by visualisation on a 1.2 % agarose mini-gel.
6. RNA was further treated prior to electrophoresis by mixing 10-20 μg of RNA (quantities were standardised for each gel to give equal amounts in each lane) with 50X MOPS and 100 % formamide to give final concentrations of 1X MOPS and 50 % formamide. This was then boiled for 10 min before electrophoresis on 1.4 % formaldehyde-agarose gels as described in chapter 2.

6.2.3.6 Transformation of Synechococcal cells with plasmid DNA.

1. Cells were grown to late log phase in Allen's medium, counted using a haemocytometer, and a total of 10^8 cells pelleted (3000 rpm) in a 10 ml sterile test tube.
2. Pelleted cells were resuspended in 100 μl of Allen's medium, and 10 μg of plasmid DNA added. The cells were then left at 30 °C for 40 min to allow DNA uptake.

3. Aliquots of transformed cells (between 5 and 40 µl) were spread on 1.5 % agar Allen's plates and incubated under conditions described previously.

4. After incubation for 16 h, Cm was added to the plates to give a final concentration of 10 µg/ml Cm. Antibiotic was added by gently lifting the agar disc from the bottom of the Petri dish using a 1 ml sterile glass pipette, and pipetting 0.5 ml of a 10X stock of the antibiotic underneath the agar. Plates used for transformation contained 50 ml of Allen's agar per plate to allow 100X dilution of the antibiotic.

5. After a further incubation period of 5-10 days, isolated single colonies were picked from the original plates and re-streaked onto fresh plates containing the selective agent. Colonies from these plates were used to set up 50 ml cultures for further sub-culture and for long term storage of transformants.

6.2.3.7 Plasmid rescue from Synechococcal cells.

In order to check that the plasmids transformed into Synechococcal cells had undergone no rearrangement, plasmids were re-isolated from the transformants and analysed by restriction digestion. In order to rescue the plasmids from Synechococcal cells, a standard DNA preparation was performed, and the total DNA used to directly transform *E. coli* cells. *E. coli* transformants were selected for Cm resistance, and plasmids were isolated from these cells using standard procedures. In no case was the plasmid DNA found to have been altered during the procedure.

6.2.3.8 Analysis of Cd tolerance of Synechococcal cells.

Prior to examining Cd-tolerance of PIM8 cells containing the construct to be tested, cells containing pUC105 (control) were exposed to levels of Cd between 0 and 7.0 µM (see legend to figure 6.13 for details). Based on these results, two levels were chosen to compare tolerance of control and test cells. As for *E. coli* cells, a concentration which completely inhibited cell growth (5.5 µM), and one at which growth was slowed but not completely inhibited (4.5 µM), were selected for Cd tolerance tests.

Cells were tested for increased tolerance to Cd using the following method:

1) Cells containing control or test plasmid were grown to mid log phase in 50 ml of Allen's medium containing 10 μM Cm.
2) Cell numbers were estimated by reading absorbance at 540 nm, and equal numbers were used to inoculate triplicate cultures containing 5 ml of Allen's medium containing 10 μg/ml Cm and the required levels of Cd.
3) Cultures were incubated under standard conditions, and A_{460} readings taken over a period of 7 days.

6.2.3.9 Analysis of metal accumulation by Synechococcal cells.
Metal accumulation by Synechococcal cells was tested using the following protocol.
1) 50 ml of cells were grown to late-log phase under standard conditions.
2) Aliquots (10 ml) were removed to 50 ml falcon tubes, and metal added.
3) Cells were incubated for a further period of 5 h, collected by centrifugation (3000 rpm), and washed twice in fresh medium.
4) The washed cells were resuspended in 1 ml of 70 % nitric acid and incubated overnight at room temperature.
5) Metal concentration of the cellular digests was analysed by AAS as outlined previously.

6.3 Results and discussion.

6.3.1 Examination of *smtA* expression in *Synechococcus* PCC7942 (PIM8).

The constructs made in this report were used to attempt to achieve metal-induction of the synthetic gene in Synechococcal cells. The strain used in these experiments, PIM8, is derived from *Synechococcus* PCC7942. It was necessary, prior to performing such experiments with PIM8, to confirm that the *smt*A gene was present in this strain and was expressed in a similar fashion to that observed for PCC7942. Cd-induction of *smt*A was therefore tested by northern analysis (figure 6.5). Since the pattern of induction by 5 μM Cd over the period of 1 h was the same as that observed for the *smt*A gene in PCC6301 (Robinson et al 1990b), experiments were continued using *Synechococcus* PCC7942 (variant PIM8).

6.3.2 Results of amplification of *smt*-synthetic gene sequences: Sub-cloning and sequencing of PCR fragments.

The products of PCR reactions using the primer and amplification strategy described in section 6.2.1 were separated on a 1.5 % agarose gel (figure 6.6). A fragment of the predicted size (651 bp) was obtained from the reaction containing the M13 forward primer, but not with the M13 reverse primer. The fragment was excised from the gel, blunt-ended using T4 polymerase, and sub-cloned into SmaI-restricted pUC18. Several plasmids were screened by *in situ* analysis, and one chosen from which the PCR fragment could be excised on a BamHI/BglII fragment (i.e. which had inserted into the vector such that the BglII site of the insert was intact and was at the opposite end of the vector multiple cloning site from the BamHI site). This clone, termed pWLNR14, was digested with EcoRI, followed by PstI, and the resulting 500 bp PstI, and 151 bp PstI/EcoRI fragments sub-cloned separately into pUC18 and sequenced. The sequence of this fragment in shown in figure 6.7.

6.3.3 Construction of pUC105-derived shuttle vectors and transformation into *Synechococcus* PCC7942 (PIM8) cells.

The PCR fragment described above was ligated into BamHI/BglII-digested pUC105. A clone from which the fragment could subsequently be released by re-digestion with these enzymes was selected such that additional DNA could be inserted 3' to the synthetic gene sequence via digestion

Figure 6.5 Northern analysis of *Synechococcus* PCC7942 (PIM8) RNA after exposure of cultures to 5 μM Cd. Cell were exposed to 5 μM Cd and RNA extracted and analysed by northern blotting using methods outlined in section 6.2. The blotted RNA was hybridised to ^{32}P-labelled *smt*A coding region. For both panels A (formaldehyde agarose gel) and B (northern blot) : lane 1, No Cd added (control); lanes 2-6, 5 μM Cd for 2, 5, 15, 30 and 60 min respectively.

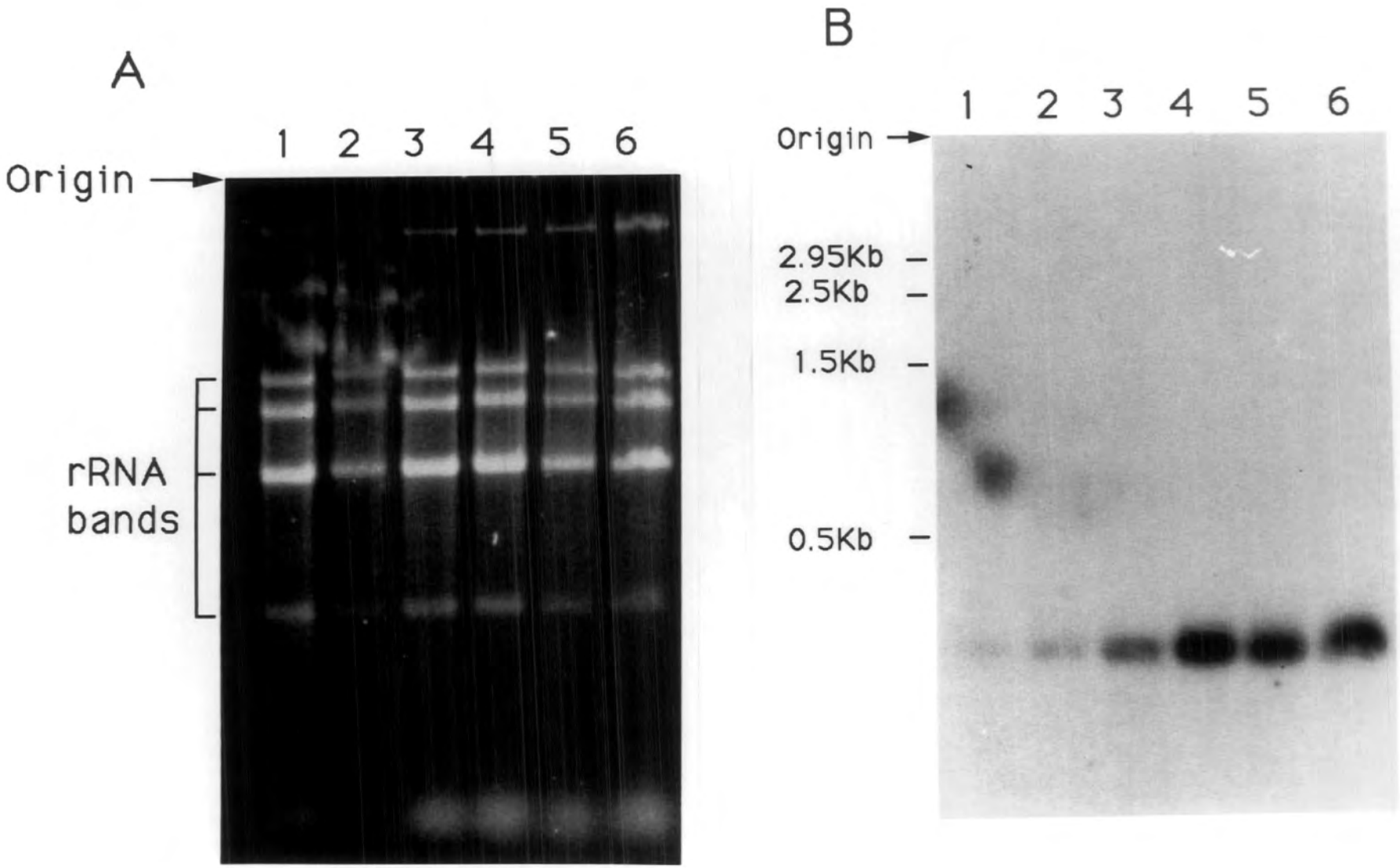

A
1 2 3 4 5 6
Origin
rRNA
bands
B
1 2 3 4 5 6
Origin
2.95Kb
2.5Kb
1.5Kb
0.5Kb

Figure 6.6 Products of the PCR separated on a 0.7 % agarose gel. Lane 1, PstI-restricted lambda DNA; lane 2, product of the PCR reaction containing the synthetic gene primer and M13 reverse primer with JHNR4.9 as template.


1
2
Origin
686bp

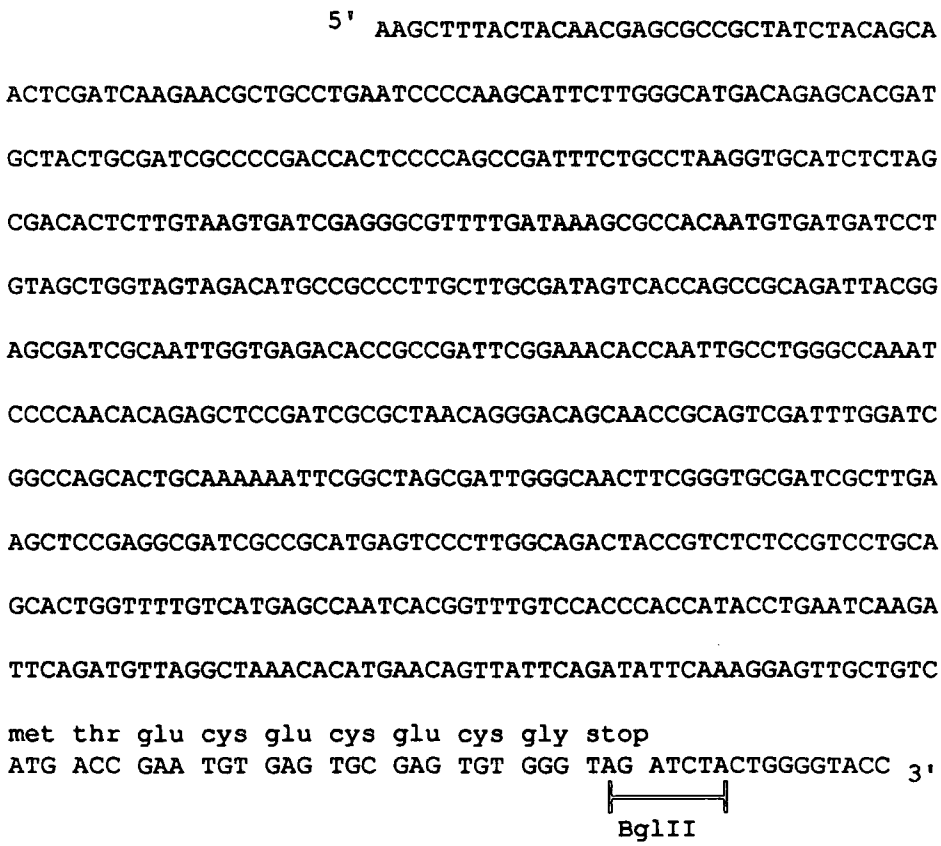


Figure 6.7 Sequence obtained from plasmid pWLNR14. For sequencing, plasmids were restricted using PstI, followed by EcoRI, yielding a PstI fragment of 500 bp, and an EcoRI/PstI fragment of 151 bp. These fragments were sub-cloned separately into pUC18 and sequenced using both forward and reverse M13 sequencing primers.

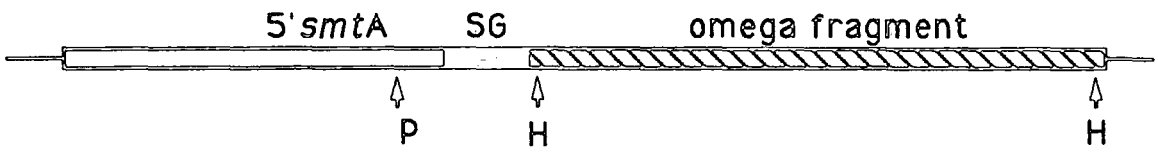
with BglII, and this clone termed pWLNR17 (figure 6.9, A). pWLNR17 was digested with BglII to allow the insertion of one of two possible sequences which could act as transcription termination signals. The first sequence inserted 3' to the synthetic gene sequence was the 2 Kb Ω fragment derived from pHP45Ω (figure 6.2). A diagram showing the predicted construct obtained by insertion of this fragment into pWLNR17 is illustrated in figure 6.8 (A). The transcription termination signals from phage T4 are present in both directions in the Ω fragment (see figure 6.2), meaning that analysis of the orientation of the cloned BamHI fragment was not necessary. Correct insertion of the Ω fragment into pWLNR17 was checked by HindIII digestion (figure 6.9, B).

A second shuttle vector was created in which the terminator sequence used was that of the *smt*A gene. In order to use this sequence, a 1.1 Kb fragment 3' to the *smt*A coding sequence was excised from plasmid pJHNR4.9 by digestion with PstI and KpnI. Restriction of this clone with KpnI retains a BamHI site from the vector polylinker sequence which is adjacent to the KpnI site (illustrated in figure 6.8). This site could then be used for subsequent manipulations. Ir order to add a BamHI site to the other end of the fragment, the excised PstI/KpnI fragment was cloned into the Bluescript vector KS^+ (named pWLNR15; see Sambrook et al [1989] for a description of the full polylinker sequence of Bluescript plasmids; also illustrated in figure 6.8). The entire sequence could then be re-excised from pWLNR15 by BamHI digestion. This BamHI fragment was cloned into BglII-restricted pWLNR17. As shown in figure 6.8, insertion of this fragment can result in two possible constructs. Although limited information is available concerning the relative positions of restriction sites of vector pUC105, it was possible to determine the orientation of the insert by digestion of the plasmids obtained with PstI. As shown in the diagram, the two possible clones obtained will release the synthetic gene sequence on diagnostically-sized PstI fragments. Thus the clones were identified following PstI digestion by Southern blotting of the fragments, and hybridizing the filter to probe made using the PCR primer as shown in figure 6.4. The restriction pattern and hybridization of the synthetic gene sequence to the blotted DNA is shown for both possible constructs in figure 6.10. The clone which

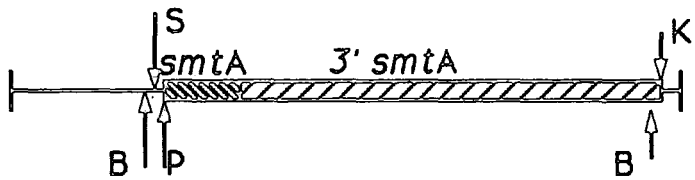
Figure 6.8 Diagram showing the design of constructs made in order to place the synthetic gene under control of presumed *smt* metal-regulatory sequences.

Two clones, pSVSG1 and pSVSG2, were created for transformation of Synechococcal cells. The arrangement of the DNA sequences of these clones is shown. Figure (A) shows the *smt*-synthetic gene fusion with the Ω fragment as a terminator (pSVSG1). Figure (B) - an intermediate step in cloning the 3' region from *smt*A into plasmid pWLNR17. The 1.1 Kb PstI/KpnI fragment of pJHNR4.9 was cloned into Bluescript KS in order to add a BamHI site to the clone. This allowed insertion of the 3' region from *smt*A into pWLNR17 as a BamHI fragment as shown in (C) and (D). (C) and (D) represent two possible products of the ligation of the 3' region from *smt*A into pWLNR17. In (C) the insert is in the correct orientation (plasmid pSVSG2), in (D) the insert is in the incorrect orientation. The approximate positions of the restriction sites which can be used to identify the clones are shown. (H - HindIII; P - PstI; B - BamHI; S - SstI).

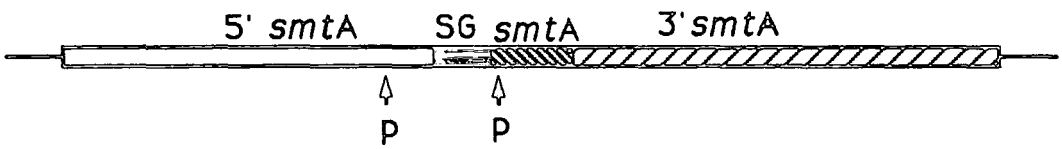
(A)


5'smtA
SG
omega fragment
P
H
H

(B)


S
smtA
3' smtA
K
B
P
B

(C)


5' smtA
SG
smtA
3'smtA
P
P

(D)

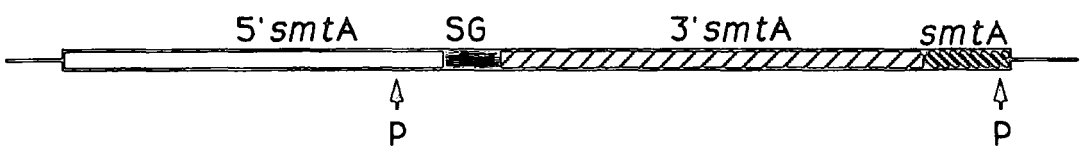
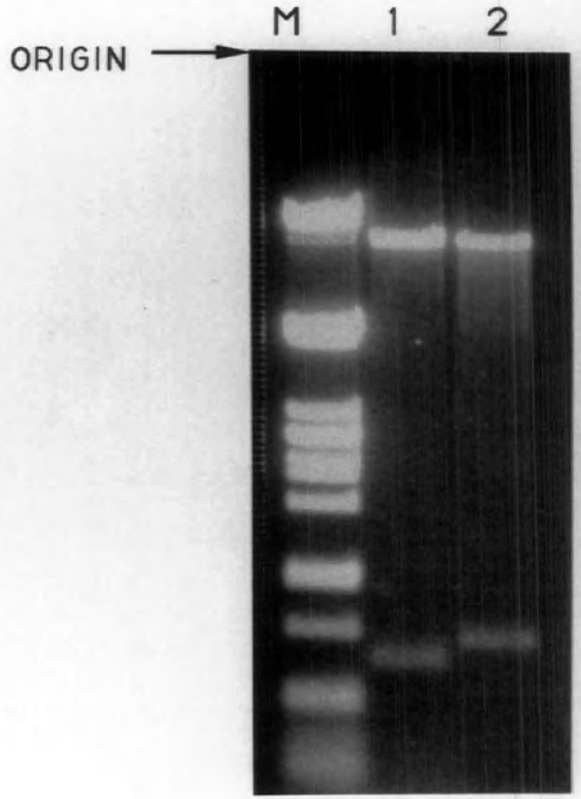

5'smtA
SG
3'smtA
smtA
P
P

Figure 6.9 Restriction analysis of plasmids pWLNR17 and pSVSG1.
Plasmids pWLNR17 and pSVSG1 were created as described in section 6.3.3. Photograph (A) shows that the BamHI/BglII fragment cloned from pWLNR14 can be re-excised after insertion into pUC105 (pWLNR17). Lane 1 - pUC105 restricted with BamHI and BglII; Lane 2 - pWLNR17 restricted with BamHI and BglII. pWLNR17 was subsequently re-digested with BglII for insertion of the BamHI-excised Ω fragment from pHP45omega. Photograph (B) shows the result of HindIII restriction of the clone resulting from insertion of the Ω fragment (pSVSG1) (pUC105 and pWLNR17 are included for comparison). Lane 3 - pUC105; lane 4 - pWLNR17; lane 5 - pSVSG1 (all restricted with HindIII).

(A)



(B)

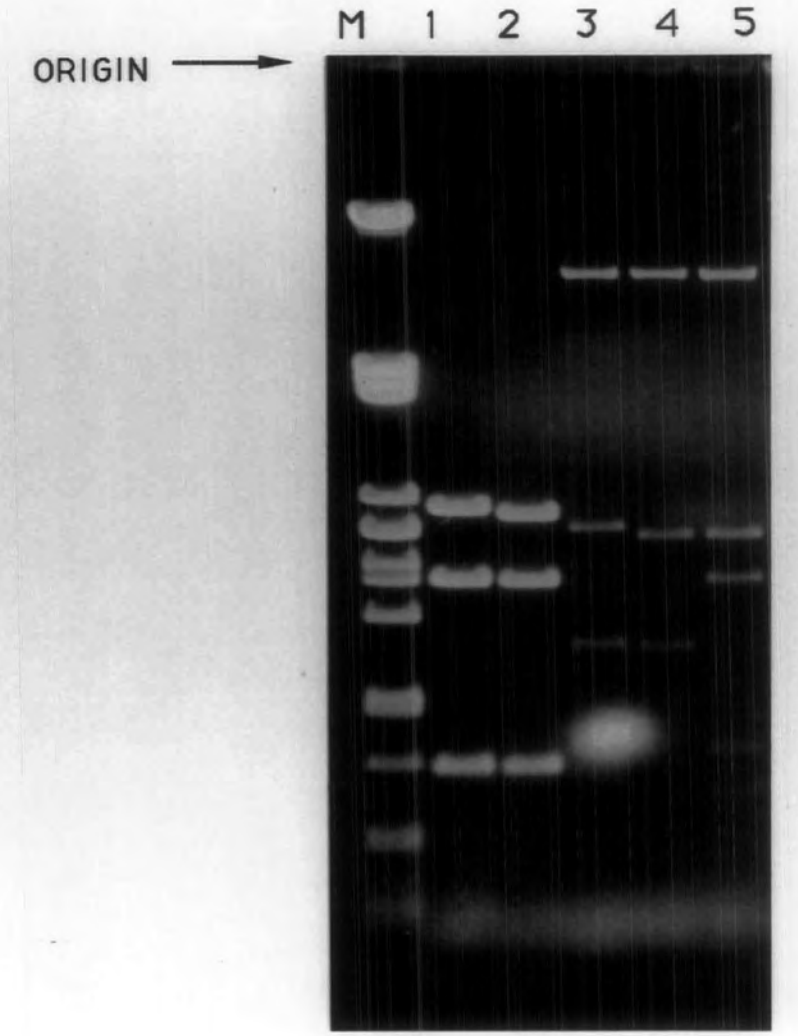
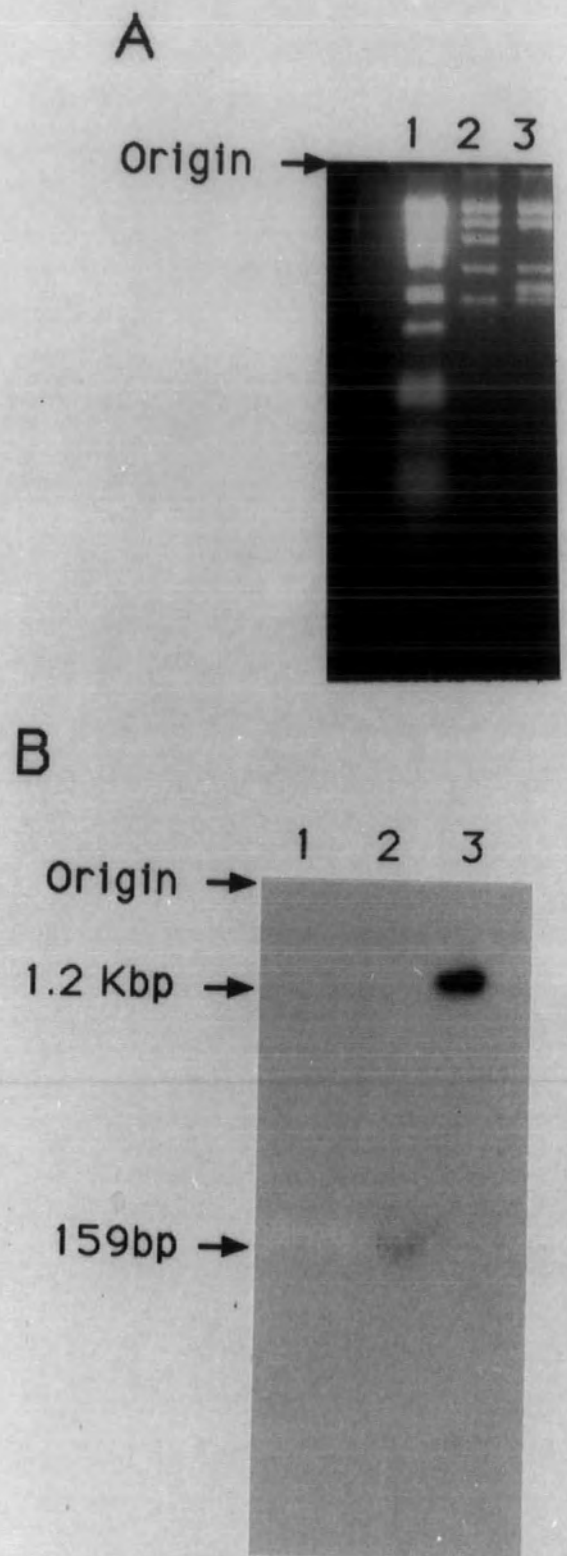


Figure 6.10 Restriction digestion and Southern analysis of the plasmids resulting from insertion of the BamHI fragment from pWLNR15 into BglII-restricted pWLNR17.

The final construct used to transform *Synechococcus* PCC7942 (PIM8), pSVSG2, comprises the 5' sequences of *smt*A, followed by the synthetic gene coding sequence, followed by part of the *smt*A coding sequence and 1.1 Kb of 3' sequence, as shown in figure 6.6. After insertion of the 1.1 Kb 3' BamHI fragment, several positive clones were selected on the basis of hybridisation to probe which was made using the synthetic gene PCR primer, and digested with PstI. Digestion with this enzyme gives the synthetic gene coding sequence on a predicted 159 bp fragment (correct orientation) or on a 1.2 Kb fragment (incorrect orientation - see figure 6.8 for details). For panels (A) and (B) : Lane 1, lambda / PstI marker; lane 2, pWLNR17 containing BamHI fragment from pWLNR15; Lane 3, as lane 2 (opposite orientation).


A
Origin
1 2 3
B
1 2 3
Origin
1.2 Kbp
159bp

released the synthetic gene sequence on a 159 bp fragment was designated pSVSG2, and used in further experiments along with pSVSG1.

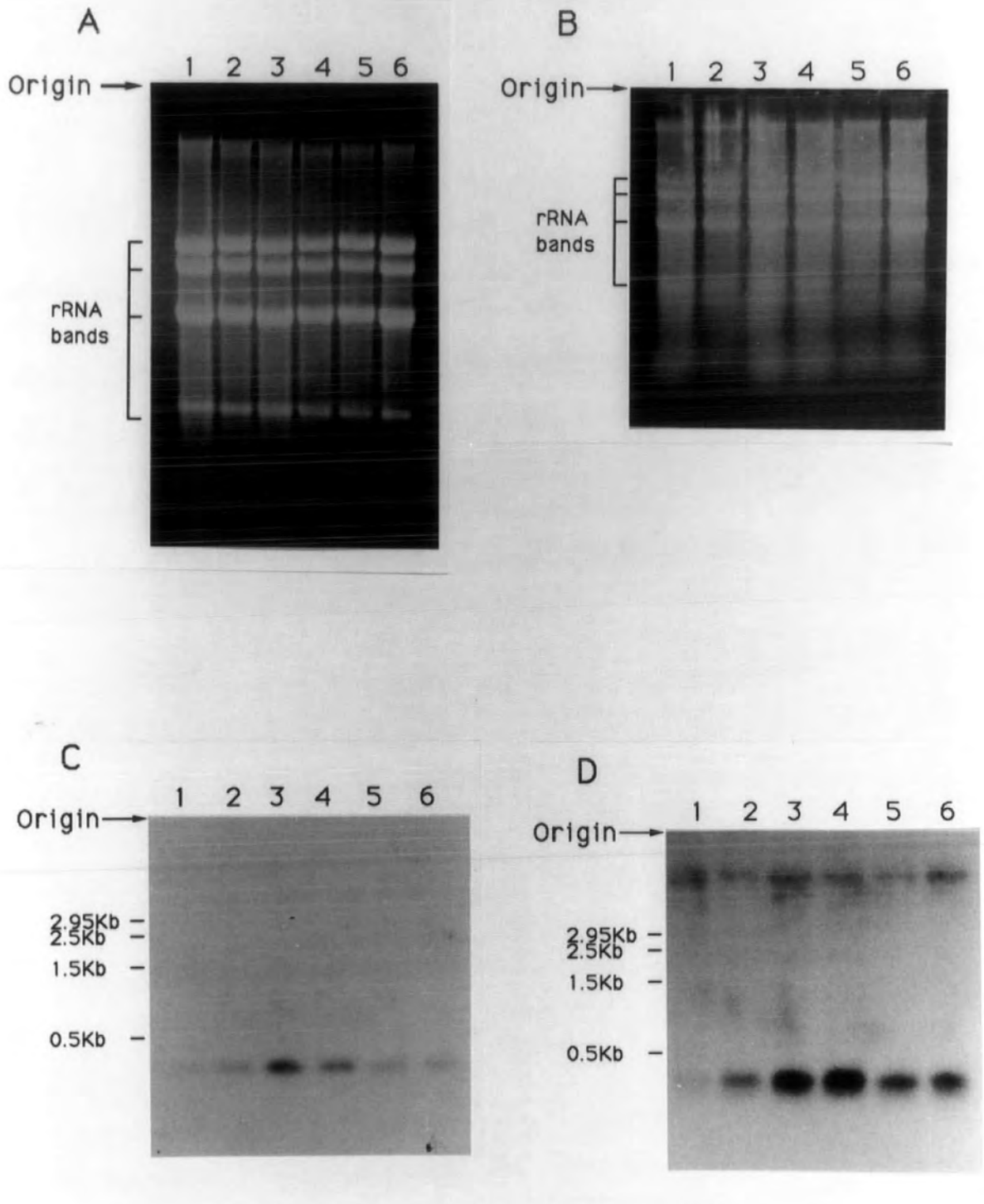
Both plasmids described above, pSVSG1 and pSVSG2, were used to transform *Synechococcus* PCC7942 (PIM8) cells, using methods as outlined in section 6.2.2.6, and transformants were selected for resistance to Cm. It was considered important to analyse the plasmids to ensure that no rearrangements had occurred during transformation since these organisms, unlike the *E. coli* cells which are routinely used in genetic manipulations, are not modified in any of the genes relating to recombination. Homologous recombination is known to be extremely common in Synechococcal cells (Hanawalt et al 1979). The presence of *smt*-derived sequences in the plasmid could therefore potentially lead to rearrangements in the construct via homologous recombination with smt sequences present on the chromosome. Since insufficient plasmid DNA could be isolated directly from Synechococcal cells to allow in vitro analysis, the plasmid was first re-transformed into *E. coli* cells using methods as described in section 6.2.2.7, followed by re-isolation from *E. coli* using standard methods. Plasmids re-isolated from *E. coli* were analysed by restriction digestion and agarose gel electrophoresis. In each case, the shuttle vector DNA was found to remain intact throughout the transformation procedures, and experiments were continued with transformed PIM8 cells.

6.3.4 Northern analysis of metal-exposed Synechococcal cells containing pSVSG1 and pSVSG2.

In order to ascertain whether the *smt*A sequences were sufficient to allow metal induced-transcript accumulation of the synthetic gene, cultures of PIM8 containing plasmids pSVSG1 and pSVSG2 were exposed to 5 μM Cd for times ranging between 2 and 60 min (see legend to figure 6.11). Exposure to Cd was exactly as described previously for non-transformed Synechococcal (PIM8) cells. RNA was extracted and analysed by northern hybridisation. Figure 6.11 shows the results of northern analysis of Cd-induced PIM8 cells containing the synthetic gene under control of smt sequences. Formaldehyde-agarose gels of RNA isolated from cells containing pSVSG1 (figure 6.11, A) and pSVSG2 (figure 6.11, B) illustrate that similar amounts of RNA were loaded into each lane. These gels were blotted onto nylon membranes for hybridisation analysis. Firstly, each northern blot was hybridised to a probe which

Figure 6.11 Formaldehyde-agarose gels and the resulting northern blots of RNA extracted from *Synechococcus* PCC7942 (PIM8) cells containing pSVSG1 or SVSG2 exposed to 5 μM Cd.

Gel photographs (A) and (B) show RNA extracted from *Synechococcus* cells transformed with pSVSG1 and pSVSG2 respectively. (C) Northern analysis of gel (B) using the synthetic gene coding sequence as a probe. (D) Northern analysis of gel (B) using the *smt*A coding sequence as a probe. Synthetic gene-specific sequences were obtained by digestion of clone pWLNR14 (section 6.3.2.3) using BspHI followed by BglII, separation on a 20 % polyacrylamide gel, and end labelling using the method described in section 2.3.3.9. (A BspHI site is situated at the ATG initiation codon of the synthetic gene, and a BglII site just following the termination codon, so providing the coding sequence on a 28 bp fragment - figure 6.7). For figures A, B, C and D: lane 1, 0 Cd (control); lanes 2-6, 5 μM Cd for 2, 5, 15, 30 and 60 min respectively.


A
Origin
1 2 3 4 5 6
rRNA
bands
B
Origin
1 2 3 4 5 6
rRNA
bands
C
Origin
1 2 3 4 5 6
2.95Kb
2.5Kb
1.5Kb
0.5Kb
D
Origin
1 2 3 4 5 6
2.95Kb
2.5Kb
1.5Kb
0.5Kb

was specific to the coding sequence of the synthetic gene (preparation of the probe for this analysis is described in the figure legend). As shown in figure 6.11 (C), the probe hybridised to a sequence corresponding to the expected size of the transcript which would be produced by transcription of the gene in pSVSG2-containing cells. By reference to the cloning strategy as presented in figure 6.8, it can be calculated that this transcript should be 49 bases smaller than the endogenous smtA transcript, giving 229 bases in total. Northern analysis of RNA from pSVSG1-containing cells failed to show any hybridisation to the same probe. Subsequent washing of both blots to background levels (less than 5 cps), and hybridisation to a probe specific to the smtA coding sequence, resulted in the appearance of the expected bands at the correct size for the smtA transcript (shown for gel B; figure 6.11, D). The results also demonstrate that synthetic gene-specific transcripts increased in abundance in response to administration of Cd in cells containing pSVSG2 but not in cells containing pSVSG1. The reason why transcripts were not detected in cells containing plasmid pSVSG1 is not known. The subsequent analysis for smtA transcripts in these cells, which indicated normal transcription of the smtA gene in response to Cd, shows that the Cd exposure and resultant northern blot should have given transcription levels of the synthetic gene similar to those seen in cells containing pSVSG2. The most likely explanation of the lack of such transcripts is that the mRNA produced from these constructs was much less stable than pSVSG2 transcripts.

The rate of mRNA turnover in both eukaryotic and prokaryotic cells is highly variable, the half-life of *E. coli* transcripts, for example varying from between 20 seconds to 40 minutes. The half-life of a particular mRNA species relates to the degree to which the molecule is protected from the cellular mRNases which are responsible for RNA turnover. Both endo- and exonucleases are responsible for this process in bacterial cells. Several features of the RNA molecule determine its susceptibility to nuclease degradation. One of the major factors in protection of mRNA from exonucleolytic degradation has been found to be the stem-loop structure, which also functions as the transcript termination signal (Chen et al 1988). A number of other factors, such as specific endonucleolytic attack (Belasco et al 1986) and the rate

of mRNA translation (Cole and Nomura 1986), have been shown to have roles in the rate of mRNA decay. The importance of each of these factors appears to vary according to particular mRNA species and the organism concerned (reviewed by Belasco and Higgins 1988).

In relation to the effect on message stability of different sequences used as transcript terminators in this report, it is possible to hypothesise that any of the aforementioned features, could have affected the stability of the mRNA molecule produced from clone pSVSG1. The 3' stem-loop structures are similar in terms of the length of the stem structure, and so there is likely to be minimal difference in transcript half-life due to this feature. Other factors, such as the length of the mRNA produced, and different sequence specificity for endonuclease attack may have decreased the half-life of the transcript produced from pSVSG1. Conversely, the transcript from pSVSG2, is very similar to the natural mRNA produced by transcription of *smt*A and may form similar secondary structure to the *smt*A transcript. This would be likely to provide the molecule with maximal protection from endonuclease digestion.

Certain features of eukaryotic mRNA molecules have been shown to have a role in allowing alteration of transcript half-life in response to external physiological stimuli. Best characterised in this regard are the genes involved in Fe uptake and storage in mammalian cells, in which both the translation of mRNA encoding ferritin (which is concerned with intracellular Fe transport and storage) and the stability of the transcript encoding the transferrin receptor (involved in Fe uptake) are both modulated by Fe levels. Transferrin receptor mRNAs are relatively stable in normal Fe levels, are highly stable in Fe-depleted cells, and are rapidly degraded when Fe levels are high (reviewed by Raghow 1987). The observed effect is due to the presence of a stem-loop structure in a non-translated region of the transcript, which is responsible for the rapid turnover of the transcripts in the presence of Fe. A protein which binds to this region in the absence of Fe causing increased stability of the transcript has reduced affinity in the presence of Fe, resulting in a rapid increase in mRNA degradation. Several other systems have been partially characterised in which altered physiological conditions affect transcript stability. Aida and Negishi (1991) demonstrated that coumarin-7-hydroxylase transcripts were stabilised by treatment of

mice with pyrazole, though the mode of action has yet to be investigated. Similarly, alteration in transcript stability in response to physiological stimuli has been demonstrated to occur in casein (Guyette et al 1979) and vitellogenin (Brock and Shapiro 1983) genes.

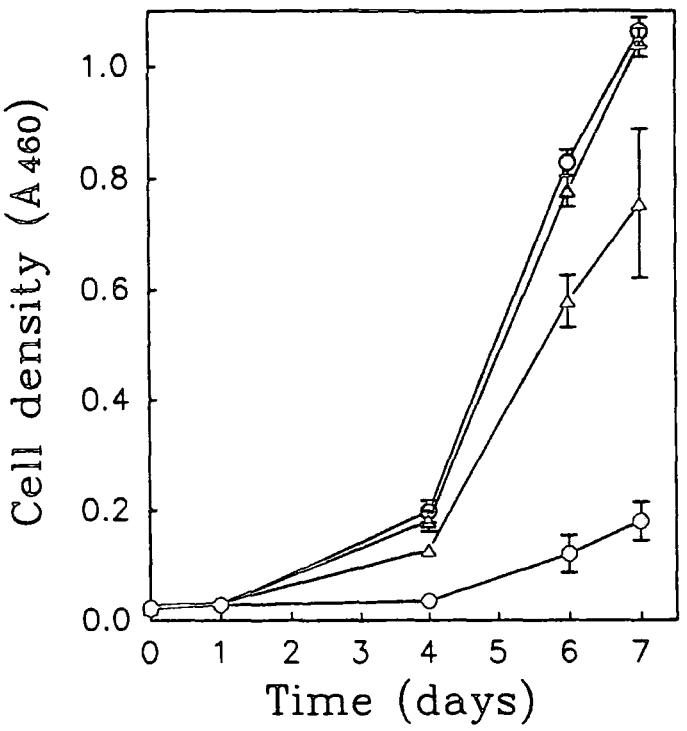
It is possible that the stability of *smt*A transcripts may be affected by metal ions, thus partially explaining the difference in amounts of synthetic gene-specific transcripts detected in cells containing pSVSG1 (which does not have *smt* 3' sequences) and pSVSG2 (from which the transcripts contain most of the normal *smt* sequences). The rapid increase in transcript abundance in response to the presence of metal ions, however, strongly indicates that the primary mechanism of *smt*A activation is at the level of transcriptional activation of the gene, and most recent results utilising constructs containing *lacZ* sequences fused to *smt*A sequences indicate transcriptional activation of the gene (J.W. Huckle pers. commun.). Experiments are currently being performed in order to elucidate whether or not increased message stability may have a secondary effect in the response of *smt*A to the presence of metal ions.

6.3.5 Analysis of phenotypic effects of the synthetic gene construct in Synechococcal cells.

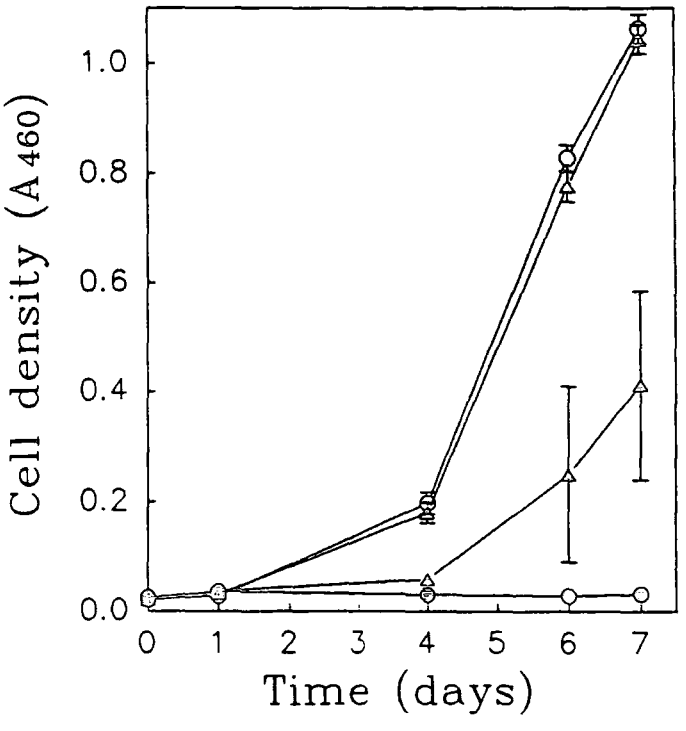
Figure 6.12 shows the results of Cd-tolerance testing of Synechococcal (PIM8) cells containing pSVSG2 compared to cells containing pUC105 (control). Tests were performed at both the minimal concentration causing cell death in control cells (5.5 μM), and a higher concentration which did not completely inhibit cell growth (4.5 μM; concentrations were chosen, as for *E. coli* cells, by growth of cells in a range of concentrations to determine inhibitory levels). This experiment was performed only once (in triplicate), due to time constraints, therefore final conclusions regarding the effectiveness of the synthetic gene in conferring Cd-resistance in Synechococcal cells await further investigation. The results of this preliminary investigation, however indicate that the cells containing pSVSG2 were significantly more tolerant to the toxic effects of Cd than control cells, consistent with the observed effect in *E. coli*. No effect of expression of the synthetic gene on Cd accumulation by Synechococcal cells was observed, which is consistent with the observed effect of

Figure 6.12 Growth of *Synechococcus* PCC7942 (PIM8) cells containing either plasmid pSVSG2 or pUC105 in Cd. Growth of cells containing pSVSG2 and pUC105 in either 4.5 (graph A) or 5.5 μM (graph B) Cd was compared using methods outlined in section 6.2.2.8. (Symbols ○ - cells containing pUC105 without Cd; ○ - cells containing pUC105 with Cd; Δ - cells containing pSVSG2 without Cd; △ cells containing pSVSG2 with Cd).

A


Cell density (A 460)
1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4 5 6 7
Time (days)

B


Cell density (A 460)
1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4 5 6 7
Time (days)

the gene in *E. coli*. Two experiments, comprising three replicate cultures, gave values of 7.52 ($^{+}$/_0.31) pmoles/ODU and 7.83 ($^{+}$/_0.18) pmoles/ODU for cells containing pUC105 and pSVSG2 respectively.

There is a clear indication of an effect on Cd tolerance of the expression of αPC_3 in Synechococcal cells. Cd accumulation was not significantly altered, although measured against the background of endogenous *smt*A induction, the contributory effect, if any, of the product of the synthetic gene may be obscured. Recently, *smt*A-deficient mutants have been produced. Further studies may therefore be carried out using this host organism in order to determine whether the constructs described in this report are capable of complementing the functions of *smt*A.

CHAPTER 7 GENERAL DISCUSSION.

The metal binding characteristics of the different ligands which have been expressed in *E. coli* and Synechococcal cells are compared and contrasted in this chapter.

Expression of these molecules in *E. coli* and *Synechococcus* PCC7942 (PIM8) presented seemingly anomalous results in relation to alterations in metal-accumulation and -tolerance observed in *E. coli* and Synechococcal cells. Factors which may be of importance in determining the observed phenotypes of these cells are therefore addressed.

A final summary of the work and of the main conclusions is presented in section 7.4.

7.1 Metal coordination characteristics of SmtA- and $PsMT_A$-fusion proteins.

The order of metal affinities exhibited by both SmtA and $PsMT_A$ (Cu>Cd>Zn), as described in previous chapters, is the same as that observed for mammalian MT. This order of metal-affinity in MT-like molecules is attributed to the affinity of these metals for sulphydryl groups, for which the order of metal binding is identical (Nielson and Winge 1983; Gurd and Wilcox 1956). In mammalian MT, and other MT-like molecules, the observed high affinity of the protein for metal is attributed to cluster formation, which implies chelation (bridging of metal ions between two or more liganding atoms of the same molecule). The formation of chelate metal complexes results in a much higher affinity for metal ions than would result from single, non-chelate, metal/ligand associations (Gurd and Wilcox 1956). The results of *in vitro* analysis of the fusion proteins produced by the expression of *smt*A (chapter 3) and *PsMT*$_A$ (appendix 1) demonstrate that these molecules have affinities for Cu, Cd and Zn, which are comparable with the affinities of known MTs. Therefore it is likely that both SmtA and $PsMT_A$ exhibit chelate metal-ligand associations, possibly involving cluster formation.

Spectral analysis of Synechococcal MT (Olafson et al 1988) indicated that a metal-cluster could potentially exist in this protein, although the manner in which the protein could fold to accommodate such a cluster could not be predicted by computer

alogorithm of 2° from 1° protein structure. In the case of $PsMT_A$, two distinct types of cluster may be proposed. The two cysteine-rich domains of the protein could potentially coordinate metal ions separately, or, as has been proposed earlier (section 4.3.4.1) the protein could form a single domain, potentially with digestion of the linker segment of the molecule. The current data is insufficient to support either model, and results of NMR analyses are therefore awaited to resolve the 2° structure of the molecule.

7.2 Metal-accumulation and -tolerance in *E. coli*.

It is noted that the order of binding affinity described in section 7.1 is not reflected in the degree of accumulation of each of these metals in *E. coli* cells expressing $PsMT_A$ and *smt*A (chapters 3 and 4; summarised in figure 7.1).

Additionally, despite an increase in accumulation of metals, indicating metal ion coordination, no enhancement of metal tolerance was observed in cells expressing $PsMT_A$ or *smt*A. Cd tolerance was observed, however, in cells expressing the PC analogue $(\alpha PC)_3$. The factors which may influence the observed metal tolerance phenotypes are discussed in section 7.2.2.

7.2.1 Factors affecting metal accumulation in *E. coli*.

Several factors may be proposed to influence metal accumulation in *E. coli* cells expressing heterologous ligands. These may be summarised as follows:

1) The affinity of the expressed protein for a particular metal ion, relative to other cellular ligands.
2) The mechanism of metal ion uptake and its regulation.
3) The degree to which production of the ligand in *E. coli* is coupled to intracellular levels of metal ions.

7.2.1.1 The metal-affinity of the ligand.

Table 7.1 provides a comparison of the affinities of SmtA, $PsMT_A$ and equine renal MT for Cd, Cu and Zn, in terms of pH of half dissociation from the GST- fusion proteins described in previous chapters and from commercially supplied equine renal MT. It is noted from examination of these data that whereas, relative to the other ligands, $PsMT_A$ has the lowest pH of half dissociation and hence the highest affinity for Cu,

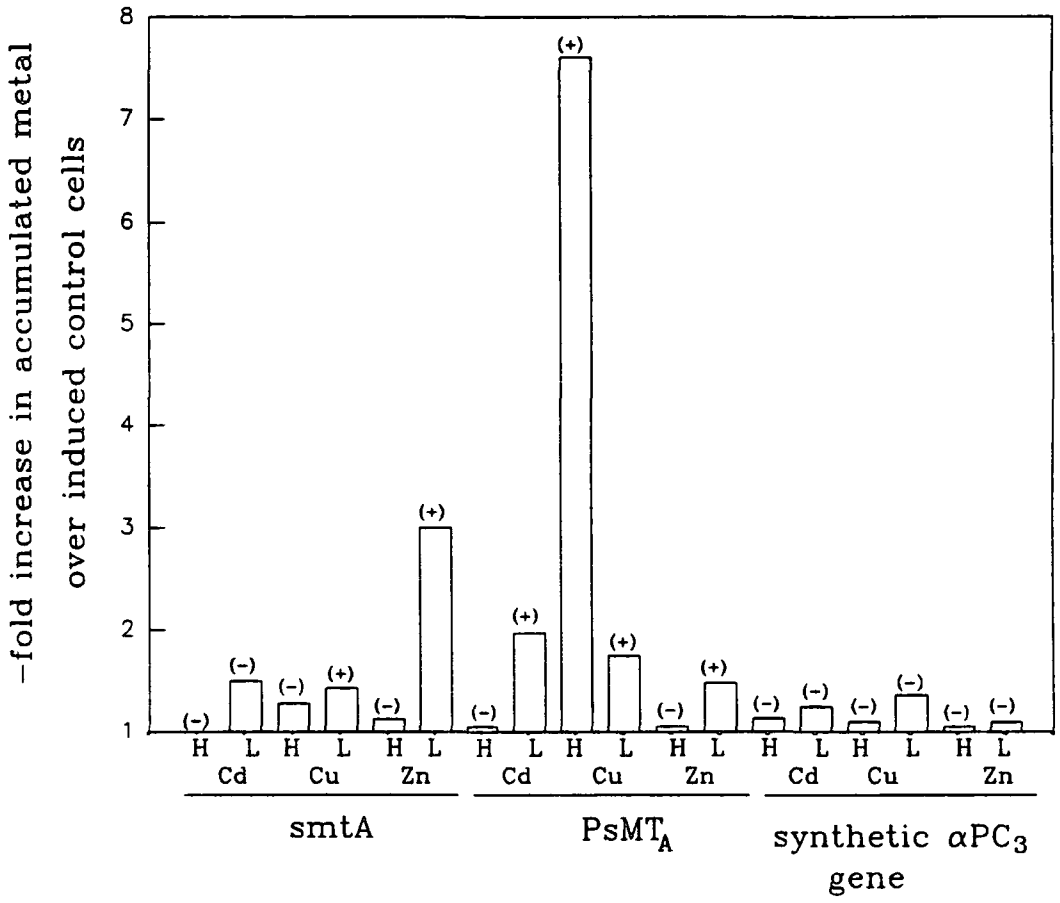
SmtA has the lowest pH of half dissociation and hence the highest affinity for Zn.

	Metal ion		
Ligand	Zn	Cd	Cu
SmtA[a]	4.10	3.50	2.15
$PsMT_A$[b]	5.35	3.95	1.46
equine MT[a]	4.50	3.00	1.80

Table 7.1 Estimated pH of half dissociation of Zn, Cd and Cu from SmtA, $PsMT_A$ and equine MT.
(a) from chapter 3; (b) appendix 1.

The data presented in figure 7.1, reveal that at both concentrations of Cu, expression of $PsMT_A$ resulted in higher accumulation of Cu than did expression of *smt*A. No significant increase in Zn accumulation was observed by cells expressing either ligand when cells were exposed to high Zn concentrations. Cells expressing *smt*A exhibited greater accumulation of Zn than did cells expressing *$PsMT_A$* when cells were exposed to lower concentrations of Zn. These data suggest a correlation between the affinity of the proteins, as presented in table 7.1, and metal accumulation by *E. coli* expressing these ligands. It is noted, however, that the concentration of metal to which the cells were exposed had an influence on the degree of accumulation. Additionally, accumulation of Cd, for which SmtA was shown to have a higher affinity than $PsMT_A$, was higher in cells expressing *$PsMT_A$* than in cells expressing *smt*A. Thus, although the data suggest that the affinity of the ligand is of importance in determining metal accumulation, other factors must also influence the degree to which this effect is observed. The metal-affinities of heterologous ligands may affect accumulation of metals by expressing cells via competition with endogenous ligands. Sequestration of essential metals, such as Zn and Cu from molecules involved in the homoeostatic control of these ions may cause the activation of transport mechanisms, and hence result in increased metal accumulation. A high affinity relative to such molecules could therefore be of importance in determining the degree to which the

Figure 7.1 Comparison of metal accumulation by *E. coli* expressing *smtA*, *PsMTA* and a synthetic gene encoding αPC_3. The data presented in previous chapters are summarised in bar-chart form. H and L on the X-axis refer to high and low metal exposure respectively (as defined in previous chapters). Symbols: (-) - no statistically significant increase in metal accumulated relative to control cells (for mean and standard deviation for nine replicates); (+) - statistically significant increase.


-fold increase in accumulated metal over induced control cells
8
7
6
5
4
3
2
1
(-)
(-)
(-)
(+)
(-)
(+)
(-)
(+)
(-)
(+)
(+)
(-)
(+)
(-)
(-)
(-)
(-)
(-)
H L H L H L H L H L H L H L H L H L H L H L
Cd Cu Zn Cd Cu Zn Cd Cu Zn
smtA
PsMT$_A$
synthetic αPC$_3$ gene

metal is accumulated. Thus, although, for example, SmtA appears to have a higher affinity for Cu than for Zn, it may not compete for Cu with endogenous ligands.

7.2.1.2 The mechanism of metal ion uptake.

The mechanism of metal ion uptake may influence the degree to which a metal will be accumulated by *E. coli* cells expressing a heterologous ligand. Relatively little is known, however, concerning the mechanisms of uptake of metals such as Cu, Zn or Cd into *E. coli* cells (discussed in section 1.8). It has been demonstrated that uptake of Cd into the cytoplasm of *E. coli* cells represents only a small percentage of the total Cd associated with Cd-exposed cells. Mitra et al (1975) reported that 2 % of Cd was associated with cell walls of non-accomodated *E. coli* cells, 75 % was associated with the cell membrane fraction, and 23 % with the cytoplasm, when cells were exposed to Cd. This indicates that in these cells much of the Cd does not reach the cytoplasm. Conversely, in pre-adapted cells the amount altered to 56 %, 13 % and 31 % for the cell wall, membrane and cytoplasm respectively.

The mechanisms involved in the regulation of metal uptake may also influence metal accumulation by cells expressing heterologous metal-ligands. As discussed in section 7.2.1.1, disruption of normal homoeostatic mechanisms may occur due to competition for metal ions between the heterologous ligand and endogenous ligands. Molecules involved in "sensing" the internal metal ion concentration, and thereby controlling uptake, may no longer be in contact with the metal ion. Such a situation would result in increased metal uptake. Currently, there is little information available concerning the molecules which control the uptake and storage of essential metals such as Cu and Zn in *E. coli*, though such controls are presumed to exist (Brown et al 1992).

7.2.1.3 Ligand/metal coupling.

Several reports have demonstrated that expression of a metal ligand, uncoupled from cellular metal ion concentration, can induce metal accumulation in various systems (cited in section 1.8). There are no reports, however, directly comparing metal accumulation in a system in which ligand expression is uncoupled (constitutive) to one in which induction of the ligand is coupled to metal exposure (via metal-

induced transcription). There have been reports, however, confirming that correct coupling of ligand expression to metal ion concentration may not necessarily result in increased accumulation, relative to non-ligand-expressing cells.

Lin and Kosman (1990) examined Cu uptake and accumulation in *S. cerevisiae* cells which were deleted in CUP1, compared to wild-type (wt) cells. It was noted in this study that for cells pre-grown in non-copper-supplemented medium, and subsequently exposed to 10 μM Cu, there was no difference in Cu accumulation in wt relative to CUP1-deleted cells. Growth of cells in 10 μM Cu following pre-growth in 5 μM Cu resulted in a short period of rapid Cu-exchange (isotope exchange between ^{64}Cu and "cold" Cu) during which the net Cu uptake in both cell lines was similar. The net accumulation of Cu was not affected by the CUP1 gene in any of the experiments presented. It is possible therefore, that the close coupling of CUP1 induction to intracellular Cu levels, or the manner by which the protein associates with the ion, avoids excess cellular accumulation of Cu.

Jackson et al (1984), selected *D. innoxia* cell cultures for Cd resistance. A difference between sensitive and tolerant cell lines was subsequently shown to be enhanced Cd-PC complex formation in tolerant cells relative to sensitive cells (although the level of total PC induction [Cd-PC + apo-PC] was similar in each case [Delhaize et al 1989]). It was also found that Cd accumulation was identical for both cell lines. This may further demonstrate that in a system where ligand synthesis and metal ion concentration are coupled, metal exposure may not necessarily result in increased metal accumulation in cells in which the ion associates with that ligand compared to cells in which less metal-liganding is observed.

7.2.2 Factors affecting metal tolerance of cells expressing metal-ligands.

As discussed above, cells expressing heterologous metal-ligands, although exhibiting increased metal accumulation, do not necessarily exhibit increased levels of tolerance to these ions. Conversely, in some cases, cells expressing heterologous ligands do not exhibit increased accumulation but do exhibit increased tolerance to certain metal ions. Factors affecting tolerance toward different metal ions in such cells may be summarised as follows:

1) The high level of endogenous resistance to certain metal ions observed in *E. coli* cells.
2) The site of metal ion toxicity.
3) The metal-affinity of the ligand.
4) The degree of uncoupling of ligand from metal ion concentration.

7.2.2.1 Metal tolerance in *E. coli*.

As previously discussed, *E. coli* have a high natural tolerance to certain metal ions, although the nature of the mechanisms of metal tolerance and homoeostasis are not well defined (section 1.8). This high background level of metal tolerance may be one factor contributing to the fact that in the majority of reports examining the effect of MTs in *E. coli* cells, no alteration in tolerance has been observed. It was observed in this thesis and in a report by Hou et al (1988), however, that by defining the inhibitory concentrations of metal ions, alterations in tolerance could be observed in cells expressing heterologous ligands. A number of other factors may therefore play a role in determining whether or not the metal tolerance phenotype is altered by expression of a metal ligand.

7.2.2.2 The site of metal ion toxicity.

As described in section 7.2.1.2 concerning Cd accumulation by *E. coli*, a large amount of Cd is associated with the membrane fraction when cells are exposed to this ion, and it is conceivable that Cd exerts a toxic effect at this site. Whether or not a (presumably) cytoplasmic heterologous ligand enhances metal tolerance via sequestration of metal ions will depend upon the major site at which the metal exerts its toxic effect. The mechanism of toxicity (binding to cellular ligands, destabilisation of transport mechanisms, alterations in membrane permeability or involvement in redox reactions) will also influence the effectiveness of the ligand in enhancing tolerance.

7.2.2.3 Ligand metal-binding affinity.

In order to protect sensitive sites from metals, a ligand must have a sufficiently high affinity to allow successful competition with sensitive sites for binding to the metal ion. Conversely, competition with endogenous ligands for essential metals could be deleterious.

7.2.2.4 Metal/ligand coupling.

Kay et al (1991) note that whereas a high level of expression of MT molecules in *E. coli* did not enhance metal tolerance (Berka et al 1988; Kille et al 1990), much lower expression of MT in *E. coli* resulted in significant increases in tolerance toward a range of metal ions (Hou et al 1988). Similarly, the data presented in this thesis revealed that a low level of expression of aPC_3 (as indicated by northern analysis) resulted in increased tolerance toward Cd, whereas higher levels of expression of $PsMT_A$ and *smt*A did not affect metal tolerance. These data indicate that the level of expression of a heterologous ligand in *E. coli* may be a critical factor in determining the phenotype of cells expressing the ligand. This may relate to the degree to which the production of the ligand is coupled to intracellular metal ion concentrations. In Synechococcal cells in which production of αPC_3 was coupled to the intracellular concentration of Cd, tolerance toward Cd was also observed.

One report has examined alteration in metal tolerance brought about by the expression of a metal ligand in a coupled, as opposed to an uncoupled system. Silar and Wegnez (1990) examined Cu tolerance in CUP1-deleted *S. cerevisiae* cells expressing the *Drosophila melanogaster* MT gene (MTn), using both an uncoupled (constitutive) expression system, and a coupled (Cu-induced) system (using CUP1 *cis* elements). These authors reported that whereas growth of constitutively-expressing cells was inhibited at 250 μM Cu, Cu-induced cells survived up to 1 mM Cu. This was despite the observation that the level of transcript abundance (as measured by northern analysis) was much higher in constitutively-expressing cells compared to Cu-induced cells when both were exposed to 250 μM Cu. Thus it is apparent that correct coupling of MT expression in this system was much more effective in achieving a Cu-resistance phenotype than was uncoupled expression of the gene.

7.3 Summary: Phenotypic effects of the expression of heterologous metal-ligands in prokaryotic cells.

The results of this study, and of other reports as cited above, reveal the various factors which determine the phenotypic effects of expression of metal-ligands in microbial cells. Uncoupled expression of a metal-liganding molecule may actively promote metal uptake and

accumulation, without having an effect on metal tolerance, as observed in several reports (section 1.8). Coupled expression of a ligand may not significantly affect metal uptake, while providing protection from intracellular metal ions (section 7.2.1.3; section 7.2.2.4). It may be proposed that in an uncoupled system, a high number of available metal-liganding atoms may "scavenge" cellular metal ions, causing the activation of metal transport systems, and hence increased influx of metal ions. Uptake of specific ions may be affected by the affinities of the heterologous ligand, and of endogenous ligands for that ion. Subsequent binding of a proportion of the ions entering the cell will not necessarily provide protection for the cell for reasons as outlined above. Conversely, coupling of ligand production to intracellular metal ion concentration may allow binding of those ions inside the cell, without necessarily promoting the influx of more metal ions. Such an hypothesis is consistent with the results presented in this thesis regarding metal accumulation and tolerance in *E. coli* and *Synechococcus* PCC7942 (PIM8) expressing heterologous ligands.

7.4 Summary and conclusions.

The following is a summary of the main findings of the research presented in previous chapters and of the conclusions which may be drawn.

7.4.1 Expression of *smtA* in *E. coli*.

1) SmtA, expressed in *E. coli* as a fusion protein, could subsequently be isolated in association with metal ions (Hg, Cd, Cu, Zn) when cells were grown in medium supplemented with these ions.

2) The metal-loaded protein, when isolated from *E. coli* and analysed *in vitro* was found to bind metals with affinities of the order Hg>Cu>Cd>Zn>Ni,Cr,Co.

3) pH displacement of metals from the protein *in vitro* gave pH values of 4.10, 3.50 and 2.15 for half dissociation of Zn, Cd and Cu respectively. This revealed a high affinity for Zn relative to the other molecules which were studied.

4) The relative affinities of the protein for metal ions (Zn>Cd,Cu) was reflected in the degree to which *E. coli* expressing GST-SmtA fusion protein accumulated metals from growth medium, although the

amount of metal accumulated was dependent on the level to which the cells were exposed.

5) IPTG-induced expression of *smt*A did not alter the ability of *E. coli* cells to tolerate toxic concentrations of metal ions (Cu, Cd, Zn).

7.4.2 Expression of $PsMT_A$ in *E. coli*.

1) *E. coli* cells expressing $PsMT_A$ accumulated metals (Cu, Cd, Zn) from medium supplemented with these ions, suggesting metal binding *in vivo*.

2) The relative metal-affinities of the protein (Cu>Cd,Zn) was reflected in the degree to which these ions were accumulated from growth medium, although this was dependent upon the concentration of metal to which these cells were exposed.

3) No effect on metal tolerance was observed in *E. coli* as a result of IPTG-induced expression of $PsMT_A$.

7.4.3 Expression of αPC_3 in *E. coli*.

1) Initial attempts to create a construct for expression of this molecule resulted in rearrangements and mutations in the gene and surrounding DNA sequence. This indicated that the gene product may have exerted a toxic effect on *E. coli* cells due to a relatively high level of background (non-IPTG-induced) expression from the *lac*Z promoter during cloning.

2) Subsequent cloning of the gene encoding αPC_3 in a heat-inducible system was successful, and heat-induction of transcripts encoding αPC_3 was achieved.

3) Association of ^{35}S (believed to be incorporated into ^{35}S-labelled αPC_3) with endogenous proteins was observed when the gene encoding this molecule was expressed at a high level.

4) Expression of αPC_3 at low levels in *E. coli* resulted in increased tolerance of *E. coli* cells toward Cd.

5) No consistently observed increase in metal accumulation (Cu, Cd, Zn) was observed via the expression of αPC_3 in *E. coli*.

7.4.4 Expression of αPC_3 in *Synechococcus* PCC7942 (PIM8).

1) Fusion to putative *cis*-acting regulatory sequences derived from the Synechococcal gene *smt*A conferred Cd-inducible increases in the abundance of transcripts of a gene encoding αPC.

2) Fusion of 3' sequences derived from the *smt*A gene 3' to sequences encoding αPC_3 resulted in increased transcript stability, relative to constructs which utilised an alternative transcript terminator.
3) Increased Cd tolerance was observed in Synechococcal cells expressing the gene encoding αPC_3.
4) No increase in Cd accumulation was observed in Synechococcal cells expressing the gene encoding αPC_3.

7.4.5 Concluding remarks.

The main conclusions inferred by the results described above are as follows:
1) Both of the recombinant proteins produced as a result of expression of the *smt*A and *PsMT*A genes exhibit metal binding qualities which are consistent with the classification of these genes as metallothionein genes.
2) SmtA, having a high relative affinity for Zn, may have evolved primarily as a Zn-thionein. Association of the protein with Cd, Cu and Hg, also indicates a possible role in homoeostasis and/or detoxification of Cu, and in detoxification of Cd and Hg.
3) The high affinity for Cu, relative to the other molecules studied, of the product of the $PsMT_A$ gene indicates a possible role for this protein in Cu homoeostasis.
4) Increased metal tolerance in both *E. coli* and Synechococcal cells expressing αPC_3, is consistent with the hypothesis that PC molecules have a role in detoxification of Cd.
5) Sequences derived from the *smt*A gene were shown to confer Cd-inducible expression of αPC_3 fused to these sequences.

Additionally, the results of this study indicate that the level of expression of metal ligands, and coupling of expression of such ligands to intracellular metal ion concentrations, may be a critical feature in determining whether or not metal-tolerance or -accumulation is observed.

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