Functional analysis of the prokaryotic metallothionein locus, smt

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"Functional Analysis of the Prokaryotic Metallothionein Locus, *smt*"

by

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ABSTRACT

"Functional Analysis of the Prokaryotic Metallothionein Locus, *smt*"

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The localisation of the prokaryotic metallothionein (MT) divergon *smt* (which includes the MT gene *smtA* and a divergently transcribed gene *smtB*) was examined, and *smt* deficient mutants of *Synechococcus* PCC 7942 (strain R2-PIM8) have been generated by insertional inactivation/partial gene deletion mediated by homologous recombination. The structure and homozygosity (of the *smt* region) of these mutants, designated R2-PIM8(*smt*), was confirmed by Southern analyses and plasmid recovery in *Escherichia coli* (involving the generation of a ca. 7.8 kb plasmid from *Sal*I digested R2-PIM8(*smt*) DNA). Furthermore, *smtA* transcripts were not detected in R2-PIM8(*smt*) RNA.

Viability of R2-PIM8(*smt*) reveals that *smt* performs no essential role in *Synechococcus* under these culture conditions. R2-PIM8(*smt*) has reduced tolerance to Zn^{2+} and Cd^{2+}, and short term reduced resistance to Ag^{+}. Restoration of Zn^{2+} tolerance was used as a phenotypic selection to isolate recombinants derived from R2-PIM8(*smt*) after reintroduction of a linear DNA fragment containing an uninterrupted *smt* divergon. These *smt*-restored cells also exhibited restored Cd^{2+} tolerance. Hypersensitivity to Cu^{2+} or Hg^{2+} was not detected in R2-PIM8(*smt*) indicating independence of Cu^{2+} and Hg^{2+} resistance to *smt*-mediated metal tolerance.

Sequences upstream of *smtA* (including *smtB* and/or the *smt* operator-promoter) fused to a promoterless *lacZ*, conferred metal-dependent β-galactosidase expression in R2-PIM8. At maximum permissive concentrations for growth, β-galactosidase assays revealed Zn^{2+} to be a more potent elicitor of metal-dependent expression from the *smtA* operator-promoter than Cd^{2+}. Equivalent experiments, in R2-PIM8(*smt*) and R2-PIM8(*smtA+/B-*) (containing functional chromosomal *smtA* and non-functional chromosomal *smtB*), revealed that *smtB* encodes a repressor of *smtA* transcription. In addition, it is demonstrated that SmtB can act in *trans*. It is proposed that Zn^{2+} is the most potent (metal ion) inducer of SmtB mediated derepression of *smtA* transcription. Furthermore, β-galactosidase assays indicated that, in addition to SmtB, other regulatory elements (including a transcriptional activator) are involved in the regulation of expression from the *smt* operator-promoter.

Restoration of Zn^{2+} tolerance was also used as a phenotypic selection to isolate recombinants derived from R2-PIM8(*smt*) after reintroduction of a linear DNA fragment, containing functional *smtA* and non-functional *smtB*. The resulting transformants, R2-PIM8(*smtA+/B-*), exhibited increased (early) tolerance to Zn^{2+} and Cd^{2+} as compared to R2-PIM8(*smt-* reintroduced) (equivalent to R2-PIM8).

The work presented in this thesis proposes a role for SmtA in Zn^{2+} homoeostasis/metabolism and Cd^{2+} detoxification. SmtB is confirmed to be a *trans*-acting inducer- (metal ion) responsive negative regulator of *smtA*. The phenotype of R2-PIM8(*smtA+/B-*) (with respect to metal tolerance) has significance regarding previous work (Gupta *et al.*, 1993. Molecular Microbiology 7, 189-195), in which analysis of the *smt* region of *Synechococcus* PCC 6301 cells selected for Cd^{2+} resistance, by stepwise adaptation, revealed the functional deletion of *smtB*. It was proposed that loss of *smtB* may be beneficial for continuously metal challenged cells. Loss of *smtB*, now shown to encode a repressor of *smtA* transcription, is shown to confer constitutive derepressed expression from the *smtA* operator-promoter and determine an (early) increase in metal (Zn^{2+}/Cd^{2+}) tolerance.

MEMORANDUM

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

Turner, J.S., Morby, A.P., Whitton, B.A., Gupta, A. and Robinson, N.J. 1993. Construction and characterisation of Zn^{2+}/Cd^{2+} hypersensitive cyanobacterial mutants lacking a functional metallothionein locus. The Journal of Biological Chemistry **268**, 4494-4498.

Huckle, J.W., Morby, A.P., Turner, J.S. and Robinson, N.J. 1993. Isolation of a prokaryotic metallothionein locus and analysis of transcriptional control by trace metal ions. Molecular Microbiology **7**, 177-188.

Morby, A.P., Turner, J.S., Huckle, J.W. and Robinson, N.J. 1993. SmtB is a metal-dependent repressor of the cyanobacterial metallothionein gene *smtA*: identification of a Zn inhibited DNA-protein complex. Nucleic Acids Research **21**, 921-925.

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

AAS	atomic absorption spectrophotometry;
bla	β-lactamase gene;
bp	base pair(s);
BSO	buthionine sulphoximine;
cat	chloramphenicol acetyl transferase gene;
Cd^r	Cd^{2+} resistant;
Cm^r	chloramphenicol resistant;
Cm^s	chloramphenicol sensitive;
Cu^r	Cu^{2+} resistant;
°	degrees;
°C	degrees Celsius;
dCTP	2'-deoxycytidine-5'-triphosphate;
DMSO	dimethyl sulfoxide;
DNA	deoxyribonucleic acid;
EDTA	ethylenediaminetetra-acetic acid (disodium salt);
g	gravity(s);
g	gram(s);
mg	milligram(s);
µg	microgram(s);
GRE	glucocorticoid-responsive element;
GSH	glutathione;
IPTG	isopropylthio-β-D-galactoside;
kb	kilo base(s);
l	litre(s);
ml	millilitre(s);
µl	microlitre(s);
LB	Luria-Bertani;
M	molar;
mM	millimolar;
µM	micromolar;
µm	micrometre(s);
nm	nanometre(s);
MRE	metal-responsive element;
MT	metallothionein;
MTs	metallothioneins;
NAD(P)	nicotinamide adenine dinucleotide (phosphate);
ONPG	o-nitrophenyl-β-D-galactopyranoside;
ORF(s)	open reading frame(s);

PPi	pyrophosphate;
PCR	polymerase chain reaction;
%	percent;
pH	hydrogen ion potential;
r_{ave}	average radius;
R2-PIM8	*Synechococcus* PCC 7942 (strain R2-PIM8).
RNA	ribonucleic acid;
SDS	sodium dodecyl sulphate;
Tris	tris(hydroxymethyl)methylamine;
v/v	volume for volume;
w/v	weight for volume;
Xaa	an amino acid other than cysteine;
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactoside;
Zn^r	Zn^{2+} resistant.

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CHAPTER 1

INTRODUCTION

Cyanobacteria are widely distributed, surviving in both metal ion impoverished/limited and metal ion abundant environments (reviewed by Whitton, 1984; 1992). Furthermore, the concentration of metal ions in a particular environment may fluctuate (Whitton, 1984; 1992). Mechanisms of adaptation must therefore exist in cyanobacteria to deal with changing concentrations of such ions. Attention has been paid, at the molecular level, to the effect of iron and copper starvation in cyanobacteria (recently reviewed by Tandeau de Marsac and Houmard, 1993), but very little is known about the molecular mechanisms controlling "Zn^{2+} buffering" and cellular responses to high concentrations of trace metal ions.

Trace metal compounds enter the biosphere through human activity (including industrial processes and the use of pharmaceuticals) and to some extent natural weathering. Trace metal ions can be classified into two groups; those (which include Zn^{2+} and Cu^{2+}) that are required for the growth and maintenance of living organisms but are toxic in excess; and those (such as Cd^{2+}, Hg^{2+} and Ag^{+}) that are purely toxic and have no known beneficial biological function (although, Cd^{2+} has been observed to stimulate growth of a Zn^{2+} deficient marine diatom by substituting for Zn^{2+} in certain macromolecules (Price and Morel, 1990)). Organisms have evolved a number of mechanisms which protect against the deleterious effect of toxic metals but allow the accumulation of essential yet potentially toxic metals (Hamer and Winge, 1989; Silver, 1992).

In eukaryotes metallothioneins (MTs) are involved in cellular responses to elevated concentrations of certain metal ions, and have attracted interest from researchers involved in a wide range of disciplines. The isolation and properties of MTs are the subject of an extensive literature (Hamer, 1986; Kägi and Kojima, 1987; Kägi and Schäffer, 1988; Andrews, 1990; Riordan and Vallee, 1991). Despite this interest there is still considerable debate regarding the function of MTs, and proposed functions vary for the structurally distinct MTs in different organisms. The regulation of MT genes have

also attracted considerable interest primarily due to these genes being induced by high concentrations of metals. Prokaryotes are often more tractable for the analysis of gene function, and a prokaryotic MT gene had recently been isolated from a cyanobacterium at the time this research commenced.

This thesis describes the functional analysis of the MT of the cyanobacterium *Synechococcus* PCC 7942, and an examination of its transcriptional regulation.

1.1. EUKARYOTIC MT

MTs are cysteine rich metal binding proteins and polypeptides of low molecular weight. They are structurally diverse, some MTs are gene products, while others are secondary metabolites. A common feature of all MTs is an abundance of cysteine-Xaa-cysteine sequences (where Xaa is an amino acid other than cysteine), which are involved in binding metal ions in metal thiolate clusters. MTs lack histidine and aromatic acids, and their synthesis increases in organisms exposed to elevated concentrations of certain trace metal ions (Kägi and Kojima, 1987).

MT was first isolated from equine kidney cortex (Margoshes and Vallee, 1957; Kägi and Vallee, 1960) and has since been isolated from a variety of eukaryotes including vertebrates, invertebrates, plants and microorganisms (Hamer, 1986). A number of studies have indicated the presence of MT-like proteins in prokaryotes, however only one prokaryotic MT has been isolated and characterised (refer to section 1.3.).

1.1.1. MT classification

Taking into account structural relationships, MTs have been subdivided into three classes (Fowler *et al.*, 1987). Class I and II MTs are proteins encoded by structural genes. MT genes have been sequenced from a number of organisms including several animals (Karin and Richards, 1982), invertebrates (Nemer *et al.*, 1985; Marconi *et al.*, 1986; Mokdad *et al.*, 1987) and fungi (Butt *et al.*, 1984a; 1984b; Munger *et al.*, 1987; Mehra *et al.*, 1989; 1990). Recently a number of plant genes have been isolated which encode predicted proteins with sequence similarity to some class I and II MTs (Evans *et al.*, 1990; De Miranda *et al.*, 1990; De Framond, 1991; Kawashima *et al.*, 1991; Okumura *et al.*, 1991). Class III MTs are secondary metabolites and have been identified in higher plants (Grill *et al.*, 1987; Jackson *et al.*, 1987), eukaryotic algae (Gekeler *et al.*, 1988; Shaw *et al.*, 1989) and fungi (Murasugi *et al.*, 1981; Kondo *et al.*, 1985; Mehra *et al.*, 1988; Kneer *et al.*, 1992). The three classes of MT are defined:

Class I, proteins with locations of cysteine closely resembling those of equine renal MT;

Class II, proteins with locations of cysteine only distantly related to those in equine renal MT;

Class III, atypical non-translationally synthesised metal thiolate polypeptides referred to as phytochelatin (Grill *et al.*, 1985), cadystin (Murasugi *et al.*, 1981; Kondo *et al.*, 1985), phytometallothionein (Rauser, 1987), γ-glutamyl metal binding peptide (Reese *et al.*, 1988) and poly(γ-glutamylcysteinyl)glycine (Robinson and Jackson, 1986; Jackson *et al.*, 1987).

1.1.2. MT structure and metal binding characteristics

Class I and II

All class I and II MTs characterised so far are single chain proteins. Mammalian MT is a 61 or 62 amino acid peptide containing 20 cysteines, 6 to 8 lysines, 7 to 10 serines, a single acetylated methionine at the amino-terminus and no aromatics or histidines. Chicken MT and sea urchin MTa contain 63 and 64 residues, respectively. Shorter chains are found in invertebrates and certain fungi (Kägi and Schäffer, 1988). Class I MTs correspond in the alignment of the cysteines along the chain. In mammals all 20 cysteines are invariant, and there is extensive sequence similarity with arthropod and certain fungal MTs. The basic residues lysine and arginine are also highly conserved in mammalian MT. No obvious sequence relationships are discernible among members of the class II MTs (Hamer, 1986).

All animal species examined, with the exception of chicken, contain two or more distinct MT isoforms which are readily resolved by ion exchange chromatography and grouped into two classes, designated MT-I and MT-II, differing at neutral pH by a single negative charge. In many cases each class consists of several different isoMTs which are designated MT-I_a, MT-I_b, MT-I_c, etc. As many as ten isoMT genes are expressed in humans, some of them tissue specifically. Classification of proteins as members of the

MT-I or MT-II class does not necessarily imply structural or functional homology, especially when comparing proteins from divergent species (Hamer, 1986; Kägi and Schäffer, 1988).

The metal content of purified MT is highly variable and depends on organism, tissue and history of metal exposure (Hamer, 1986; Kägi, 1991). In mammalian MTs 20 cysteine thiolates serve as ligands to 7 divalent metal ions; whereas in crab MTs there are 18 cysteine thiolates and 6 divalent metal ions (Kägi and Kojima, 1987). Besides divalent metal ions, Cu^{+} is often associated with the native protein, for which increased metal-to-protein ratios have been found (Winge, 1991). In most mammals, not exposed to experimental pretreatment, Zn^{2+} tends to prevail over Cd^{2+} and Cu^{+}. In mammalian and crab MTs the metal ions are contained in two distinct metal-thiolate clusters (cited in Kägi and Schäffer, 1988). In mammals the A cluster contains 11 cysteines, binds 4 atoms of Zn^{2+} or Cd^{2+}, or 5 to 6 atoms of Cu^{+}, and is contained within the carboxy-terminal α-domain extending from amino acid 31 to 61. The B cluster contains 9 cysteines, binds 3 atoms of Zn^{2+} or Cd^{2+}, or 6 atoms of Cu^{+}, and is contained in the amino-terminal β-domain extending from amino acid 1 to 30 (Hamer, 1986). In both clusters, the polypeptide chain makes 3 turns to spiral around the metal atoms. Zn^{2+} and Cd^{2+} first fill the A cluster then the B cluster, whereas Cu^{+} first fills the B cluster then the A cluster. The binding reaction is highly cooperative. The affinity of metal ions for the binding sites in mammalian MTs follow the order typical of thiolate complexes, i.e., $Hg^{2+} > Cu^{+}, Ag^{+}, Bi^{3+} > Cd^{2+} > Pb^{2+} > Zn^{2+} > Co^{2+} > Fe^{2+}$ (cited in Vasák, 1991).

Class I MTs have been isolated from two fungi *Neurospora crassa* and *Agaricus bisporus*. These two Cu^{+}-MTs are shorter than mammalian MTs, and the location of the cysteine residues match the location of the cysteine residues at the amino-terminus of mammalian MT. These two proteins correspond to the β-binding domain of mammalian MT, with the α-domain being absent (Nielson and Winge, 1984; Kägi and Kojima, 1987).

Class III

The class III MTs are often oligomeric structures made up of two or more polypeptide chains of variable length. Derived from glutathione (GSH), they are of the general

structure (γ-glutamylcysteine)$_n$Xaa, where n=2 to 11 (depending upon the source) and Xaa is most often glycine (Robinson, 1990).

Many metal ions (including Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+} and Ag^{+}) induce synthesis of (γ-glutamylcysteine)$_n$glycine (Rauser, 1990). However, *in vivo* binding has only been shown for Cd^{2+} and Cu^{+} with the virtual exclusion of all other inducing metal ions (Grill (1989) although, also reported Pb^{2+} and Zn^{2+} binding complexes in *Rauvolfia serpentina*) (Wagner, 1984; Steffens, 1990; Rauser, 1990). The metal ions are coordinated to a cluster containing several (γ-glutamylcysteine)$_n$glycine aggregates (Reese *et al.*, 1988). Two different forms of Cd^{2+}-(γ-glutamylcysteine)$_n$glycine are produced. One form contains acid labile S in the cluster, present as reduced sulphide (S^{2-}) (Murasugi *et al.*, 1983). Aggregates containing S^{2-} have both a higher affinity and capacity for Cd^{2+} (Reese and Winge, 1988). S^{2-} has not been identified as a component of isolated Cu^{+}-aggregates (Reese *et al.*, 1988).

1.1.3. MT function

The function of most MTs is not certain. A role proposed for MTs in all of the organisms in which they have been detected is the sequestration of excess amounts of certain metal ions. The specific metals sequestered by MTs vary for the structurally distinct proteins/polypeptides occurring in different organisms. Several lines of evidence also suggest other functions for some MTs, in particular those in higher eukaryotes where the inducing, and coordinated, metal ions include Zn^{2+}.

Class I and II

The metal binding properties of cytosolic MT account for resistance of cultured cells to metals that are sequestered by the protein. It has been demonstrated, using numerous cell lines, that elevations in MT correlate with the tolerated concentrations of metal ions, such as copper and Cd^{2+}, that induce and bind to MT (Templeton and Cherian, 1991).

The ability of MT to act as a Cd^{2+} detoxifying agent in cultured mammalian cells has been demonstrated. Cell lines that fail to produce MT are unusually sensitive to Cd^{2+}

(Compere and Palmiter, 1981), whereas cell lines selected for Cd^{2+} resistance overproduce MT (at least in part) due to gene amplification (Durnam and Palmiter, 1984; Crawford *et al.*, 1985). These Cd^{2+} resistant (Cd^r) cell lines have also shown increased resistance to Zn^{2+}, Hg^{2+}, Cu^{2+} and Bi^{3+}. Introduction of a plasmid borne mammalian MT gene into yeast cells deficient in the Cu^+-MT gene, *CUP1*, confers copper and Cd^{2+} resistance in a copy-dependent manner (cited in Templeton and Cherian, 1991).

Although higher eukaryotic MT binds to (and its synthesis is induced by) many metal ions, Zn^{2+} and Cu^+ are the only ones of nutritional importance. The others, including Cd^{2+} and Hg^{2+}, are generally considered to be nonessential, and appear to have reduced cytotoxic effects by binding to MT. Such detoxification could be a consequence of the ability of these metal ions to bind to (and induce) a protein that is primarily concerned with the metabolism of Zn^{2+} and copper (Bremner, 1991). It has been argued that toxic trace metals are not present at high or fluctuating concentrations in most biotopes and would not exert a sufficiently high selection pressure to cause the widespread existence of a specialised detoxification system (Karin, 1985). As a homoeostatic mediator MT could provide a reservoir for donating metal ions in the biosynthesis of Zn^{2+} and copper containing metalloenzymes and metalloproteins.

Zn^{2+} associated with animal MT is highly labile, a necessary attribute for an intracellular Zn^{2+} donor (cited in Kägi, 1991). Donation of Zn^{2+} to some apo-enzymes has been demonstrated *in vitro* (cited in Zeng *et al.*, 1991b). In addition thionein (apo-MT) can reversibly inactivate the Zn^{2+} requiring transcription factor Sp1 (human) and also acquire Zn^{2+} from *Xenopus laevis* transcription factor IIIA *in vitro*, thereby interfering with DNA binding properties (Zeng *et al.*, 1991a; 1991b). It has thus been proposed that modulation of thionein biosynthesis, or intracellular distribution, could affect DNA binding by Zn^{2+} requiring transcription factors and thereby regulate expression of a large subset of genes in higher eukaryotes (Zeng *et al.*, 1991a).

Lower eukaryotic (fungal) MTs bind Cu^+ *in vivo* (not Cd^{2+} or Zn^{2+}, although *in vitro* Cd^{2+} binding has been shown for *Saccharomyces cerevisiae* MT (CUP1) (cited in Tohoyama *et al.*, 1992)) and their synthesis is regulated by copper. Mutants of *S. cerevisiae* deficient in the MT gene, *CUP1*, have been constructed (Thiele *et al.*, 1987).

CUP1 performs no essential role(s) required for cell growth, differentiation or normal copper metabolism. CUP1 mutants grow with normal doubling times in standard low Cu^{2+} media and are capable of mating, diplophase growth, sporulation, germination, accumulation of copper and accumulation/activation of a copper requiring enzyme, copper-dependent superoxide dismutase (Thiele *et al.*, 1987). However, MT deficient *S. cerevisiae* are hypersensitive to elevated concentrations of Cu^{2+}. The role of fungal Cu^{+}-MT in Cu^{2+} tolerance is further demonstrated by selection of Cu^{2+} resistant (Cu^{r}) *S. cerevisiae* and *Candida glabrata* involving a stable chromosomal amplification of the *CUP1* and *MT-II* genes, respectively (Fogel *et al.*, 1983; Mehra *et al.*, 1990).

In addition to metal ions, MT synthesis can be induced in higher eukaryotes by many physiological and nutritional factors, including starvation and imposition of various types of physical or inflammatory stress (cited in Bremner, 1991). This has implied that MT could have physiological roles such as in the acute phase response, the scavenging of free radicals (see below), the regulation of cell differentiation and the storage of sulphur.

Generators of free radicals, which include types of radiation, chemicals, certain metals (copper and iron) and inflammatory stress (reviewed by Halliwell and Gutteridge, 1984; Bremner, 1987), induce MT synthesis (cited in Bremner, 1991). MT is a potent hydroxyl scavenger (Thornally and Vasák, 1985), but its antioxidant properties *in vivo* have not been defined. It was reported that cells in which MT was induced by Cd^{2+} became more resistant to ionising radiation (Bakka *et al.*, 1982). However, further studies indicated that cross-resistance to H_2O_2 observed in Cd^{r} cells was due to an increase in GSH content, and cells overexpressing MT (without concomitant increase in GSH) do not show increased resistance to oxidative stress (Chubatsu and Meneghini, 1993). The presence of MT has been reported in the nucleus (Tsujikawa *et al.*, 1991), and a protective role of MT to the DNA-strand-breaking effect of hydroxyl radical attack has been reported (Chubatsu and Meneghini, 1993).

Class III

There is evidence that (γ-glutamylcysteine)$_n$glycine detoxifies Cd^{2+} and excess copper in some cells, and indirect evidence that these polypeptides detoxify excess Ag^{+} (cited in Robinson, 1989; Grill *et al.*, 1987).

Synthesis of (γ-glutamylcysteine)$_n$glycine increases after exposure of cells to Cd^{2+} and Cu^{2+} (Grill *et al.*, 1986; 1987; Kneer *et al.*, 1992), and (γ-glutamylcysteine)$_n$glycine unequivocally binds Cd^{2+} and Cu^{+} *in vivo* (Reese *et al.*, 1988; Grill, 1989; Rauser, 1990). Furthermore, production of (γ-glutamylcysteine)$_n$glycine metal complexes correlates with resistance to Cu^{2+} and Cd^{2+} in plant cell cultures (Jackson *et al.*, 1987). Buthionine sulphoxamine (BSO) is a potent inhibitor of the enzyme γ-glutamylcysteine synthetase (the first enzyme of the GSH biosynthetic pathway), involved in (γ-glutamylcysteine)$_n$glycine synthesis. In a number of species, concentrations of this inhibitor which have little or no effect on growth in the absence of Cd^{2+}, cause substantial reductions in growth following exposure to Cd^{2+} (Reese and Wagner, 1987; Rauser, 1990). There is dramatic synergism between Cd^{2+} and BSO in the inhibition of growth and this has been directly correlated with decreased levels of (γ-glutamylcysteine)$_n$glycine (Reese and Wagner, 1987). In cultured tobacco cells, synergism between Cu^{2+} and BSO was not detected (Reese and Wagner, 1987), although it has since been suggested that this might be due to chelation of Cu^{2+} by BSO thereby reducing uptake (N.J. Robinson, personal communication).

Mutants of *Schizosaccharomyces pombe* unable to synthesise (γ-glutamylcysteine)$_n$glycine are Cd^{2+} sensitive, and mutants unable to produce Cd^{2+}-(γ-glutamylcysteine)$_n$glycine aggregates containing S^{2-} also show reduced Cd^{2+} resistance (Mutoh and Hayashi, 1988). A gene, *hmt1*, encoding a polypeptide associated with the vacuolar membrane, complements a Cd^{2+}-sensitive *S. pombe* mutant deficient in production of Cd^{2+}-(γ-glutamylcysteine)$_n$glycine aggregates containing S^{2-}. Yeast cells harbouring an *hmt1*-expressing multicopy plasmid exhibit enhanced metal tolerance along with a higher intracellular level of Cd^{2+}, implying a relationship between HMT1 mediated transport and formation, or stabilisation, of S^{2-} containing Cd^{2+}-(γ-glutamylcysteine)$_n$glycine aggregates (Ortiz *et al.*, 1992).

By analogy to animal MT, it could be speculated that (γ-glutamylcysteine)$_n$glycine plays a role in essential metal ion homoeostasis (Rauser, 1990). (γ-glutamylcysteine)$_n$glycine synthesis is induced by micronutrient concentrations of Cu^{2+} and Zn^{2+} in various fresh media (Grill *et al.*, 1987; Rauser, 1990). Cu^{+}- and Zn^{2+}-(γ-glutamylcysteine)$_n$glycine complexes have been shown to be capable of efficiently activating metal-depleted apoenzymes *in vitro* (Thumann *et al.*, 1991). However, *in vivo* Zn^{2+} binding of (γ-glutamylcysteine)$_n$glycine has not been unequivocally demonstrated.

Enzymes involved in (γ-glutamylcysteine)$_n$glycine biosynthesis are constitutively produced in the absence of elevated levels of trace metals, suggesting a constitutive function for (γ-glutamylcysteine)$_n$glycine, or an alternative function for the biosynthetic enzymes (cited in Robinson, 1989). Other proposed functions for (γ-glutamylcysteine)$_n$glycine include roles in assimilatory SO_4^{2-} reduction (Steffens, 1986), and protection against free radicals (Hayashi *et al.*, 1991).

1.1.4. MT gene organisation and expression

Mammalian

Mammalian MT genes are encoded by three exons which are interrupted by two large introns at conserved positions (Palmiter, 1987). The synthesis of MTs is controlled mainly at the level of transcription, and their synthesis can be induced by a wide range of factors (refer to section 1.1.3.; Kägi, 1991). In murine cell cultures activation of the two iso-MT genes, *MT-I* and *MT-II*, is coordinate (Griffith, 1985). In human cells, expression of some of the iso-MT genes are regulated differentially by metals and glucocorticoids in a cell or tissue specific manner (Foster and Gedamu, 1991; Heguy *et al.*, 1986; Jahroudi *et al.*, 1990).

Transcriptional activation by metals is dependent on the presence of a *cis*-acting 15 bp consensus sequence (metal-responsive element, MRE) present in the MT gene promoter region (cited in Radtke *et al.*, 1993; Labbé, 1993). The optimal metal concentration for induction varies in different systems but is generally at the maximum permissive concentration for cell viability (Hamer, 1986). The mouse *MT-I* promoter

contains six MREs (MREa-f) within the first 200 bp 5' of the transcriptional start site. MREa-d confer metal-responsive transcription when tested independently in association with a reporter gene. The ability to mediate metal-activated transcription varies between the different MREs; MREd is the strongest MRE of the mouse *MT-I* promoter (Stuart *et al.*, 1985) and responds to the same spectrum of metal ions as the complete promoter (Cizewski Culotta and Hamer, 1989). The different transcriptional efficiencies may reflect variations in the sequences of the MREs outside the conserved core element TGCPuC (where Pu is a purine) (Séguin and Hamer, 1987).

MRE binding proteins have been characterised (cited in Radtke *et al.*, 1993; Labbé *et al.*, 1993). These proteins act in *trans* and specifically bind in a metal-dependent manner to the MREs of the mouse *MT-I* promoter *in vitro* and *in vivo*. They may be responsible for metal-induced MT transcription.

An MRE binding factor of HeLa cell nuclear extracts designated MTF-1, binds most strongly to MREd (Westin and Schaffner, 1988). MTF-1 binding is inactivated/reactivated *in vitro* by Zn^{2+} withdrawal/addition, and the amounts of MTF-1 DNA complexes are elevated several fold in Zn^{2+} treated cells (Radtke *et al.*, 1993). The cDNA of a mouse MTF-1 has also been isolated and characterised (Radtke *et al.*, 1993). MTF-1 contains six zinc fingers and separate transcriptional activation domains with high contents of acidic and proline residues. Ectopic expression of MTF-I in primate or rodent cells strongly enhances transcription of a reporter gene that is driven by four consensus MREd sites, or by the complete mouse *MT-I* promoter, even at normal Zn^{2+} levels.

An MRE binding factor designated MEP-1, has been purified from metal resistant mouse L cells (Labbé *et al.*, 1993). MEP-1 binds with high affinity to MREd, and to the other MRE sequences with affinities that are proportional to their relative transcriptional strength *in vivo*. MEP-1 binding can be abolished by a point mutation in the MRE core consensus sequence. Consistent with MTF-1, MEP-1 binding is inactivated/reactivated *in vitro* by Zn^{2+} withdrawal/addition. It is suggested that amino acids with affinity for Zn^{2+} are exposed on the MEP-1 protein surface. MEP-1 also binds to MREs of the human *MT-II$_a$* and trout *MT-B* genes.

Other identified MRE binding factors include (cited in Labbé, 1993); a HeLa-cell nuclear factor, MREBP, that recognises MREs of the human *MT-II$_a$* gene and is indicated to bind specifically to several MREs present upstream of *MT-II$_a$*; MRE-BF1 and MRE-BF2, have been found in human cells; ZAP and p39, have been detected in rat cells.

It was found that regions outside the MREs could also play an important role in determining the efficiency of transcription of human *MT-II$_a$* (Karin *et al.*, 1987). Another element, the glucocorticoid-responsive element (GRE), present further upstream, was found to be responsible for steroid hormone induction of human *MT-II$_a$*. The GRE, which coincides with a binding site for the glucocorticoid receptor (a *trans* regulator of gene expression), acts like a hormone-dependent enhancer element. Unlike MREs, GREs were capable of operating like enhancer elements on their own and did not seem to require interaction with additional elements, suggesting the existence of two basically different modes of transcriptional control (Karin *et al.*, 1987).

Fungal

The *Saccharomyces cerevisiae* Cu^+-MT, CUP1, is encoded on chromosome VIII (cited in Butt and Ecker, 1987). Cu^r *S. cerevisiae* cells contain 10 or more tandemly repeated copies of *CUP1* (Welch *et al.*, 1983). *CUP1* transcription is induced in response to copper and Ag^+ (which is physiologically irrelevant). Transcription induction is mediated through the action of a *trans*-acting Cu^+ or Ag^+ activated sequence-specific DNA binding protein, designated ACE1/CUP2, encoded on chromosome VII (Welch *et al.*, 1989; Buchman *et al.*, 1989; Thiele and Hamer, 1986; Casas-Finet *et al*, 1992; Thiele, 1992). *S. cerevisiae* cells exposed to Cu^{2+} or Ag^+ foster the rapid binding of metallated monomeric ACE1 to four distinct regulatory sites within a region of the *CUP1* promoter, which is located between -105 and -230 with respect to the *CUP1* transcription initiation site (the upstream activation sequence) (Thiele and Hamer, 1986; Thiele, 1992). Once bound, ACE1 activates *CUP1* transcription in large part through the carboxy-terminal acidic region (Thiele, 1988; Hu *et al.*, 1990).

The Cu^+ or Ag^+ activated DNA binding domain of ACE1 resides within the amino-terminal 100 residues, is highly positively charged, and contains 11 cysteine residues

(arranged in cysteine-Xaa-cysteine or cysteine-Xaa-Xaa-cysteine motifs) which are individually of critical importance for function (Thiele, 1988; Hu *et al.*, 1990; Buchman *et al.*, 1990). The ACE1 DNA binding domain cooperatively binds Cu^{+} in a 1:6 stoichiometry as a sulphur-coordinated polynuclear cluster (Fürst and Hamer, 1989; Dameron *et al.*, 1991). ACE1 has been shown to contribute to basal level (in the absence of added Cu^{2+}) transcription of *CUP1* (although this basal level of transcription could be the result of activation by residual copper) (Welch *et al.*, 1989; Butler and Thiele, 1991). Two other transcription factors, ACE2 and heat shock transcription factor, have also been demonstrated to activate *CUP1* transcription (Butler and Thiele, 1991; Silar *et al.*, 1991).

Candida glabrata harbours an MT gene family composed of a single *MT-I* gene, multiple tandemly amplified *MT-II*$_a$ genes and a single unlinked *MT-II*$_b$ gene (Mehra *et al.*, 1988; 1989; 1990; 1992). These MT genes are transcriptionally activated in response to copper and Ag^{+}. A *C. glabrata* gene, designated *AMT1*, has been isolated and encodes a Cu^{+} or Ag^{+} activated DNA binding protein (Zhou and Thiele, 1991). AMT1 synthesised in *Escherichia coli, in vitro* forms multiple Cu^{+}-dependent, sequence specific DNA protein complexes with both *MT-I* and *MT-II*$_a$ promoter DNA fragments. AMT1 was indicated to recognise two binding sites in a *MT-1* promoter fragment, and five major binding sites and one low-affinity site in a *MT-II*$_a$ promoter fragment (Zhou *et al.*, 1992). Comparable to ACE1, AMT1 preferentially binds to the promoter sequences in the monomeric form. The DNA sequences recognised by AMT1 in the *MT-1* and *MT-II*$_a$ promoters and by ACE1 in the *CUP1* promoter are similar in that they contain a four nucleotide core sequence (5'-GCTG-3'), with a T- or A-rich region immediately 5' (Thiele, 1992).

AMT1 shares several features at the primary structural level with ACE1 from *S. cerevisiae*. The amino-terminal 110 amino acids of AMT1 are 73 % similar and 55 % identical to the 100 amino-terminal residues encompassing the ACE1 Cu^{+} activated DNA binding domain, this region being positively charged and containing 11 similarly arranged cysteine residues (Zhou and Thiele, 1991). AMT1 also contains an abundance of acidic residues in the carboxy-terminal region, corresponding to a potent ACE1 *trans-*

activation domain (Hu *et al.*, 1990; Zhou and Thiele, 1991). Analogous to the induction of *CUP1* transcription by *ACE1*, *AMT1* is required in *trans* for both basal and Cu^{+}-dependent activation of *MT-I* and *MT-II*$_a$ gene transcription *in vivo*, indicating that a single metalloregulatory transcription factor activates the family of MT genes in *C. glabrata* (Zhou *et al.*, 1992). However, both the mRNA and protein levels for *MT-II* are higher than those for *MT-I* (Mehra and Winge, 1991). This may reflect a combination of the number or organisation of *cis*-acting elements, the *MT-II* gene dosage effect, and perhaps differential stabilities of the mRNAs or proteins (Zhou *et al.*, 1992). AMT1-mediated *trans* activation of the *C. glabrata* MT genes is essential for high level Cu^{2+} resistance, however AMT1 is completely dispensable for Cd^{2+} tolerance. Cd^{2+} has been shown to reduce the basal levels of both *MT-I* and *MT-II* mRNAs (Mehra *et al.*, 1989).

1.2. PROKARYOTIC METAL RESISTANCE DETERMINANTS

Metal resistance determinants have frequently been found on plasmids and transposons of Gram-negative and Gram-positive bacteria (Silver and Misra, 1988). Specific resistances have been discovered to a range of metal ions, including Ag^{+}, AsO_2^{-}, AsO_4^{3-}, Bi^{3+}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, SbO_2^{-}, TeO_3^{2-}, Tl^{+}, Pb^{2+}, Zn^{2+} and other metals of environmental concern (Silver, 1992). The known mechanisms of bacterial metal resistances can be grouped into four categories (cited in Silver, 1992):

1. Keeping the toxic ion out of the cell by altering a membrane transport system involved in influx.
2. Intracellular or extracellular sequestration of the metal ion by specific metal binding proteins or non-proteinaceous ligands (analogous to MTs, but generally at the cell wall level in bacteria).
3. Highly specific cation or anion efflux systems encoded by resistance genes (this is the most common mechanism of plasmid controlled bacterial metal ion resistance).
4. Detoxification of the toxic cation or anion by enzymatically converting it from a more toxic to a less toxic form.

The plasmid encoded bacterial metal resistances for the ionic species of arsenic, antimony, cadmium (zinc and cobalt), mercury (and organomercurials), copper and silver are described.

1.2.1. Arsenic and antimony

The arsenic oxyanion of +V oxidation state, arsenate, is toxic to bacteria because it is an analogue of phosphate. It is transported by bacterial phosphate transport systems and interferes intracellularly with the formation of phosphorylated intermediates (e.g. arsenylated sugars hydrolyse spontaneously) (Kaur and Rosen, 1992). The oxyanion of +III oxidation state, arsenite, is considerably more toxic than arsenate. Arsenite and antimonite (the +III oxyanion of antimony) react with protein sulphydryl groups to

inactivate enzymes (Knowles and Benson, 1983; cited in Silver *et al.*, 1981). The pathways for arsenite and antimonite uptake into cells are unknown.

Arsenic and antimony resistance determinants are found on plasmids of both Gram-negative and Gram-positive bacteria. In most cases these provide inducible resistance to antimonite, arsenite and arsenate. The presence of the resistance determinant does not alter the existing phosphate uptake systems (Silver *et al.*, 1981). In both Gram-negative and Gram-positive bacteria, resistance is correlated with lowering of the intracellular concentration of these toxic anions by efflux from cells (Kaur and Rosen, 1992).

Structure and metalloregulation of the *ars* operon

Arsenate, arsenite and antimonite resistances are coded for by an arsenical resistance (*ars*) operon in both Gram-negative and Gram-positive bacteria. In each case the *ars* operon is inducible *in vivo* by all three salts (Silver *et al.*, 1981).

In *Escherichia coli* the *ars* operon is located on the resistance plasmid R773 (Hedges and Baumberg, 1973), and confers resistance by encoding an ATP-driven pump which extrudes the toxic anions out of cells (Kaur and Rosen, 1992). The operon encodes two regulatory genes, *arsR* and *arsD* (San Francisco *et al.*, 1990; Wu and Rosen, 1993b), followed by three structural genes, *arsA*, *arsB* and *arsC* (Chen *et al.*, 1986). The structural genes of the operon comprise an anion-translocating ATPase (Wu and Rosen, 1991). The *arsA* and *arsB* gene products form the basic pump for arsenite and antimonite (Rosen and Borbolla, 1984). ArsA is the catalytic subunit, and shown to be an oxyanion-stimulated ATPase (Rosen *et al.*, 1988). ArsB, which is located in the inner membrane of *E. coli* (San Francisco *et al.*, 1989), forms the anion-conducting subunit as well as the anchor for ArsA. *arsC* encodes an arsenate to arsenite reductase (Ji and Silver, 1992b).

A regulatory region of the *ars* operon has been localised and consists of a single promoter sequence followed by the regulatory gene *arsR* (San Francisco *et al.*, 1990). In the presence of inducer the genes of the *ars* operon (including *arsR*) are transcribed from the promoter as a single 4 400-nucleotide polycistronic mRNA (Owolabi and Rosen, 1990). However, the genes of the *ars* operon are differentially expressed. The transcript

is rapidly converted into two smaller species, and the site of cleavage of the transcript is within the *arsB* sequence. The half life of the 4 400-nucleotide transcript is much less than that of the two smaller derivatives. Inefficient translational initiation of mRNA coupled with a rapid loss of the *arsB* message could result in low production of the membrane protein (Owolabi and Rosen, 1990).

ArsR, in spite of an apparent lack of DNA binding motifs, is a *trans*-acting inducer-responsive negative regulator (San Francisco *et al.*, 1990; Wu and Rosen, 1993a). *arsD* is situated between *arsR* and *arsA*, and encodes an inducer-independent *trans*-acting regulator (ArsD mediated down-regulation being dependent on the ArsD intracellular concentration) (Wu and Rosen, 1993b). The concerted action of ArsR and ArsD establishes the regulatory circuit that controls the basal and maximal levels of expression of the *ars* operon (Wu and Rosen, 1993b). In the absence of inducer, constitutively produced ArsR binds as a dimer to an operator site proximal to the *ars* promoter and represses transcription (Wu and Rosen, 1993a). Under uninduced conditions there would be little ArsD. Addition of inducer releases the repressor from the DNA and permits transcription of the *ars* operon. ArsR and ArsD are produced in addition to ArsA, ArsB and ArsC. Only when the level of ArsD reaches a critical concentration in the cell would it prevent further *ars* expression (Wu and Rosen, 1993b). ArsD is postulated to prevent over-production of ArsB, which is toxic when produced in high amounts. *In vivo*, the operon was derepressed by oxyions of +III oxidation state of arsenic, antimony and bismuth, as well as arsenate. However, *in vitro* studies indicated that arsenate must be reduced to arsenite *in vivo* to induce (Wu and Rosen, 1993a). Recent data indicated direct interaction of ArsR cysteine residues with inducers (Rosen *et al.*, 1993; refer to section 4.7.).

In *Staphylococci* the *ars* operon is located on the penicillinase resistance plasmids, pI258 and pSX267, of *Staphylococcus aureus* and *Staphylococcus xylosus*, respectively (Novick and Roth, 1968; Götz *et al.*, 1983). The nucleotide sequence of the *ars* operon of both *Staphylococcus* plasmids have been determined. Only three open reading frames (ORFs) were found, and correspond to the *arsR*, *arsB* and *arsC* genes of the R773 operon, having similarities in sequence and function. The staphylococcal plasmid *ars*

operons therefore lack *arsA* and *arsD* genes (Ji and Silver, 1992a). The *S. aureus ars* system, although missing the ArsA ATPase, determines energy-dependent arsenic efflux (Ji and Silver, 1992b), and two hypotheses have been proposed for the mechanism of the *S. aureus* arsenic resistance system: Efflux by ArsB in the absence of ArsA might be driven by a chemiosmotic mechanism in response to the cell membrane potential; alternatively, the staphylococcal *ars* system might function as an ATPase, with the ATPase subunit provided by a chromosomal equivalent to the plasmid *arsA* gene (Bröer *et al.*, 1993).

1.2.2. Cadmium (zinc and cobalt)

Zn^{2+} and Co^{2+} are essential nutrients for bacteria, required at nanomolar concentrations. However Cd^{2+}, and higher concentrations of Zn^{2+} and Co^{2+}, without nutritional roles are toxic (Nies, 1992a). These cations are transported into the cell by constitutively expressed divalent cation uptake systems of broad specificity, basically magnesium transport systems. The uptake of Cd^{2+} by Gram-positive bacteria occurs by the energy-dependent manganese transport system (Weiss *et al.*, 1978).

The mechanism of Cd^{2+} toxicity is unclear. It has been shown that Cd^{2+} interacts with thiol groups, and competes with Zn^{2+} for sulphydryl or imidazole groups of proteins (Vallee and Ulmer, 1972). Cd^{2+} has also been shown to uncouple oxidative phosphorylation and to interact with phospholipids in membranes. Furthermore, loss (85-95 %) of viability in Cd^{2+} exposed *Escherichia coli* was accompanied by single strand breakage of DNA (Mitra and Bernstein, 1978). A direct correlation appeared to exist between the number of single strand breaks and the concentrations of Cd^{2+} to which the cells were exposed.

There are several systems for bacterial Cd^{2+} resistance. Three of these have been characterised in detail and are described below; the *cadA* and *cadB* operons of *Staphylococcus aureus* penicillinase plasmids (Smith and Novick, 1972; Silver and Misra, 1988) and the *czc* (Cd^{2+}, Zn^{2+} and Co^{2+}) resistance system of *Alcaligenes eutrophus* (Nies and Silver, 1989). Other mechanisms of Cd^{2+} resistance in bacteria include; a

chromosomal encoded energy-dependent Cd^{2+} efflux system of *S. aureus*, which differs from *cadA* in that it confers Cd^{2+} resistance alone (*cadA* confers both Cd^{2+} and Zn^{2+} resistances) (Witte *et al.*, 1986); a chromosomal mutation in *Bacillus subtilis* that resulted in a change in the membrane manganese transport system so that Cd^{2+} is no longer accumulated (Laddaga *et al.*, 1985); and the synthesis of a polythiol Cd^{2+} binding protein analogous to MT of animal cells (refer to section 1.3.).

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

Two distinct Cd^{2+} resistance determinants, *cadA* and *cadB*, have been identified on *S. aureus* penicillinase plasmids (Smith and Novick, 1972). *cadA* encoded resistance confers high level resistance (100 fold increase) to Cd^{2+} and Zn^{2+} by lowering the intracellular concentration of these ions by efflux from cells (Weiss, *et al.*, 1978; Tynecka *et al.*, 1981). The *cadB* gene product may confer low level resistance (10 fold increase) to Cd^{2+} and Zn^{2+} by enhanced binding, but the mechanism is unclear (Perry and Silver, 1982). Some plasmids (e.g. pI258) in *S. aureus* contain only the *cadA* determinant; others (e.g. pII147) contain the second determinant *cadB*.

Structure and metalloregulation of the *cadA* operon

The Cd^{2+} and Zn^{2+} resistance determinant of *S. aureus*, *cadA*, located on plasmid pI258 (and related plasmids) (Novick *et al.*, 1979), contains two ORFs, *cadC* and *cadA*, which code for an ATP-dependent pump (Nucifora *et al.*, 1989a; Tsai *et al.*, 1992). *cadC* overlaps the second ORF, *cadA*, for eight nucleotides and the two genes are cotranscribed as a polycistronic mRNA (Yoon *et al.*, 1991). The product of the longer ORF, CadA, is membrane bound with strong sequence similarity to the P-type ATPases (Yoon and Silver, 1991). Cd^{2+} (and probably Zn^{2+}) efflux is catalysed by CadA. The product of the smaller ORF, CadC is also required for efflux, although its role is not clear. Both CadA and CadC are required for full Cd^{2+} and Zn^{2+} resistance (Yoon and Silver, 1991). *cadC* functions both in *cis* and in *trans*.

The *cadA* operon is regulated at the transcriptional level. Cd^{2+}, Bi^{3+} and Pb^{2+} were found to be good inducers, while Co^{2+} and Zn^{2+} were weak inducers (Yoon *et al.*, 1991). A *trans*-acting regulatory protein in the *cadA* operon has not been identified. However, the existence of a *trans*-acting, chromosomally encoded regulatory CadR protein has been postulated (Yoon *et al.*, 1991). The transcript start of the *cadA* operon lies in the middle of an inverted repeat sequence, and is a candidate binding site for the hypothetical CadR protein (Yoon *et al.*, 1991).

Structure and metalloregulation of the *cadB* operon

The *cadB* resistance system is less well characterised than the *cadA* system, and is thought to encode an inducible Cd^{2+} binding protein (Perry and Silver, 1982). Cd^{2+} resistance determined by *cadB* is independent of, and not additive to, the action of *cadA* (Elsolh and Ehrlich, 1982). The *cadB* operon contains two ORFs, *cadB* and *cadX* (Silver and Walderhaug, 1992). CadX is thought to be a required structural component of the CadB system as CadC is required for the CadA system (Yoon and Silver, 1991).

The predicted amino acid sequences of CadC and CadX show similarity to each other, and to the *trans*-acting repressor of the *ars* operon, ArsR, of plasmids of both *Escherichia coli* and *Staphylococcus* species (described in section 1.2.1.; refer to section 4.4.1.). Although these proteins have sequence similarities, how these relate to the functional roles and evolutionary histories of the genes is uncertain.

1.2.2.2. The *czc* operon

The Gram-negative bacterium *Alcaligenes eutrophus* strain CH34, isolated from a zinc decantation tank (Mergeay *et al.*, 1978), contains at least seven determinants encoding metal resistances. These resistance determinants are located on the bacterial chromosome or on one of the two indigenous large plasmids designated pMOL28 and pMOL30 (Dressler, *et al.*, 1991; Gerstenberg *et al.*, 1982; Mergeay *et al.*, 1985). The *czc* resistance determinant of CH34, encoded by plasmid pMOL30, provides inducible Cd^{2+},

Zn^{2+} and Co^{2+} resistance by lowering the intracellular concentration of these cations by energy-dependent efflux from cells (Nies and Silver, 1989).

CH34 resistance to Co^{2+} is also provided by the energy-dependent (Co^{2+} and Ni^{2+}) efflux resistance system, *cnr*, encoded by plasmid pMOL28 (Nies *et al.*, 1989b); this system is not described here. Furthermore, a Zn^{2+} binding protein has been detected in CH34, the synthesis of which was induced during growth in high concentrations of Zn^{2+} (Remacle and Vercheval, 1991). This protein has a low content of cysteine and a high content of acidic amino acids, and therefore differs from MTs. It is not known whether this metalloprotein is involved in metal resistance of CH34.

Structure and metalloregulation of the *czc* operon

The *czc* operon from CH34 encodes five genes, coding for proteins required for Co^{2+}, Zn^{2+} and Cd^{2+} efflux (CzcA, CzcB and CzcC) and regulation of the *czc* determinant (CzcD and CzcR) (Nies *et al.*, 1989a; Nies, 1992b). The largest of the four proteins CzcA, is essential for cation transport and is the core of the efflux protein complex, it may function as a cation/proton antiporter (Nies, 1992a). The second largest protein, CzcB, may function as a cation binding subunit (Nies, 1992a). CzcA and CzcB function together as a Zn^{2+} pump (Nies *et al.*, 1989a). CzcC is proposed to function as a modifier protein required to change the substrate specificity of the system from Zn^{2+} only to Co^{2+}, Zn^{2+} and Cd^{2+}. CzcC is dependent on CzcB for function (Nies, 1992a).

The *czcR* gene product, transcribed in a divergent orientation to that of the other *czc* genes, is essential for induction of *czc*, and is hypothesised to act as an activator of transcription (Nies, 1992b). CzcR is essential for full expression of resistance to Co^{2+}, Zn^{2+} and Cd^{2+}. The best effector of CzcR is Zn^{2+} (with induction by Co^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+} and Al^{3+} also observed) (Nies, 1992b). Zn^{2+} is also the best substrate of the CzcABC efflux complex (Nies *et al.*, 1989a). The membrane bound *czcD* gene product is also necessary for activation of the *czc* efflux system, although it is not essential for the activation of *czc* by CzcR (Nies *et al.*, 1989a; Nies, 1992b). CzcD is needed for regulation of *czc*, only if the efflux pump is active, and might function as a sensor for the extracellular cation concentration (Nies, 1992b).

1.2.3. Mercury

Mercuric ion (Hg^{2+}) and organomercurial compounds, which have strong affinities for the thiol groups in proteins and for other substituent groups in proteins, lipids, nucleic acids and polysaccharides are toxic to cells (Brown *et al.*, 1991; Misra, 1992). Mercury resistance determinants also encode transport proteins, involved in the uptake of mercurials (Nakahara *et al.*, 1979; Griffin *et al.*, 1987).

Essentially all bacterial mercury resistance systems tested have the same single basic mechanism; enzymatic transformation of mercury from the toxic form (either ionic or organic), to the less toxic elemental form, Hg^0, which is chemically inert, water soluble and volatilises due to its high vapour pressure. Two classes of mercury resistance determinants have been characterised; broad and narrow spectrum. Broad spectrum determinants confer resistances to a wide range of organomercurials, as well as inorganic Hg^{2+} salts. The enzyme organomercurial lyase catalyses the cleavage of C-Hg bonds to yield Hg^{2+} (in addition to the appropriate organic compound), a separate enzyme, mercuric ion reductase, catalyses the reduction of Hg^{2+} to Hg^0. Narrow spectrum determinants lack the gene for organomercurial lyase and do not confer resistance against most organomercurials (cited in Misra, 1992). The mercury resistance determinants in bacteria are usually encoded by genes on plasmids or transposons (Silver and Misra, 1988).

Structure and metalloregulation of *mer* operons

The genes conferring resistances to mercurial compounds are clustered *mer* operons in most known systems, and can be subgrouped into three categories based on function (cited in Misra, 1992); those conferring transport of mercurials into the cell; those encoding enzymes involved in transformation of mercurials to Hg^0; and those encoding *mer* regulatory proteins.

The organisation of the *mer* genes from Gram-negative bacteria are similar. Mercury resistance determinants of Tn*21* (from *Shigella flexneri* plasmid R100), Tn*501* (from

Pseudomonas aeruginosa plasmid pVS1) and pDU1358 which contains a broad and a narrow spectrum operon that can function independently (Griffin *et al.*, 1987) (from *Serratia mercescens*), have been studied in detail and are described below. A chromosomally located *mer* operon of *Thiobacillus ferrooxidans* has also been sequenced and is the most different of the Gram-negative operons (Silver and Walderhaug, 1992).

Precise mapping of the genes of the Tn*21*, Tn*501* and pDU1358 *mer* operons was achieved from DNA sequence analysis (Misra *et al.*, 1984, 1995; Brown *et al.*, 1986; Nucifora *et al.*, 1989c; Griffin *et al.*, 1987). The *mer* operon starts with a divergently transcribed regulatory gene, *merR*, whose product binds as a dimer to the adjacent operator-promoter site (Silver and Walderhaug, 1992). Adjacent to this are the other *mer* genes, which are transcribed as a single polycistronic mRNA (Misra, 1992). This begins with a series of two (*merT* and *merP*) or three (an additional *merC* in Tn*21*) genes whose products are involved in the transport of mercurials. MerT and MerC are inner membrane proteins, whereas MerP (after cleavage of a signal polypeptide) is located in the periplasmic space, and is suggested to increase resistance by acting as an external scavenger of Hg^{2+} (Brown *et al.*, 1991). *merA* is located next to *merP* (or *merC*) and encodes the mercuric reductase, a NAD(P)H-dependent flavin adenine dinucleotide-containing disulphide oxidoreductase (Silver and Walderhaug, 1992). In pDU1358 (broad spectrum operon) *merB* follows and encodes the organomercurial lyase. *merD* is distal to the promoter in each case and encodes a secondary regulatory protein. The DNA sequence of the operator-promoter region is the most conserved among the different *mer* operons and is approximately 95 % identical, the genes with common function are approximately 85 % identical (Misra, 1992).

A model for Hg^{2+} detoxification encoded by Tn501 in *Escherichia coli* has been proposed (Brown, 1985). Initially Hg^{2+} ions are sequestered by the pair of thiol groups on MerP in the periplasmic space. The Hg^{2+} is then transferred across the cytoplasmic membrane by a series of ligand exchange reactions between paired thiol groups of the transmembrane protein MerT to the amino-terminal cysteines of mercuric reductase. Hg^{2+} bound to the carboxy-terminal cysteine pair of mercuric reductase is reduced to

Hg^0 and diffuses out of the cell. The role of Tn*21 merC* remains obscure (Hamlett *et al.*, 1992).

Expression of the *mer* operon is regulated and is inducible by mercurials. Broad spectrum resistance loci, containing the gene encoding organomercurial lyase (*merB*) are inducible by both inorganic and organic mercurial compounds, whereas narrow spectrum loci are only inducible by inorganic Hg^{2+} (Nucifora *et al.*, 1989b). MerR represses operon expression from the promoter P*mer*T in the absence of inducers and activates transcription in the presence of inducers. In both cases MerR represses its own transcription from the divergent promoter P*mer*R (cited in Summers, 1992). The -10 σ-70 RNA polymerase recognition hexamers of the divergent promoters overlap, but the -35 hexamers do not. MerR-mediated transcription occurs in nanomolar concentrations of Hg^{2+} (Frantz and O'Halloran, 1990). MerR also activates transcription *in vitro* in response to Cd^{2+}, Zn^{2+} and Au^{+}, but to a lesser extent than with Hg^{2+}. MerR has a dual role as the metal receptor and transcriptional effector (Foster and Brown, 1985; Lund *et al.*, 1986).

The *mer* operator has a 7-4-7 bp perfect dyad symmetrical repeat which lies between the -10 and -35 σ-70 RNA polymerase recognition hexamer of the structural gene promoter P*mer*T (O'Halloran *et al.*, 1989). MerR contains a predicted helix-turn-helix motif associated with DNA binding, and this is involved in recognising the dyad symmetry region. MerR binds as a dimer to the operator site encompassing the P*mer*T and an overlapping region that is transcribed from the P*mer*R (cited in Misra, 1992). MerR binds only one Hg^{2+} per dimer, and *in vitro* experiments revealed that MerR alone or MerR in the presence of Hg^{2+} binds to the same operator site (O'Halloran and Walsh, 1987; Heltzel *et al.*, 1987). MerR has three conserved cysteine residues that are required for specific binding of Hg^{2+} and efficient activation of transcription by the bound complex and the RNA polymerase (cited in Misra, 1992).

The sequence of the *mer* operator-promoter region is unusual, the -10 and -35 sequences of P*mer*T are separated by 19 bp, 2 bp longer than the optimal distance for efficient transcription by the *E. coli* σ-70 RNA polymerase. The -10 and -35 transcript initiation sites are consequently offset by approximately 70°. The 19 bp spacing and the

relative positions of the 7 bp dyad sequences with respect to the -10 and -35 sequences are important for operon induction and repression from P*mer*T (Parkhill and Brown, 1990; Lund and Brown, 1989). Experiments, altering the spacing between the -10 and -35 sequences, have revealed that a spacing of 20 or 21 bp prevents transcription of the operon, while a spacing of 17 or 18 bp confers expression from P*mer*T in the absence of MerR, but MerR represses expression in both the presence and absence of mercury (Parkhill and Brown, 1990).

In the absence of Hg^{2+}, transcription from P*mer*R and P*mer*T is inhibited. RNA polymerase can still bind to the promoter region, but DNA footprinting experiments suggest that MerR prevents the formation of an open complex by RNA polymerase (cited in Park *et al.*, 1992; Summers, 1992). In the presence of Hg^{2+}, MerR is allosterically modified and the MerR-Hg(II) complex alters the spacing between the -35 and -10 transcript initiation sites, by reducing supercoiling between them and causing them to lie on the same face of the DNA. This facilitates the bound RNA polymerase to form a transcriptionally active open complex at the P*mer*T and initiate transcription (reviewed by Misra, 1992; Ansari *et al.*, 1992; Lee *et al.*, 1993; Livrelli *et al.*, 1993). Furthermore, specific MerR mutants that stimulate transcription in the absence of Hg^{2+} have been created, and there is a direct correlation between the degree of activation caused by the mutants and DNA distortion (Parkhill *et al.*, 1993).

MerD has sequence similarities to MerR at the amino-terminus, spanning the putative DNA binding domain and both MerR and MerD bind to the same operator site, although MerD has a considerably lower affinity than MerR (Brown *et al.*, 1986; Mukhopadhyay *et al.*, 1991). The critical amino acids required for activation of transcription by MerR and Hg^{2+} are absent in MerD and deletion of MerD has little or no effect on the expression of mercury resistance (Brown *et al.*, 1986). MerD down regulates transcription from the P*mer*T *in vivo* and is involved in fine tuning the expression of the *mer* genes (Nucifora *et al.*, 1989c; Mukhopadhyay *et al.*, 1991).

In addition to the control of *mer* expression at the initiation of transcription, the *mer* operon of Gram-negative bacteria is also subject to modulation of transcriptional elongation (Gambill and Summers, 1992). Comparison of the relative abundance of the

5' and 3' ends of the *merTPCAD* transcript revealed a strong transcriptional gradient in the operon, with a lower relative abundance of the more promoter-distal gene products. The mRNA half lives of all genes are quite similar, however the rates of mRNA synthesis varies considerably from the beginning to the end of the operon.

The *mer* operons for three Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus* sp. and *Streptomyces lividans*) have also been sequenced. The genes of the operons from *S. aureus* (located on plasmid pI258) (Laddaga *et al.*, 1987) and *Bacillus* sp. (chromosomally located) (Wang *et al.*, 1989) are organised similarly. The operons start with operator-promoter sequences, which are highly conserved with those of Gram-negative bacteria, followed by *merR*. Next is a region that encodes proteins involved in mercury transport (Babich *et al.*, 1991; Wang *et al.*, 1989), followed by the mercuric reductase gene, *merA*, and the organomercurial lyase gene *merB*. For the *S. aureus* operon *merB* is contiguous with *merA*, however for the *Bacillus* operon there is a gap of several kb of apparently unrelated DNA between *merA* and *merB*. The operon of *S. lividans* is different from those previously studied (Silver and Walderhaug, 1992). The genes are arranged in two divergently transcribed clusters; one containing a gene weakly recognisable as *merR* and another weakly recognisable as *merT*; the other containing *merA* and *merB*.

The MerR proteins from Gram-positive sources have sequences about 36 % identical to those from Gram-negative sources (Helmann *et al.*, 1989; Silver and Walderhaug, 1992). The *Bacillus* MerR has been shown to bind Hg^{2+} and positively regulate transcription of the *Bacillus mer* operon in the presence of Hg^{2+} (Helmann *et al.*, 1989; 1990). Furthermore, the pI258 encoded MerR has been demonstrated to be a *trans*-acting activator of the *mer* operon transcription in the presence of inducer Hg^{2+} (and to a lesser extent Cd^{2+}) (Chu *et al.*, 1992).

1.2.4. Copper

Copper is an essential trace element which is required by certain metalloproteins, but is also highly toxic if levels of free ions are not controlled. The chemical properties of

copper underlie its role in metalloproteins and its toxicity (cited in Brown *et al.*, 1992): Copper can undergo redox reactions between Cu^+ and Cu^{2+} under physiological conditions, and can therefore act as an electron donor and acceptor in the electron transport chain (e.g. in cytochrome oxidases) and in redox-active enzymes which use molecular oxygen as a substrate. Copper can also catalyse adverse redox reactions in the cell, such as the generation of hydroxyl radicals in the reaction

$$Cu^+ + H_2O_2 \rightleftharpoons Cu^{2+} + OH^- + OH^\cdot$$

The peroxide species in the reaction inevitably arise from partial reduction of molecular oxygen. Hydroxyl radicals are highly reactive and can participate in a number of deleterious reactions, such as the peroxidation of lipids, and the oxidation of proteins. Copper can also bind to, and modify, functional groups in proteins, nucleic acids, polysaccharides and lipids.

Different bacteria have adopted diverse strategies for copper resistance. However, recent molecular analysis suggest similarities between copper resistance genes from several bacterial genera that were described as having different resistance mechanisms (cited in Cooksey, 1993). Most known bacterial determinants of copper resistance are plasmid encoded, however efficient chromosomal encoded systems for uptake and control of trace levels of copper also exist. In *Escherichia coli*, isolated from pigs (fed diets containing copper sulphate), an efflux mechanism encoded by the *pco* genes from plasmid pRJ1004 is tightly coupled to chromosomal copper transport functions (Rouch *et al.*, 1989a; Brown *et al.*, 1991). In the plant pathogen *Pseudomonas syringae* pv. *tomato*, copper exclusion from the cytoplasm and copper uptake are determined by components of the *cop* operon encoded by plasmid pTD23D (Bender and Cooksey, 1986; 1987). The mechanisms of copper homoeostasis in these two bacterial strains will be described below. Other reports of copper resistance in bacteria include; a copper resistance operon, related to *cop*, encoded by plasmid pXV10A of another plant pathogen *Xanthomonas campestris* pv. *vesicatoria* (Bender *et al.*, 1990; Cooksey, 1993); and the production of hydrogen sulphide and concomitant precipitation of copper sulphide in *Mycobacterium scrofulaceum* (Erardi *et al.*, 1987).

Structure and metalloregulation of the *pco* operon and the copper uptake system, *cut*

Two determinants contribute to the inducible copper resistance system encoded by plasmid pRJ1004 in *E. coli* (Rouch *et al.*, 1989a); *cdr*, which encodes DNA repair functions and *pco*, which encodes an inducible mechanism which results in decreased intracellular copper accumulation. The *pco* gene products are proposed to modify the action of the proteins for normal copper metabolism (encoded by *cut*) to both increase energy-dependent copper efflux from cells, and to modify copper such that it is unavailable for uptake (the form of copper modification is not known) (Brown *et al.*, 1991).

Initially four *pco* genes, *pcoARBC*, were identified. All four genes were required for expression of copper resistance, expression being controlled largely at the transcriptional level (Rouch *et al.*, 1989a). The *pcoC* gene product was identified as an inducible copper binding protein, whereas *pcoR* encodes a *trans*-acting regulator (Rouch *et al.*, 1989a). The roles of *pcoA* and *pcoB* were not identified.

Sequence analysis has subsequently shown that *pco* encodes seven genes, *pcoABCDRSE*. Four structural genes *pcoABCD* (previously part of a single long *pcoA* complementation unit) show amino acid sequence similarity to the *copABCD* operon of *Pseudomonas syringae* (described below) (Mellano and Cooksey, 1988a) and the *E. coli* operon has been renamed *pcoABCD* to be consistent with *cop* designations (Cooksey, 1993; Silver *et al*, 1993). Two regulatory genes that are required for copper induction, *pcoR* (previously identified at that position) and *pcoS* (previously identified as two complementation groups, *pcoB* and *pcoC*), follow the *pcoABCD* genes. A fifth structural gene required for copper resistance, *pcoE*, follows *pcoABCDRS* (Cooksey, 1993).

The predicted amino acid sequences of PcoR and PcoS show strong similarities to the "response regulators" and "sensory kinases", respectively, of two-component regulatory systems (refer to section 1.4.3) (Brown *et al.*, 1992; Mills *et al.*, 1993). PcoS is indicated to be a membrane protein as is frequently the case with such autophosphorylating proteins (Silver *et al.*, 1993). It is suggested that PcoS will autophosphorylate and the phosphate will subsequently be transferred to PcoR. Phosphorylated PcoR is then

predicted to activate several operator-promoter regions in the *pco* determinant, increasing transcription of mRNA. It is proposed that the "C-portion" of *pcoS* (originally designated *pcoC*) encodes a soluble polypeptide that binds Cu^{2+}, and internal as well as extracellular Cu^{2+} may be sensed by PcoS (Rouch *et al* 1989a; Silver *et al.*, 1993). The *pco* resistance genes are only induced by high levels of copper (cited in Mills *et al.*, 1993). It is uncertain whether regulation will be positive (as is frequently the case for two-component phospho-protein gene regulation) or negative (Silver *et al.*, 1993).

The *pco* resistance mechanism is dependent on a set of chromosomal genes, *cut*, apparently required for normal copper uptake and management. Six structural *cut* genes have been identified, *cutABCDEF* and assigned functions (Rouch *et al.*, 1989b; Brown *et al.*, 1992): *cutA* non-specific copper uptake; *cutB* copper-specific uptake; *cutC* and *cutD* copper efflux; *cutE* intracellular copper storage; *cutF* intracellular copper carrier. The predicted translation product of *cutE* has a small region with sequences that are repeated in the copper binding protein CopA from *P. syringae* and the predicted protein encoded by *pcoD* (cited in Cooksey, 1993). These conserved sequences may participate in copper binding. *cutR* has also been identified and its product regulates the expression of *cutABCDEF* (Brown *et al.*, 1991). The expression of the *cut* genes is regulated to maintain copper homoeostasis.

Regulation of the chromosomal *cut* genes appear to be coupled to regulation of the plasmid encoded *pco* genes (which act in high external concentrations of Cu^{2+}) to maintain homoeostasis at both high and low external Cu^{2+} concentrations (Brown *et al.*, 1992). Mutations in *pcoR* can be complemented by the chromosomal gene *cutR*, which may have an analogous function in regulation of the chromosomal *cut* genes (Brown *et al.*, 1992). The corresponding sensor, assuming that this is a typical two-component system, has been designated CutS, and has been identified by mutation (Brown *et al.*, 1992).

Structure and metalloregulation of the *cop* operon

In *Pseudomonas syringae* pv. *tomato* carrying the plasmid encoded *cop* operon, copper is excluded from the cytoplasm by proteins that bind copper in the periplasm and outer

membrane (Cha and Cooksey, 1991). The *cop* operon encodes four genes *copABCD* transcribed from a single copper-inducible promoter (upstream of the first gene, *copA*), as a single polycistronic mRNA (Mellano and Cooksey, 1988a; 1988b). The *cop* encoded proteins have been characterised; CopA, CopB and CopC have leader sequences, CopD has extensive internal regions of hydrophobic amino acids (Mellano and Cooksey, 1988a). CopA and CopC are periplasmic proteins, CopB is an outer membrane protein, and CopD is (probably) an inner membrane protein.

CopA binds multiple Cu^{2+} atoms, whereas CopC binds only one Cu^{2+} atom per polypeptide. Additional copper is bound in the outer membrane, probably involving CopB (Cooksey, 1993). A model for Cu^{2+} resistance in P. *syringae* pv. *tomato* in which free copper ions are prevented from entering the cytoplasm by compartmentalisation in the periplasm and outer membrane (involving sequestration by CopA, CopC and probably CopB) is suggested (Cooksey, 1993). The strong copper sequestering activity may require a compensatory transport function to assure an adequate supply of copper for copper-dependent enzymes. CopB may be involved in cross membrane transport as well as copper binding (Silver *et al.*, 1993). There is also evidence suggesting *copC* and *copD* function in copper transport (Cha and Cooksey, 1991).

Copper resistance in *P. syringae* is specifically induced by copper (Mills *et al.*, 1993), and the regulation of *cop* operon expression was shown to be at the transcriptional level (Mellano and Cooksey, 1988b). Two genes, *copR* and *copS*, have been identified immediately downstream of *copABCD* (Mills *et al.*, 1993). These genes are expressed in the same orientation as the *cop* operon, but from a separate constitutive promoter located 5' to *copR* (they may also be expressed from the *cop* promoter). The deduced amino acid sequences from *copR* and *copS* have similarities to known two-component regulatory and sensor proteins respectively, and CopR has strong similarity to the copper resistance regulatory protein, PcoR, from *E. coli* (Mills *et al.*, 1993).

Regulation of copper resistance in *P. syringae* pv. *tomato* involves an interaction between plasmid and chromosomal genes (Cooksey, 1993). Negative regulation has been proposed, involving a chromosomally-encoded repressor that binds to the *cop* promoter in the absence of copper but is released in the presence of copper (Cooksey, 1993). This

inducible release of the repressor requires the plasmid encoded *trans*-acting CopR and CopS activator/sensor proteins. Functional chromosomal homologues to *copRS* also activate the *cop* promoter in a copper-inducible manner (Mills *et al.*, 1993).

1.2.5. Silver

Ag^+ is biologically non-essential, and is toxic due to its complexation to membranes, enzymes, nucleic acids and other cellular components. Ag^+ complexes strongly to electron donor groups containing nitrogen, oxygen and sulphur (cited in Slawson *et al.*, 1992a).

Ag^+ resistant strains of *Enterobacteriaceae* and *Pseudomonas stutzeri* have been identified (cited in Slawson *et al.*, 1992a; 1992b) and a Ag^+ resistance plasmid, pKK1, of *P. stutzeri* has been isolated. It is likely that Ag^+ is excluded from certain bacteria, or immobilised intracellularly to prevent toxic effects being exerted (Slawson *et al.*, 1992a). These mechanisms of silver resistance have not yet been elucidated.

1.3. PROKARYOTIC MT

Metal binding MT-like proteins have been described in only two prokaryotic species; *Pseudomonas putida* (Higham *et al.*, 1984) and *Synechococcus* sp. (MacLean *et al.*, 1972; Olafson *et al.*, 1980; Olafson, 1984; Takatera and Watanabe, 1992). A series of three related low molecular weight cysteine-rich metal binding proteins were isolated from a Cd^r strain of *Pseudomanas putida* (Higham *et al.*, 1984). NMR analysis of these proteins showed sulphur-cation domains similar to eukaryotic MTs. Unfortunately the strain lost its ability to produce these proteins before they were fully characterised (Higham *et al.*, 1985). The MT-like proteins of *Synechococcus* sp. have been characterised in detail and are described below.

In addition to these MT-like proteins, a high molecular weight Cd^{2+} binding protein was isolated from *Esherichia coli* cells accommodated to growth-inhibiting concentrations of Cd^{2+} (Mitra *et al.*, 1975; Khazaeli and Mitra, 1981). This protein was associated with 60 % of cytoplasmic Cd^{2+} in accommodated cells. However a similar protein was not detected in non-accommodated cells.

Prokaryotic MT from *Synechococcus* species

The production of an MT-like protein of *Anacystis nidulans* was correlated with Cd^{2+} resistance (MacLean *et al.*, 1972). Class II MTs have since been isolated from a marine strain of *Synechococcus*, *Synechococcus* RRIMP NI (Olafson *et al.*, 1979; 1980), and freshwater strains *Synechococcus* UTEX-625 and *Synechococcus* TX-20 (these strains, and very closely related strains, are also referred to as *Anacystis nidulans*, *Synechococcus* PCC 6301, *Synechococcus* PCC 7942 and *Synechococcus* R2 (refer to section 2.1.2.)) (Olafson, 1984).

In common with eukaryotic MTs, chemical characterisation of the three isolated *Synechococcus* MTs indicated a high cysteine content, low molecular weight, high metal binding capacity and essentially no 280 nm absorption (Olafson *et al.*, 1979; 1980; Olafson, 1984). However the histidine content was high for MTs and there was an abundance of hydrophobic residues, making these the most hydrophobic MTs to be

described. The MT from *Synechococcus* TX-20 was sequenced and compared with several eukaryotic MTs (Olafson *et al*, 1988). Despite the high frequency of cysteine residues increasing the probability of chance alignments, database comparisons indicated no significant similarity (aside from characteristic cysteine-Xaa-cysteine arrangements).

In common with eukaryotic MT, metal induction of *Synechococcus* MT is indicated to be regulated at the level of transcription. This MT increased in abundance following exposure of *Synechococcus* cells to elevated concentrations of Cd^{2+} or Zn^{2+}, but not Cu^{2+}, and the purified protein was associated with either Cd^{2+} or Zn^{2+} (dependent upon the metal administered to the cells) with copper as a minor component (Olafson *et al.*, 1988). Furthermore, a metal binding MT-like protein recently detected in Cd^{2+} stressed *Anacystis nidulans* R2 contained predominantly Cd^{2+} as well as smaller amounts of Zn^{2+} and copper ions (Takatera and Watanabe, 1992). Spectral data obtained for Zn^{2+}-MT and MT substituted with copper ions suggested that the *Synechococcus* MT may have a metal-thiolate cluster structure similar to that of eukaryotic MT, but in a single domain (Olafson *et al.*, 1988).

Polymerase chain reaction fragments corresponding to the *Synechococcus* MT gene have been generated from *Synechococcus* PCC 6301 genomic DNA (Robinson *et al.*, 1990), and the MT divergon, designated *smt*, from *Synechococcus* PCC 7942 has recently been cloned and structurally characterised (Huckle *et al.*, 1993). The MT divergon includes the MT gene, *smtA*, and the divergently transcribed gene *smtB*. The predicted *Synechococcus* PCC 7942 *smtA* gene product is identical to the polypeptide previously purified and sequenced by Olafson and co-workers, with the exception of a serine substitution for cysteine$_{32}$ and two additional codons at the carboxy-terminus. The sites of transcription initiation of *smtA* and *smtB* have been mapped (Huckle *et al.*, 1993). A 100 bp operator-promoter region lies between the *smtA* and *smtB* protein coding regions and contains divergent promoters (with similarity to *E. coli* -10 promoter consensus sequences) that border a 7-2-7 hyphenated inverted repeat. An imperfect 6-2-6 hyphenated inverted repeat and a 6-2-6 hyphenated direct repeat are located between the sites of transcriptional and translational initiation in *smtA* (Huckle *et al.*,

1993; Morby *et al.*, 1993). The repeat sequences are putative sites for the interaction of DNA binding regulatory proteins.

The abundance of *smtA* transcripts increased in response to elevated concentrations of a range of trace metal ions (including, Cd^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Co^{2+} and Ni^{2+}) (Huckle *et al.*, 1993), and there was no detectable effect of elevated metal (Cd^{2+}) concentration on *smtA* transcript stability. This is consistent with *smtA* being regulated at the level of transcription. Examination of other known metal responsive proteins in cyanobacteria has revealed that; in comparison to SmtA, metal- (iron) dependent expression of ferredoxin I is determined by differential mRNA stability in *Synechococcus* PCC 7942 and *Anabaena* PCC 7937 (Bovy *et al.*, 1993); expression of the iron stress induced putative photosystem II chlorophyll binding protein and flavodoxin (encoded by *isiA* and *isiB*, respectively (refer to section 1.4.2.)), in *Synechococcus* PCC 7942 and *Synechococcus* PCC 7002, involves increased mRNA abundance (due to increased transcription and/or mRNA stability) (Laudenbach and Straus, 1988; Leonhardt and Straus, 1992); and expression of both plastocyanin and cytochrome c_{553} is regulated by copper via changes in mRNA abundance in *Anabaena* PCC 7937 (indicated to be at the level of transcription initiation in this case) and *Synechocystis* PCC 6803 (*Synechococcus* PCC 7942 has no gene for plastocyanin and expresses the cytochrome c_{553} irrespective of the amount of copper (Laudenbach *et al.*, 1990)) (Bovy *et al.*, 1992; Zhang *et al.*, 1992).

Sequences upstream of *smtA* (including *smtB* and the operator-promoter region), fused to a promoterless *lacZ* gene, conferred metal-dependent β-galactosidase activity in *Synechococcus* PCC 7942 (strain R2-PIM8, refer to section 1.2.2.). At maximum permissive concentrations for growth, Zn^{2+} was the most potent elicitor *in vivo*, followed by copper and Cd^{2+} with slight induction by Co^{2+} and Ni^{2+} (Huckle *et al.*, 1993). Following expression of SmtA as a recombinant fusion protein in *E. coli*, the pH of half dissociation of different metal ions indicated a particularly high affinity for Zn^{2+}, in comparison to mammalian MT (Shi *et al.*, 1992). A *smtA* homologue has also recently been reported in *Synechococcus vulcanus* and designated *mtnA* (Shimizu *et al.*, 1992).

1.4. REGULATION OF GENE EXPRESSION IN PROKARYOTES

Bacteria rapidly respond to fluctuations in their environment, such as the supply of nutrients and the presence of toxins, by modulating the expression of specific sets of genes. Regulation of protein abundance can, of course, involve processes such as; transcription, mRNA stability, translation and protein stability. Regulation at the level of transcription is discussed.

1.4.1. Regulation at the level of transcription

Many studied genes show a component of regulation at the level of transcription, in particular initiation of transcription. Transcription initiation depends on the direct interaction of RNA polymerase with a promoter sequence on the DNA. The major *Escherichia coli* RNA polymerase interacts with most promoters by recognising two consensus hexameric sequences located around 35 bp (TTGACA) and 10 bp (TATAAT) to the 5' of the transcription start (reviewed by McClure, 1985). Recognition is primarily due to the interactions between these sequences and the RNA polymerase σ-70 subunit (Waldburger *et al.*, 1990).

The degree of adherence to the -10 and -35 consensus sequences is correlated with promoter strength (Schneider *et al.*, 1991). The spacing between the -10 and -35 sequences should be 16 to 18 bp (as discussed in the context of P*mer*T (section 1.2.3.)), and the transcript start site is usually 6 to 8 bp from the -10 sequence. The distance between the consensus sequences also contributes to promoter strength (Hawley and McClure, 1983; Harley and Reynolds, 1987). The DNA structure in the spacer region is important, and base substitutions in the spacer region, which change the structural characteristics of the DNA, can also affect promoter strength (Auble *et al.*, 1986).

The activity of a promoter can be modulated by the action of regulatory molecules, which bind to specific sequences in the promoter. Regulators can interfere with different steps in the initiation of transcription such as; binding of the RNA polymerase to the promoter sequence, or isomerisation of the polymerase-promoter complex to a

transcription-competent open complex. Examination of *E. coli* σ-70-dependent promoters revealed that most activators and repressors bind close to the -10 and -35 consensus sequences (Gralla, 1991; Collado-Vides *et al.*, 1991).

Some promoters can function with no apparent -35 elements (Keilty and Rosenberg, 1987; Harley and Reynolds, 1987). These promoters usually have weak constitutive activities and are activated by positively acting proteins which seem to substitute for the -35 element (Raibaud and Schwartz, 1984; Collado-Vides *et al.*, 1991). The overall structure of open complexes may differ according to whether or not a -35 hexamer resembling the consensus sequence is present. In the absence of a -35 sequence, the RNA polymerase fails to make contact in the -35 region and appears to make upstream compensatory contacts that involve distortion of the DNA (Chan *et al.*, 1990). Activators bind to the region between -35 and -80 relative to the transcription start sites. A direct interaction of the activator with the RNA polymerase is suggested for activation (Gralla, 1991; Collado-Vides, 1991) and the interaction of an activator with RNA polymerase may be required for stable promoter recognition and binding (Reznikoff, 1992). Alternatively, an activator may be required for the formation of a transcription-competent open complex.

Several studies have suggested that the sequence motif 5'-TGN-3' found immediately upstream of the -10 hexamer in some cases, creates an "extended -10" region that can compensate for a poor -35 sequence (Keilty and Rosenberg, 1987; Kumar *et al.*, 1992). It has been demonstrated that in the absence of "correct" -35 contacts, the formation of transcriptionally competent complexes is dependent on both a contact provided by the "extended -10" sequence and distortion of upstream sequences around RNA polymerase (Minchin and Busby, 1993). It is not clear whether both types of contacts are needed at all "extended -10" promoters.

Repressor binding sites for σ-70-dependent promoters are generally located between +20 and -20 relative to the transcription start site, and repression may occur by direct interference with the polymerase. In almost all cases, the repressor is close enough to touch either the polymerase or an activator which in turn is close enough to touch the polymerase (Gralla, 1991; Collado-Vides, 1991). The position of the regulator binding

site can be very critical for the function of the regulator (e.g. the global regulators Crp and Fnr activate transcription from a normal position near -40, but act as repressors when their binding sites are downstream of -20) (Gralla, 1991; Collado-Vides, 1991).

There are a number of other σ factors that direct the recognition of promoters with different DNA sequences (Gralla, 1991; Collado-Vides, 1991). These minor σ factors fall into two distinct classes; those that are very similar to σ-70 and appear to behave similarly (most); and σ-54 not at all similar to the σ-70 family, having different basal elements located at -12 and -24, and a different arrangement of regulatory elements. Sequence conservation among the σ-70 class of proteins suggests that this class can be divided into three groups; the primary σs in each organism reponsible for most RNA synthesis (group 1), closely related but non-essential σs (group 2), and alternative σs responsible for transcription of specific regulons (group 3) (Lonetto *et al.*, 1992). Modification of RNA polymerase by alternative forms of the σ subunit allows regulation of the temporal and spatial expression of discrete sets of genes.

Transcription initiation in cyanobacteria

Transcription initiation in the filamentous cyanobacterium *Anabaena* 7120 is the most characterised cyanobacterial system. The RNA polymerase from vegetative cells of *Anabaena* 7120 has been purified (Schneider *et al.*, 1987). The core enzyme is composed of four subunits, three of which, α, β and β', correspond to the core enzyme common to prokaryotes (Schneider *et al.*, 1987). The additional subunit, γ, corresponds to the amino-terminal half of the *E. coli* β' subunit, and is common to the other characterised cyanobacterial RNA polymerases (Schneider and Haselkorn, 1988; Brahamsha and Haselkorn, 1991). The holoenzyme also contains the σ-subunit, which confers promoter-specific activity on the core enzyme (Schneider *et al.*, 1987). The principle σ subunit resembles σ-70 of *E. coli* (Brahamsha and Haselkorn, 1991).

A comparison of several promoters of *Anabaena* 7120 vegetative cells (Schneider *et al.*, 1991) did not reveal a common consensus sequence. However they share some, but not all, of the *E. coli* consensus promoter. They particularly depart from the *E. coli* consensus elements around the -35 sequence. *In vitro* transcription assays using the

Anabaena 7120 holoenzyme showed that promoters most similar to the consensus *E. coli* promoter gave the greatest rates of transcription. It was concluded that the *Anabaena* 7120 genes expressed in vegetative cells, characterised to date, have weak promoters or that they require activators (Schneider *et al.*, 1987; 1991).

Promoters, of *nif* genes (encoding proteins involved in nitrogen fixation), in *Anabaena* 7120 are only active in heterocysts (Elhai and Wolk, 1990). The sequences of *nif* promoters differ from vegetative cell promoters in that they have no homology to the *E. coli* consensus elements (Tumer *et al.*, 1983). The *Anabaena glnA* gene (encoding glutamine synthetase) provides an example of a gene that is expressed in vegetative cells as well as in heterocysts. This gene has multiple promoters used under different circumstances. Under normal growth conditions this gene was predominantly transcribed from an *E. coli* σ-70 like promoter, while in heterocysts nearly all the transcripts initiated at a *nif* like promoter (Tumer *et al.*, 1983). It therefore appears that different RNA polymerase σ subunits are used in *Anabaena* 7120, that direct the recognition of promoters with different DNA sequences. *Anabaena* 7120 has been shown to contain a number of genes with similarity to *sigA* (encoding the principal σ factor) (Brahamsha and Haselkorn, 1992). Two of these genes, *sigB* and *sigC*, have been sequenced and further characterised, and are non-essential for growth on N_2 as nitrogen source (Brahamsha, 1993; reviewed by Buikema and Haselkorn, 1993).

1.4.2. Metalloregulatory proteins

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

Iron is required for a wide range of metabolic pathways. Due to the low solubility of iron above neutral pH the biological availability of iron is often limited, and bacteria must adapt their physiology to survive during iron deprivation. Furthermore, surplus iron may find its way into coordination sites where it can generate oxidising radicals (Bagg and Neilands, 1987). Iron uptake therefore requires regulation. The best

understood iron-responsive metalloregulatory protein in microbial systems is Fur (ferric iron uptake regulation), and Fur-like regulatory systems are ubiquitous in Gram-negative bacteria (cited in O'Halloran, 1993). In *Escherichia coli*, the product of the *fur* gene is responsible for regulating the expression of a number of genes in response to intracellular iron levels. Fur negatively controls genes coding for multiple high-affinity iron-uptake pathways and for enzymes concerned with the biosynthesis of siderophores (small iron binding chelates), as well as genes whose products are not required for low iron survival (Hennecke, 1990). In the presence of iron, Fur complexes with Fe^{2+} and represses transcription by binding to a 19 bp consensus DNA recognition sequence exhibiting dyad symmetry (designated the 'iron box') within the promoter regions of these genes (cited in Silver and Walderhaug, 1992). The *iucA* promoter of the aerobactin operon is the most extensively studied promoter under the regulation of Fur. This promoter requires the occupation of two contiguous repressor binding sites for full repression. The primary Fur binding site overlaps the -35 region of the σ-70-dependent promoter, blocking access to RNA polymerase. The secondary binding site overlaps the -10 region and the transcription start site (de Lorenzo *et al.*, 1988). At low iron concentrations Fur has a weak affinity for the operator DNA and transcription of the aerobactin promoter occurs. At high iron concentrations, Fur binds tightly to the operator DNA and transcription is blocked. The Fur complex is thought to wrap around the DNA in a spiral fashion (de Lorenzo *et al.*, 1988).

The iron stress induced genes *isiA* and *isiB* (encoding a putative photosystem II chlorophyll binding protein and flavodoxin, respectively) form a dicistronic operon, and have been cloned and sequenced from *Synechococcus* PCC 7942 and *Synechococcus* PCC 7002 (Laudenbach *et al.*, 1988; Laudenbach and Straus, 1988; Leonhardt and Straus, 1992) (refer to section 1.3.). The upstream region of the *isiAB* operon, in *Synechococcus* PCC 7942, contains an *E. coli* like -10 consensus promoter sequence but lacks the typical -35 consensus sequence (Laudenbach and Straus, 1988). However, in *Synechococcus* PCC 7002, sequences in the -10 and -35 regions bear similarity to the *E. coli* -10 and -35 consensus sequences, respectively (Leonhardt and Straus, 1992). In *Synechococcus* PCC 7942 there are three 17 bp sequences, approximately 15, 25 and

150 bp upstream from the transcription start site of the *isiAB* mRNA, which resemble the Fur binding sequences of the aerobactin promoter (Laudenbach and Straus, 1988). *Synechococcus* PCC 7942 also contains *fur* consensus sequences upstream from *irpA*, which is thought to transcribe a product involved in the acquisition or storage of iron (Reddy *et al.*, 1988). In *Synechococcus* PCC 7002, potential *fur* consensus sequences have been located 5 bp downstream, and 29, 183 and 229 bp upstream from the transcription start site of the *isiAB* mRNA (Leonhardt and Straus, 1992).

Metal regulatory proteins exist in which the metal ion is not the signal, but acts as the sensor for the signal (reviewed by Hennecke, 1990). The positive regulatory proteins, NifA (an activator for the expression of symbiotic nitrogen fixation genes) of the *Rhizobia* and Fnr (a regulator of genes concerned with anaerobic energy metabolism) of *E. coli*, respond directly to the oxygen concentration in the environment. They are active in microaerobic or anaerobic conditions, and inactive at high oxygen tension. Using a helix-turn-helix domain, NifA binds to an activator sequence located 100 to 200 bp upstream of the transcript start site (cited in Hennecke, 1990). Fnr also contains a helix-turn-helix domain and acts as an activator of the majority of genes, while a few are repressed. The promoter regions of the positively induced genes contain a 22 bp consensus Fnr binding site with dyad symmetry, located 39 to 49 bp upstream of the transcription start sites (Bell *et al.*, 1989). As often observed with positively controlled genes, these promoters contain -10 regions but no recognisable -35 regions (Raibaud and Schwartz, 1984). In the negatively controlled genes the Fnr binding site overlaps the transcript start sites, consistent with them being repressed (Eiglmerier *et al.*, 1989). The activity of NifA and Fnr may be metal-dependent. Both proteins contain potential metal binding domains and are proposed to function via the redox state of the bound metal ion sensing the presence of oxygen (Fischer *et al.*, 1988; Spiro *et al.*, 1989).

1.4.3. Two-component regulatory systems

Adaptive responses in prokaryotes often involve two families of signal-transduction proteins (Stock *et al.*, 1989; 1990). Striking sequence similarities have been found in

each family of signal-transduction proteins involved in a variety of adaptive responses (Ronson *et al.*, 1987). Proteins belonging to one family classified as "sensory kinases", monitor an environmental parameter and transmit a signal to the second family "response regulators", which mediate changes in gene expression in response to the signals. Members of such adaptive response systems have been identified in more than 20 different systems and more than 30 different prokaryotic species (reviewed by Parkinson and Kofoid, 1992). Sequence similarities between sensor-regulator systems suggests that, during prokaryotic evolution, a single two-component motif has been continually adapted to link expression of sets of genes with specific environmental stimuli (Ronson *et al.*, 1987).

The hydropathic profiles of members of the sensor family suggest that many are transmembrane proteins with an amino-terminal periplasmic domain, defined by two hydrophobic transmembrane regions, and a carboxy-terminal cytoplasmic domain. Members of the regulator family of proteins may consist of two potentially autonomous regions, an amino-terminal regulatory region, which acts as the receptor of signals, and the remainder of the protein, which functions in DNA binding and interaction with RNA polymerase. The only demonstrated mechanism of communication between transmitters ("sensory kinases") and receivers ("response regulators") involves phosphorylation and dephosphorylation (cited in Parkinson and Kofoid, 1992). Transmitters have an autokinase activity that attaches phosphoryl groups from ATP to a histidine residue, from which they are subsequently transferred to an aspartate residue in the target receiver (stimuli would modulate autokinase activity). Receiver phosphorylation modulates the activity of its adjoining output domain. Receiver dephosphorylation occurs by several routes and interrupts these regulatory responses (Parkinson and Kofoid, 1992).

The high affinity phosphate transport system, Pst, of the phosphate regulated *pho* regulon of *Escherichia coli* is under the control of the "response regulator" and "sensory kinase" proteins PhoB and PhoR, respectively. The "sensory kinase" and "response regulator" genes (*sphS* and *sphR*, respectively) of a two-component regulatory system have recently been characterised in the cyanobacterium *Synechococcus* PCC 7942 (Aiba

et al., 1993). The deduced amino acid sequences show a high degree of similarity to the bacterial sensory-regulator systems (in particular PhoR and PhoB). The *sphS* and *sphR* genes encoding the signal-transduction proteins are proposed to have roles in regulation of a phosphate regulon in *Synechococcus* PCC 7942. Furthermore, two ORFs have been identified in *Synechococcus* WH 7803 that contain all the diagnostic residues and domains characteristic of a "response regulator" and a "sensory kinase", and have similarity to a PhoB homologue and PhoR of *Bacillus subtilis* and *E. coli*, respectively (Mann *et al.*, 1993). It is therefore suggested that expression of the *pstS* gene of *Synechococcus* WH 7803 (induced in phosphate limited conditions), encoding a cell wall associated polypeptide (with considerable similarity to the inducible periplasmic phosphate binding protein, PstS, of the *E. coli* Pst system), may be under the control of a two-component regulatory system (Scanlan *et al.*, 1993).

1.5. CYANOBACTERIAL TRANSFORMATION

DNA can be introduced into a number of strains of cyanobacteria by transformation (mediated by direct DNA uptake or enhanced by electroporation) or conjugation (Porter, 1988; Thiel and Wolk, 1987; Thiel and Poo, 1989; Haselkorn, 1991). The cyanobacterial strain used in the study described in this thesis, is a small plasmid- (pUH24) cured derivative of the unicellular strain *Synechococcus* PCC 7942 (refer to section 2.1.2.). *Synechococcus* PCC 7942 cells are naturally competent, and can easily be transformed with both plasmid and chromosomal DNA.

Two strategies have been employed for developing host-vector systems for the transformation of *Synechococcus* PCC 7942. One uses hybrid shuttle vectors that contain both a *Synechococcus* and an *Escherichia coli* origin of replication (Shermann and Van de Putte, 1982). The other makes use of homologous recombination allowing for the stable integration of specific DNA fragments, of exogenous or endogenous origin, at a defined site on the cyanobacterial genome (e.g. an integration platform system) (Van der Plas, 1990).

Hybrid shuttle vectors

Hybrid vectors have been constructed using the replication origins of the *Synechococcus* PCC 7942 small indigenous plasmid pUH24, and an *E. coli* plasmid such as pBR322, as well as an antibiotic resistance gene. Initially a complication in the use of pUH24-derived vectors was recombination between the vector and the indigenous pUH24, however the use of pUH24 cured strains of *Synechococcus* PCC 7942 has alleviated this problem (Dzelzkalns *et al.*, 1984; Kuhlemeier *et al.*, 1985).

It has been shown that hybrid shuttle vectors, containing cloned DNA fragments of exogenous or endogenous origin, are able to replicate extrachromosomally in both *E. coli* and *Synechococcus* PCC 7942, and are genetically and structurally stable during passage through both organisms (Golden and Sherman, 1983).

Homologous recombination

Homologous recombination can be exploited to integrate heterologous DNA into the cyanobacterial chromosome, using flanking homologous chromosomal DNA to direct it to a particular site. Specific mutants can be generated by gene interruption or deletion (Williams and Szalay, 1983). Integration platform systems have been created which enable stable chromosomal insertion of any DNA fragment (unless the expression of the inserted DNA in *Synechococcus* PCC 7942 has some deleterious effect on the cell), cloned in a particular vector (homologous fragments of which are contained within the platform) (Van der Plas, 1990).

Donor DNA can be linear or circular (Williams and Szalay, 1983). The stability and efficiency of integration into the cyanobacterial chromosome depends upon the position of heterologous DNA within the donor molecule. When the homologous cyanobacterial DNA fragment is interrupted by foreign DNA, integration occurs through replacement of recipient DNA by homologous donor DNA containing the foreign insert (Kolowsky *et al.*, 1984). Transformation is efficient and stable, and occurs by reciprocal recombination (a reciprocal crossover event occurring either side of the foreign DNA) or gene conversion (non-reciprocal recombination) (Williams and Szalay, 1983; Golden *et al.*, 1987; Kuhlemeier *et al.*, 1985). Foreign DNA linked to the ends of the cyanobacterial homologous DNA fragment in a circular donor molecule (i.e. not interrupting) integrates less efficiently, and is the result of a single reciprocal crossover between donor and recipient (Williams and Szalay, 1983; Kolowsky *et al.*, 1984). The entire plasmid becomes integrated at the chromosomal locus, causing duplication of the region of homologous cyanobacterial DNA (Golden *et al.*, 1987). The duplication can be maintained by continual selection for the foreign DNA phenotype.

Cyanobacteria are polyploid. Under "normal" conditions *Synechococcus* PCC 7942 was estimated to contain approximately ten chromosomes per cell, based on a comparison of the DNA content per cell and known genome size (Herdman *et al.*, 1979). When DNA prepared from transformed (to achieve homologous recombination) cells is probed to distinguish wild type and mutant sequences, in most instances the introduced DNA is found in all chromosome copies (Brusslan and Haselkorn, 1989). It is not known whether this is due to random segregation of chromosomes, non-random segregation or

gene conversion. However in some cases, where the interrupted DNA region is vital, two populations of chromosomes can be maintained. The first contains the introduced DNA and the second remains "intact" (Murphy *et al.*, 1990; Gurevitz *et al.*, 1991).

1.6. THE AIMS OF THIS RESEARCH

Eukaryotic MTs have been extensively studied, but the precise functions of most of these molecules are not yet fully understood. The cyanobacterium *Synechococcus* PCC 7942 is amenable to genetic manipulation and may thus be a more tractable system in which to study the role of a Zn^{2+}-MT (CUP1, although in an amenable organism, does not bind Zn^{2+}).

The aim of this research was to generate mutants of *Synechococcus* PCC 7942 deficient in the MT divergon, *smt*, and thereby assign function to the prokaryotic MT gene, *smtA*, and the divergently transcribed gene *smtB*.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. *Escherichia coli* strains

The *Escherichia coli* (K12) strains used in this research were: JM101 [*supE* (*lac-proAB*) {F'*traD*36, *proAB*, *lacIqZ* M15} (rk+, mk+), *mcrA*(+)]; and DH5α [F'/*endAI*, *hsd*17 (rk-, mk+), *supE*44, *thi*1, *recA*1, *gyrA* (NaIr), *lacZYA*, *-argF*, U169, 80d*lac* (*lacZ* M15)]. Both strains were obtained from Northumbria Biologicals Ltd., Cramlington, U.K..

2.1.2. *Synechococcus* strains

R2-PIM8, a small plasmid- (pUH24) cured derivative of *Synechococcus* PCC 7942 (Pasteur Culture Collection) (Van der Plas *et al.*, 1990), was used throughout this research. The *metF* (5,10 methylene-tetra-hydrofolate-reductase) gene of R2-PIM8 is interrupted by an integration platform containing the *aad* (streptomycin resistance) gene.

Synechococcus DNA fragments used for genetic manipulation of R2-PIM8 were isolated from *Synechococcus* D 33 and *Synechococcus* D 839 (Durham University Culture Collection). These strains had been obtained from sources other than the Pasteur Culture Collection, although they were originally subcultured from similar stocks to the cultures held in the Pasteur Culture Collection. In this thesis (for simplicity), *Synechococcus* D 33 and *Synechococcus* D 839 are referred to by their Pasteur Culture Collection designations *Synechococcus* PCC 6301 and *Synechococcus* PCC 7942, respectively.

The taxonomy and origin of these strains is confused due to the different names applied to the same strain: *Synechococcus* PCC 6301, *Anacystis nidulans* TX-20, *Anacystis nidulans* UTEX 625, *Anacystis nidulans* UTEX 1550 and *Synechococcus leopoliensis* CCAP 1405/1 (all originate from the same isolate of Kratz and Myers (1955) and are therefore identical (Herdman, 1982)); *Synechococcus* PCC 7942, *Anacystis*

nidulans R2, *Anacystis nidulans* CALU 895, *Synechococcus* R2 and *Synechococcus leopoliensis* UTCC 100. *Synechococcus* PCC 6301 and *Synechococcus* PCC 7942 have been said to belong to one and the same species (Wilmotte and Stam, 1984), a major difference between the two strains being the superior transformation properties of *Synechococcus* PCC 7942 (Golden *et al.*, 1989). *Synechococcus* PCC 7942 is the organism of choice for genetic manipulation and can act as a representative host strain for *Synechococcus* PCC 6301 gene transformation studies (Golden *et al.*, 1989). *Anacystis nidulans* strains are often considered to adapt quickly to environmental change, thus it is likely that there are genetic differences among strains held by different collections (Whitton, 1992).

2.1.3. Plasmids

The plasmids used were: Bluescript KS^+, obtained from Boehringer Mannheim, Lewes, U.K. (a full description of this plasmid is given in Sambrook *et al.* (1989)); pSU19, a derivative of pSU2719 (Martinez *et al.*, 1988); and pLACPB2 (Scanlan *et al.*, 1990).

2.1.4. Chemicals, reagents and laboratory consumables

Suppliers were as follows:

Restriction enzymes, DNA modification enzymes, IPTG and Xgal- Northumbria Biologicals Ltd., Cramlington, U.K. or Boehringer Mannheim Ltd., Lewes, U.K..

Taq polymerase- Stratagene, Cambridge, U.K. or Promega Ltd., Southampton, U.K..

Radiochemicals and hybridisation membranes ("Hybond N")- Amersham International Ltd., Aylesbury, U.K..

Phosphoramidite derivatives of all nucleotide bases- Applied Biosystems, Warrington, U.K..

Sephadex G-50- Pharmacia LKB, Milton Keynes, U.K..

Nitrocellulose filter discs BA85 (0.45 μm)- Schleicher and Schluell, Dassel, Germany.

3MM chromatography paper, 2.5 cm GF/C filter discs- Whatman Ltd., Maidstone, U.K..

Electrophoresis grade agarose- BRL Ltd., Paisley, U.K..

Yeast extract and Bacto-Agar- Difco, Detroit, U.S.A..

Trypticase peptone- Beckton Dickinson, Maylan, France.

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

Phenol (redistilled)- International Biotechnologys Inc., Newhaven, U.S.A. or BRL Ltd., Paisley, U.K..

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

Other chemicals and antibiotics were supplied by Sigma Chemical Company, Dorset, U.K..

2.1.5. Metal salts

The following metal salts were used: $Ag(NO_3)$ (obtained from the Johnson Massey Technology Centre, Reading, U.K.), $CdCl_2.H_20$, $CuCl_2.2H_20$, $HgCl_2$, $ZnCl_2$.

2.2. MEDIA AND BUFFERS

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

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 below (prepared by "non-standard" procedures) or in individual protocols were as described by Sambrook *et al.* (1989).

2.2.1.1. NaI solution

This solution was used for the recovery of DNA onto silica fines (refer to section 2.3.1.4.).

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

2.2.2. Maintenance of *Escherichia coli* cultures

E. coli cells were grown in Luria-Bertani (LB) medium, terrific broth or 2 XL medium (Sambrook *et al.*, 1989) at 37 °C with constant shaking. Transformed cultures were supplemented with 100 μg ml^{-1} carbenicillin or 34 μg ml^{-1} chloramphenicol, as appropriate. Solid LB medium used for plating contained 1.5 % (w/v) agar. Cultures maintained in long term storage were frozen at -80 °C in 1 ml aliquots containing 50 % (v/v) glycerol.

Transformation and storage solution (TSS) for the preparation of competent cells was as described in Chung *et al.* (1989).

2.2.3. Maintenance of *Synechococcus* cultures

R2-PIM8 (a methionine auxotroph) was cultured under constant light (100 µmol of photon m^{-2} s^{-1} photosynthetically active radiation) at 32 °C in Allens medium (Allen, 1968) with 1.2 mg l^{-1} citric acid and the omission of $Na_2SiO_3.9H_20$, supplemented with 30 µg DL-methionine ml^{-1} and 5 µg streptomycin ml^{-1}. Transformed cultures were supplemented with 7.5 µg chloramphenicol ml^{-1} or 50 µg carbenicillin ml^{-1} as appropriate. Generally, cultures were maintained in 50 ml of liquid media. Larger culture volumes (500 ml) were aerated with filter sterile air. Solid medium for plating contained 1.5 % (w/v) agar. Cultures maintained in long term storage were frozen in liquid nitrogen in 1 ml aliquots, both with and without 10 % (v/v) DMSO. Modified Allens medium contains 0.77 µM Zn^{2+} and 0.32 µM Cu^{2+}.

Direct counts of cell numbers were achieved using a haemocytometer.

2.3. METHODS

2.3.1. GENERAL MOLECULAR BIOLOGY METHODS

Unless otherwise stated methods were performed as described by Sambrook *et al.* (1989).

2.3.1.1. Small scale plasmid isolation from *Escherichia coli*

Plasmid DNA was prepared from small (5 ml) overnight cultures of *E. coli* using the alkaline lysis extraction procedure described by Mierendorf and Pfeffer (1987).

2.3.1.2. Large scale plasmid isolation from *Escherichia coli*

Plasmid DNA was prepared from large (500 ml) overnight cultures of *E. coli* using the alkaline lysis procedure described:

Cells were collected by centrifugation at 1,000 *g* (r_{ave}) for 15 minutes (Beckman J2-21 centrifuge), and resuspended in 10 ml of a solution containing 50 mM glucose, 10 mM EDTA and 25 mM Tris.HCl (pH 8.0). The resuspended cells were transferred to two 30 ml glass corex tubes, and 10 ml of a solution containing 0.2 M NaOH and 1 % (w/v) SDS was added to each tube. The contents of the tubes were mixed gently by inversion and incubated on ice for 10 minutes. Ice cold potassium acetate (pH 4.8) (this solution is 3 M with respect to potassium and 5 M with respect to acetate, prepared by adding 11.5 ml glacial acetic acid and 28.5 ml H_2O to 60 ml 5 M potassium acetate), 7.5 ml, was then added to each tube, the tube contents were mixed by vigorous inversion (five or six times), and incubated on ice for 10 minutes. The tubes were then centrifuged at 12,000 *g* (r_{ave}) for 15 minutes (Beckman J2-21 centrifuge), the supernatant was transferred into fresh tubes and recentrifuged. The resulting supernatant was transferred to two further 30 ml glass corex tubes, and at least 0.6 volumes of isopropanol was added. The tube contents were mixed and incubated at room temperature for 15 minutes, the DNA precipitate was then collected by centrifugation at 12,000 *g* (r_{ave}) for 10 minutes (Beckman J2-21 centrifuge). The pellets were washed with 70 % (v/v) ethanol (70 %

(v/v) ethanol: 30 % (v/v) TE buffer (pH 8.0)) and dried in a vacuum desiccator. The dried DNA pellets were resuspended in 4 ml TE buffer (pH 8.0), the two solutions combined, and 8.6 g CsCl added. When the CsCl was completely dissolved 0.45 ml of a 10 mg ml^{-1} ethidium bromide solution was added. The solution was placed in two 0.5 x 2 inch quick-seal centrifuge tubes (a solution consisting of 8 ml H_2O and 8.6 g CsCl was used to top up the tubes when necessary), which were then heat sealed. The tubes were centrifuged at 230,000 *g* (r_{ave}) for 16 hours using a Sorval OTD65B ultracentrifuge. The nucleic acid bands within the tubes were visualised under ultra violet illumination and the plasmid band removed using a 10 ml syringe. The ethidium bromide was removed from the plasmid DNA by extraction with isoamyl alcohol saturated with CsCl, and salts removed by dialysis for 24 hours against TE buffer (pH 8.0). The plasmid DNA was then precipitated using 2.5 volumes of 100 % (v/v) ethanol at -20 °C (standard DNA precipitation protocol described by Sambrook *et al.* (1989)).

2.3.1.3. Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was performed as described by Sambrook *et al.* (1989).

Generally 0.8 % (w/v) agarose gels were used. However higher concentrations up to a maximum of 2 % (w/v) (as specified for each experiment) were used to separate small DNA fragments (to a minimum of 100 bp). Maxi-gels and mini-gels were cast using Tris-acetate buffer and Tris-borate buffer, respectively. DNA loading dye contained 0.25 % (w/v) each of bromophenol blue and xylene cyanol, and 15 % (w/v) Ficoll 400. Electrophoresed DNA was visualised by ethidium bromide staining.

2.3.1.4. Isolation of restriction fragments from agarose gels

Gel slices containing DNA fragments to be isolated were cut from agarose gels and placed in Eppendorf tubes. A volume of NaI solution three times the weight of the gel slice was added, and the tube incubated at 65 °C for 10 minutes (until all the agarose was melted). The tube was allowed to stand at room temperature for 5 minutes, and the DNA was recovered from solution by binding to 5 µl silica fines at room temperature for 10 minutes. The fines were pelleted at 12,000 *g* (r_{ave}) for 15 seconds in a

microcentrifuge (MSE Microcentaur), washed by resuspension in 1 ml 70 % (v/v) ethanol (70 % (v/v) ethanol: 30 % (v/v) TE buffer (pH 8.0)) and repelleted. The silica fines were dried in a vacuum dryer and the DNA was eluted into an appropriate volume of TE buffer (pH 8.0) at 37 °C for 10 minutes. The fines were pelleted at 12,000 *g* (r_{ave}) for 15 seconds in a microcentrifuge, and the supernatant containing the DNA was used in ligations or to make radiolabelled probes.

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

Competent *E. coli* cells were prepared and transformed according to the method of Chung *et al.* (1989). Aliquots of transformed cells were plated onto solid LB medium containing the appropriate antibiotic. Resulting colonies were screened using *in situ* hybridisation and/or restriction analysis of isolated plasmid DNA. Inserts were checked by sequence analysis.

2.3.1.6. *In situ* hybridisation of *Escherichia coli* colonies

The method used was based on that described by Sambrook *et al.* (1989). *E. coli* colonies were grown on nitrocellulose filter discs, and the filter discs were placed colony side up on a piece of 3MM paper saturated with 10 % (w/v) SDS for 3 minutes. The filter was transferred to 3MM paper saturated with denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes, then transferred to 3MM paper saturated with neutralisation solution (1.5 M NaCl, 0.5 M Tris.HCl (pH 8.0)) for 5 minutes. The filter was placed on a sheet of dry 3MM paper and allowed to dry at room temperature for 30 to 60 minutes before being baked for 90 minutes at 80 °C in a vacuum oven. Prior to hybridisation, filters were washed at 65 °C for 1-2 hours (with shaking) in 100 ml of washing solution (1 M NaCl, 1 mM EDTA, 0.1 % (w/v) SDS, 50 mM Tris.HCl (pH 8.0)). Prehybridisation and hybridisation were carried out as described in section 2.3.1.10..

2.3.1.7. Oligonucleotide Synthesis

Oligonucleotides were synthesised using an Applied Biosystems 381A DNA synthesiser operated with a standard synthesis programme. After cleavage and deprotection the

oligonucleotides were dried under vacuum, and resuspended in H_2O and vacuum dried twice. Oligonucleotides were stored at -20 °C either dry or as aqueous solutions, and were used without further purification.

2.3.1.8. Use of the polymerase chain reaction (PCR) for *in vitro* amplification of *smtA*

PCR reactions were carried out essentially as described by Saiki *et al.* (1988) with minor modifications by Fordham-Skelton *et al.* (1990). Genomic DNA isolated from R2-PIM8 was used as template. Reactions were carried out using a Hybaid Intelligent Heating Block and subjected to 30 cycles of; denaturation at 92 °C for 1.5 minutes, annealing at 55 °C for 1.5 minutes, and extension at 72 °C for 2 minutes. Control reactions lacking template DNA were also carried out.

2.3.1.9. Preparation of radiolabelled DNA probes

DNA probes were prepared from an *smtA* PCR product (described by Robinson *et al.* (1990)) and from restriction fragments derived from pJHNR49 (a *Sal*I/*Hin*dIII *smt* fragment from *Synechococcus* PCC 7942, in the vector pGEM4z (Huckle *et al.*, 1993)). Double stranded DNA fragments were radiolabelled by random priming using [α-^{32}P]dCTP with Klenow polymerase, according to the procedure of Feinberg and Vogelstein (1983). 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. ^{32}P-labelled probes were stored at -20 °C and boiled for 10 minutes immediately before use.

2.3.1.10. Hybridisation of radiolabelled DNA probes to filter-immobilised nucleic acids

All prehybridisation and hybridisation reactions were carried out in heat-sealed polythene bags which were contained in plastic boxes.

Southern blots and *in situ* filters - Filters were incubated for 1 hour at 65 °C in prehybridisation solution (6 x SSC, 1 x Denhardts solution, 0.5 % (w/v) SDS, 0.05 %

(w/v) PPi and 0.01 % (w/v) herring sperm DNA). The solution was replaced with 65 °C hybridisation solution (6 x SSC, 1 x Denhardts solution, 0.5 % (w/v) SDS, 0.05 % (w/v) PPi and 1 mM EDTA) and probe, and hybridisation was carried out for 16 hours at 65 °C.

Northern blots - Filters were incubated for 1 hour at 42 °C in prehybridisation solution (50 % (v/v) formamide, 5 x SSPE, 2 x Denhardts solution, 0.1 % (w/v) SDS and 0.01 % (w/v) herring sperm DNA), after which time probe was added (directly to the prehybridisation reaction). Hybridisation was carried out for 16 hours at 42 °C.

In all cases filters were washed to a final stringency of 0.5 x SSC , 0.1 % (w/v) SDS at 65 °C, after which the radioactive filter was exposed to X-ray film. Film cartridges were maintained at -80 °C for the required exposure time. After film development, the filter could be completely stripped of radioactivity by incubation at 90 °C in 0.1 % (w/v) SDS which allowed the filter to be re-probed as desired.

2.3.1.11. DNA sequence analysis

Plasmid sequencing was performed by the dideoxy-sequencing method of Sanger *et al.* (1977) using fluorescent dye-linked universal M13 primers. Sequences were analysed using an Applied Biosystems 370A DNA sequencer as described in the suppliers protocol (model 370A DNA sequencing system, Users Manual version 1.3A, October 1988, pp. 3.22-3.25).

2.3.2. INSERTIONAL INACTIVATION OF THE *smt* DIVERGON

The scheme for inactivation of the *smt* divergon is given in figure 3.1.2.. R2-PIM8 was transformed (refer to section 2.3.5.) with linearised plasmid pRECSU which consists of *smt* flanking sequences interrupted by *Escherichia coli* plasmid pSU19 containing the chloramphenicol acetyl transferase gene (*cat*). The *smt* flanking sequences in pRECSU, obtained from pJHNR49 (refer to section 2.3.1.9.), include a 1,064 bp *Pst*I/*Sal*I 3' fragment and a 340 bp *Hin*dIII/*Sac*I 5' fragment, cloned into the *Pst*I/*Sal*I and *Sma*I/*Sac*I (the protruding 5' ends from *Hin*dIII digestion were filled using the polymerase activity of Klenow polymerase, as described in Sambrook *et al.* (1989)) sites, respectively of the pSU19 polylinker (figure 3.1.2.). *Xba*I was subsequently used to linearise pRECSU prior to transformation.

2.3.3. TRANSFORMATION OF pRECSU TRANSFORMANTS WITH THE *smt* DIVERGON

A 1,775 bp *Sal*I/*Hin*dIII *smt* fragment from pJHNR49 (figure 3.1.2.) was used to transform pRECSU transformants, and recombinants were selected on Allens agar plates supplemented with 20 μM Zn^{2+}.

2.3.4. GENERATION OF RECOMBINANTS WITH FUNCTIONAL *smtA* AND NON-FUNCTIONAL *smtB*

Amplification (Gupta *et al.*, 1992) and specific rearrangement (Gupta *et al.*, 1993) of the *smt* divergon has been reported in *Synechococcus* PCC 6301 cells, selected for Cd^{2+} resistance by stepwise adaptation. Characterisation of *smt* from a Cd^{2+} tolerant cell line (C3.2) has revealed the functional deletion of *smtB*, conferring elevated expression from the *smtA* operator-promoter (Gupta *et al.*, 1993; refer to section 4.4.2.).

A 1,423 bp *Sal*I/*Hin*dIII *smt* fragment (*smtB*-/*smtA*+) obtained from pAGNR12a (a *Sal*I/*Hin*dIII *smt* fragment from C3.2, in the vector pGEM4z (Gupta *et al.*, 1993)), was

used to transform pRECSU transformants, and recombinants were selected on Allens agar plates supplemented with 20 μM Zn^{2+}.

2.3.5. TRANSFORMATION OF *SYNECHOCOCCUS* CELLS

Cells were transformed using chromosomal or plasmid DNA (linear and closed circular, as stated in individual methods), essentially as described by van den Hondel *et al.* (1980).

Cells were grown to mid/late log phase (3 x 10^7 cells ml^{-1}), and 1 x 10^9 cells were pelleted by centrifugation at 1,000 *g* (r_{ave}) (MSE bench centrifuge) for 10 minutes. The pelleted cells were "washed" with fresh Allens medium, repelleted, and resuspended in 1 ml fresh Allens medium. DNA, 1 μg, was added to 200 μl (2 x 10^8) cells, and the cells were incubated under standard growth conditions for 40 minutes (to allow DNA uptake) with occasional agitation.

Aliquots (175 μl and 25 μl) of transformed cells were plated on 50 ml Allens agar plates, and incubated under standard growth conditions (aliquots of non-transformed cells were also plated). After 16 hours, the Allens agar slab of each plate was lifted with a sterile pipette and 0.5 ml of 100 X stock of an appropriate selective agent was dispensed underneath. After a further incubation period of 5 to 10 days, single colonies were picked and re-streaked onto fresh plates containing the selective agent.

2.3.6. ISOLATION AND SOUTHERN ANALYSIS OF *SYNECHOCOCCUS* DNA

Genomic DNA was isolated from liquid cultures in late logarithmic to early stationary phase using part of a protocol described previously for the isolation of nucleic acids from plant cell cultures (Robinson *et al.*, 1988), but excluding CsCl gradients. Genomic Southern blotting was performed using 10 μg or 20 μg of DNA for each restriction digest, followed by standard agarose gel (0.8 % (w/v)) electrophoresis, and transfer to nylon filters (Sambrook *et al.*, 1989). Filters were baked for 90 minutes at 80 °C in a vacuum oven prior to hybridisation (refer to section 2.3.1.10.).

2.3.7. ISOLATION AND NORTHERN ANALYSIS OF *SYNECHOCOCCUS* RNA

Liquid cultures were grown to mid-logarithmic phase before incubation for 2 hours in the presence or absence of 2.5 μM Cd^{2+}. Total nucleic acids, isolated using standard techniques (Dzelkalns *et al.*, 1988), were analysed in 1.5 % (w/v) agarose gels and visualised with ethidium bromide to allow approximate quantification. Equivalent amounts of total nucleic acid from different cell extracts were denatured with formamide at 65 °C for 10 minutes and electrophoresed in a 1.5 % (w/v) agarose gel containing formaldehyde (prepared and run as described by Sambrook *et al.* (1989)). Ribosomal RNA bands were used as approximate size markers for these gels. The size-separated RNA was transferred to nylon filters (Sambrook *et al.*, 1989), and the filters were baked for 90 minutes at 80 °C in a vacuum oven prior to hybridisation (refer to section 2.3.1.10.).

2.3.8. RECOVERY OF INTEGRATED PLASMID FROM pRECSU TRANSFORMANTS

Genomic DNA, isolated (refer to section 2.3.6.) from pRECSU transformants, was digested to completion with *SalI* to release a 7.8 kb fragment containing integrated plasmid pSU19. Total restricted DNA was ligated at low DNA concentrations ($\leq$ 0.01 μg μl^{-1}) to favour circularisation, and used to transform *Escherichia coli* to chloramphenicol resistance. Plasmid DNA was isolated from *E. coli* transformants using standard procedures (section 2.3.1.1.), and analysed as described in section 3.1.4..

2.3.9. MEASUREMENT OF METAL ION CONCENTRATIONS

Metal ion concentrations in solution were measured using atomic absorption spectrophotometry (AAS). This was carried out using a Perkin Elmer Model HGA spectrophotometer, and analyses were performed according to manufacturer's protocols. Three replicate readings, each of 3 seconds duration, were taken automatically for each

sample, and the mean determined. The metal ion concentration was quantified via the construction of a calibration graph, which was re-plotted for each set of measurements.

2.3.10. PHENOTYPIC ANALYSIS OF *SYNECHOCOCCUS* CULTURES WITH RESPECT TO METAL TOLERANCE

Synchronous logarithmic *Synechococcus* cultures were grown in Allens medium supplemented with $ZnCl_2$, $CdCl_2$, $CuCl_2$, $Ag(NO_3)$ or $HgCl_2$ under standard growth conditions (R2-PIM8(*smt*) was cultured without chloramphenicol supplementation, and integrated *cat* was stable (data not shown)), to determine the minimum inhibitory/maximum permissive concentrations of these metal salts. Cultures were maintained in 5 ml of liquid medium (in boiling tubes), and shaken twice daily. Cells were inoculated (in triplicate) at a density of 1×10^6 cells ml^{-1} and monitored for visible growth daily for 14 days, assays were carried out on at least three separate occasions (at least 9 analyses). Significant differences in the numbers of cultures surviving at a particular metal ion concentration were assessed using the significance testing tables for small sample sizes produced by Finney *et al.* (1963) (Wardlaw, 1985). Significant differences were established at 5 % ($P_{5\%}$) and 1 % ($P_{1\%}$) probability levels, using one tailed tests.

Subsequent experiments quantified the effects on growth of selected (from the previous experiment) concentrations of metal ions as a function of time by measuring optical density of the cultures at 540 nm, using a micro-titre plate reader.

2.3.11. PHENOTYPIC ANALYSIS OF *SYNECHOCOCCUS* CULTURES WITH RESPECT TO METAL TOLERANCE FOLLOWING PRETREATMENT WITH METAL SALTS

$ZnCl_2$ or $CdCl_2$ was added to synchronous logarithmic *Synechococcus* cultures (growing under standard conditions) to a final concentration of 8 μM Zn^{2+} or 1 μM Cd^{2+}. Cultures were maintained under these conditions (and with no added metal) for three days, after which time cells were pelleted by centrifugation at 1,000 *g* (r_{ave}) (MSE bench

centrifuge) for 10 minutes. The pelleted cells were "washed" with fresh Allens medium, repelleted, and inoculated (in triplicate) at a density of 1 x 10^6 cells ml^{-1} in Allens medium supplemented with selected concentrations of $ZnCl_2$ or $CdCl_2$. Growth of these cultures was then estimated as a function of time (refer to section 2.3.10.).

2.3.12. EXAMINATION OF ZINC ACCUMULATION BY *SYNECHOCOCCUS* CULTURES

Equivalent numbers of cells (estimated from measurements of optical density at 540 nm) from mid/late logarithmic *Synechococcus* cultures were exposed to a range of concentrations of $ZnCl_2$ for up to 2 hours under standard growth conditions (R2-PIM8(*smt*) was cultured without chloramphenicol supplementation). Zn^{2+} exposed cells were collected by centrifugation at 5 000 *g* (r_{ave}) (Beckman JA-20 rotor) for 10 minutes, and resuspended in 20 ml Tris.HCl (pH 7.8) or Tris.HCl (pH 7.8) and 0.1 mM EDTA (to remove loosely bound Zn^{2+}). Cells were repelleted and solubilised overnight by incubation at 37 °C in 1 ml 70 % (v/v) nitric acid. Zn^{2+} concentrations were determined by AAS, and converted to Zn^{2+} content per 1 x 10^9 cells (assuming an optical density of 1 at 540 nm is equivalent to 3 x 10^8 cells ml^{-1}). Optical densities at 540 nm were taken immediately prior to centrifugation and compared to those taken at the time of metal induction. No changes in optical density were observed during the incubation period.

2.3.13. CONSTRUCTION OF *smt-lacZ* FUSIONS

PCR was used to generate ca. 600 bp of *smtA* 5' flanking region (including *smt* operator-promoter sequences and *smtB*), introducing a *Bam*HI site at the 3' end of the PCR product (described by Huckle *et al.*, 1993). The ca. 600 bp fragment was cloned into the promoter probe shuttle vector pLACPB2 (Scanlan *et al.*, 1990), which carries a promoterless *lacZ*, creating a transcriptional fusion with *lacZ*. The resulting construct was designated pLACPB2(*smt*-5').

A derivative of pLACPB2(*smt*-5') was generated by ligating ca. 100 bp *Pst*I/*Bam*HI fragment from pLACPB2(*smt*-5'), carrying the *smt* operator-promoter region, into

Bluescript KS^+ cut with *PstI*/*Bam*HI, to create pKS(*smtB*-). Subsequently, a similar fragment was excised on a *SalI*/*Bam*HI fragment from pKS(*smtB*-) and cloned into pLACPB2, creating a transcriptional fusion with *lacZ*. The resulting construct was designated pLACPB2(*smtB*-) (figure 3.5.1.).

2.3.14. TRANSFORMATION OF *SYNECHOCOCCUS* CELLS WITH *smt-lacZ* FUSIONS

The vector pLACPB2 contains sequences allowing independent replication in both *Synechococcus* and *Escherichia coli*, and carries *cat* and the β-lactamase gene (*bla*) conferring resistances to chloramphenicol and carbenicillin respectively. *Synechococcus* cultures were transformed (refer to section 2.3.5.) with 1 μg of pLACPB2, pLACPB2(*smt*-5') or pLACPB2(*smtB*-) (as described in section 2.3.13.). In the first instance transformants were selected on Allens agar plates supplemented with 7.5 μg ml^{-1} chloramphenicol and 1 μg ml^{-1} carbenicillin. However introduced pLACPB2 constructs were not stable in *Synechococcus* strains containing integrated, genomic, *cat* (refer to section 2.3.2.), and it was found that both non-transformed and transformed (with pLACPB2 constructs) were tolerant to 1 μg ml^{-1} carbenicillin. The antibiotic concentration often stated in relevant literature for selection of *bla* transformants is $\leq$ 1 μg ml^{-1} (van den Hondel *et al.*, 1980; Kuhlemeier and Van Arkel, 1987; Golden and Sherman, 1983; Williams and Szalay, 1983). Both pLACPB2 transformed and non-transformed *Synechococcus* cultures were exposed to a range of carbenicillin concentrations. pLACPB2 transformed cultures were tolerant to 50 μg ml^{-1} carbenicillin, and no growth of non-transformed cultures was observed at this concentration following 7 days incubation. pLACPB2 transformed cells were thereafter selected and maintained with carbenicillin supplemented at 50 μg ml^{-1}.

2.3.15. RECOVERY OF EXTRACHROMOSOMALLY REPLICATING SHUTTLE PLASMID FROM *SYNECHOCOCCUS* CELLS

To check that pLACPB2 constructs introduced into *Synechococcus* cells had not undergone rearrangement, plasmids were recovered from transformants as described:

Cells were collected from 20 ml transformed culture (late logarithmic) by centrifugation at 1,000 *g* (r_{ave}) (MSE bench centrifuge) for 10 minutes. The cell pellet was resuspended in 400 µl TE buffer (pH 8.0), and the cells lysed by the addition of an equal volume of phenol (saturated with TE buffer (pH 8.0)) followed by mixing with a vortex mixer for 30 seconds. The mixture was separated for 2 minutes at 12,000 *g* (r_{ave}) in a microcentrifuge, and the upper (aqueous) layer was transferred to a fresh tube. The plasmid DNA was precipitated with 0.2 volumes of ammonium acetate and two volumes of isopropanol at room temperature for 15 minutes. The precipitate was collected by spinning at 12,000 *g* (r_{ave}) for 10 minutes in a microcentrifuge, and the DNA pellet washed with 70 % (v/v) ethanol (70 % (v/v) ethanol: 30 % (v/v) TE buffer (pH 8.0)). The pellet was dried in a vacuum desiccator and resuspended in 20 µl of TE buffer (pH 8.0). 10 µl was used to transform *Escherichia coli* cells to chloramphenicol and carbenicillin resistance (as described in 2.3.1.5.).

Plasmid DNA was isolated from *E. coli* transformants using standard procedures (section 2.3.1.1.), and analysed by restriction digestion and sequencing.

2.3.16. DETERMINATION OF β-GALACTOSIDASE ACTIVITY

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

Logarithmic (synchronous) *Synechococcus* cultures were grown under standard conditions until their optical densities at 595 nm (using a micro-titre plate reader) were ca. 0.2 (optical densities at 595 nm were recorded). Aliquots, 0.5 ml, of cultures were induced with various concentrations of $ZnCl_2$ or $CdCl_2$ for 2 hours prior to being assayed for β-galactosidase activity.

β-galactosidase activity was assayed using ONPG as the substrate, as described by Miller (1972). ONPG (colourless) is hydrolysed to galactose and o-nitrophenol (yellow) in the presence of β-galactosidase. Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol, pH 7.0), 0.5 ml, was added to 0.5 ml of

induced culture, and the cells were lysed by the addition of 1 drop 0.1 % (w/v) SDS and 2 drops chloroform followed by mixing with a vortex for 10 seconds.

Assays were carried out in triplicate using micro-titre plates, 176 µl of lysed cell solution was added to 35 µl of ONPG 4 mg ml^{-1} (in 0.1 M phosphate buffer, pH 7.0) and incubated at room temperature for a recorded time (t) over which a yellow colouration (o-nitrophenol) developed. Reactions were then terminated by the addition of 88 µl of 1 M Na_2CO_3, and optical densities at 414 nm and 540 nm of each terminated reaction were measured. The reactions were blanked against one of three values obtained for R2-PIM8 containing pLACPB2 alone, not exposed to metal ions.

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

$$\text{Units} = 1000 \times \frac{\text{OD 414 nm} - 1.75 \times \text{OD 540 nm}}{\text{t x v x OD 595 nm}}$$

OD = optical density,

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

t = the time of the reaction (minutes).

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

β-galactosidase activity was also measured using a modification to the protocol described above. Changes in the pigmentation of cultures were observed upon the addition of metal ions and could affect the results of β-galactosidase assays. This was not taken into account using the protocol of Miller (1972), but was taken into account in the modified protocol. The activity was calculated based upon the rate of change in ONP production for each sample individually. Assays performed using the protocol of Miller (1972) had been done in triplicate, and the triplicates did not show a large variation. The modified protocol involved two assays for each sample and would be unmanageable if done in triplicate, although assays were performed on three separate occasions (results could not be pooled to calculate means and standard deviations as activity rates vary from day to day (although trends remain the same) due to uncontrolled variables such as temperature).

Synechococcus cultures were grown until their optical densities at 595 nm were ca. 0.2 (as in section 2.3.16.1.) or ca. 0.08. Cultures were then induced with metal ions and lysed as described previously (section 2.3.16.1.). Lysed cell solution, 176 µl, was added to 35 µl of ONPG 4 mg ml^{-1} (in 0.1 M phosphate buffer, pH 7.0) in a micro-titre plate. Two reactions were carried out for each sample, one of which was terminated by the addition of 88 µl of 1 M Na_2CO_3 at time 0. The remaining reactions were incubated at room temperature for a recorded time (t) over which a yellow colouration developed. These reactions were then terminated by the addition of 88 µl of 1M Na_2CO_3 (time t). The optical density at 414 nm of each terminated reaction was measured using a micro-titre plate reader. The reactions were blanked against H_20.

Activity was calculated using the equation:

$$\text{Units} = \frac{300 \times [\text{OD 414 nm (t=t)} - \text{OD 414 nm (t=0)}]}{1.83 \times (t \times v \times \text{OD 595 nm})}$$

Units = nmoles o-nitrophenol/min/mg protein.

OD = optical density,

t=t = the optical density at 414 nm of the reaction terminated at time t,

t=0 = the optical density at 414 nm of the reaction terminated at time 0,

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

t = the time of the reaction (minutes).300 nmoles of ONP = 1 optical density unit at 414 nm (calculated from a standard curve),

1/1.83 = the optical density at 595 nm of a *Synechococcus* culture that is equivalent to 1 mg of protein (calculated assuming that 10^9 cells yields approximately 150 µg of protein).

CHAPTER 3

RESULTS

3.1. INTERRUPTION OF THE *smt* DIVERGON

3.1.1. Evidence of chromosomal localisation of the *smt* divergon

Synechococcus PCC 7942 has two indigenous plasmids, pUH24 (pANS) and pUH25 (pANL) of ca. 8 kb and ca. 49 kb, respectively (Lau and Dolittle, 1979; van den Hondel *et al.*, 1980; Laudenbach *et al.*, 1983).

Southern analysis of R2-PIM8 DNA, probed with *smtA*, identified the gene on unique *Sal*I, *Hin*dIII and *Bam*HI fragments (figure 3.1.1.), confirming its presence in the small plasmid-cured strain. Furthermore, the sizes of the *smtA* containing restriction fragments do not correspond to the known sizes of *Sal*I, *Hin*dIII and *Bam*HI restriction fragments of pUH25 DNA (Laudenbach *et al.*, 1983), indicating *smtA* to be chromosomal.

Probing of pLANB2 (an *Escherichia coli* plasmid carrying pUH24 (Laudenbach *et al.*, 1983)) and pLANBa1 to 7 (*E. coli* plasmids carrying the seven *Bam*HI restriction fragments of pUH25 (Laudenbach *et al.*, 1983)) with *smtA*, confirmed the chromosomal localisation of the *smt* divergon (observations of A. Gupta; Turner *et al.*, 1993).

In addition, to pUH24 and pUH25, there is some suggestion that *Synechococcus* PCC 7942 carries a megaplasmid of ca. 1,000 kb (Rebière *et al.*, 1986). Literature detailing the presence of a megaplasmid in *Synechococcus* PCC 7942 is limited, with only one manuscript detailing its existence (and is cited in several reviews). There is no published data detailing the mapping or isolation of the proposed megaplasmid, and in many reports *Synechococcus* PCC 7942 is considered to only carry two indigenous plasmids of ca. 8 kb and ca. 49 kb (pUH24 and pUH25, respectively) (van den Hondel *et al.*, 1980; Laudenbach *et al.*, 1983; Engwall and Gendel, 1985; Van der Plas *et al.*, 1992). *smt* will be considered chromosomal, with the reservation that this designation could be changed if further evidence of a megaplasmid is reported for this strain.

3.1.2. Insertional inactivation of the *smt* divergon

The method employed to generate *smt* deficient mutants of R2-PIM8 was insertional inactivation/partial gene deletion mediated by homologous recombination.

The plasmid pRECSU (figure 3.1.2.), containing *smt* flanking sequences interrupted by *Escherichia coli* plasmid pSU19, was generated, and diagnostic restriction analysis of pRECSU is shown (figure 3.1.3.). The *smt* flanking sequences in pRECSU are separated by 371 bp in the *smt* divergon which includes the *smtA* and *smtB* transcription/translation start sites and operator-promoter sequences (refer to section 1.3.), which are therefore absent from pRECSU. The orientation of the cloned *Synechococcus* PCC 7942 DNA fragments in pRECSU were confirmed by sequencing using both forward and reverse M13 primers.

R2-PIM8 was transformed to chloramphenicol resistance with linearised pRECSU. Stable chloramphenicol resistant (Cm^r) transformants (> 100) were selected on Allens agar plates containing 7.5 μg ml^{-1} chloramphenicol. After several rounds of streaking to isolate cells homozygous for an interrupted *smt* divergon, a number of transformants were grown in liquid culture and then plated to obtain single colonies. Resulting colonies were inoculated into liquid culture for analyses.

3.1.3. Confirmation of *smt* structure in pRECSU transformants (R2-PIM8(*smt*) and R2-PIM8(*smt*).2 to .4)

The structure and homozygosity (of the *smt* region) of pRECSU transformants were confirmed by Southern analyses (figures 3.1.4., 3.1.5. and 3.1.6.). Four clones were examined and are hereafter referred to as R2-PIM8(*smt*) (used for subsequent analyses) and R2-PIM8(*smt*).2 to .4.

A ca. 5.8 kb *SalI smt* fragment in R2-PIM8 DNA was not detected in R2-PIM8(*smt*) (and R2-PIM8(*smt*).2 to .4) DNA probed with part of a diagnostic-deletion region (a 213 bp *PstI* fragment) (figure 3.1.4., *panel A*). Upon prolonged exposure a faint band was visible at ca. 7.8 kb in R2-PIM8(*smt*) (and R2-PIM8(*smt*).2 to .4) DNA (figure 3.1.4., *panel B*), and was considered to be due to weak cross-hybridisation to pSU19 (figure 3.1.4., *panel C*).

A ca. 0.9 kb *Pst*I fragment containing *smtB* in R2-PIM8 DNA and a ca. 3.1 kb *Pst*I fragment in R2-PIM8(*smt*) (and R2-PIM8(*smt*).2 to .4) DNA were detected upon probing with retained *smtB* sequences (figure 3.1.5.). The latter corresponds to the anticipated size of the *smt* divergon containing pSU19 with concomitant deletion of a 371 bp region.

Probing with plasmid pSU19 identified ca. 3.1 kb *Pst*I, ca. 2.7 kb *Hin*dIII and ca. 7.8 kb *Sal*I fragments in R2-PIM8(*smt*) (and R2-PIM8(*smt*).2 to .4) DNA as expected, confirming the chromosomal site of integration of pRECSU (figure 3.1.6.). An "anomalous" additional band (also observed in R2-PIM8 DNA and double digested R2-PIM8(*smt*) DNA) was thought to result from homology between the probe and other genomic sequences.

The structure of the interrupted *smt* divergon was the same in DNA isolated from R2-PIM8(*smt*) cultured in the presence or absence of 1.5 μM Cd^{2+} (figures 3.1.4., 3.1.5. and 3.1.6.), confirming genetic stability of R2-PIM8(*smt*) with no reversion (involving undetected, retained *smt* sequences) detected upon metal exposure.

3.1.4. Plasmid recovery from R2-PIM8(*smt*)

A ca. 7.8 kb plasmid (pJSTNR4.1) was generated from a *Sal*I fragment of R2-PIM8(*smt*) DNA via ligation at low DNA concentrations and plasmid recovery in *Escherichia coli*. The restriction pattern (figure 3.1.7., *panel A*) is that expected for digestion of DNA containing the interrupted *smt* divergon. Diagnostic probing of digested pJSTNR4.1 further confirmed its structure (figure 3.1.7., *panels B*, *C* and *D*). The *smt*-derived regions of pJSTNR4.1 were sequenced using the M13 forward and reverse primer sites within pSU19. The sequences were identical to the known *Synechococcus* PCC 7942 sequences confirming no rearrangement of these regions during homologous recombination, and further confirming the correct site of integration of pRECSU.

Cloned restriction fragments of pJSTNR4.1 have enabled the determination of further R2-PIM8 chromosomal sequences to the 5' of the *smt* divergon (data not shown). Identified ORFs, showed no "substantial" sequence similarity to sequences in the GenBank DNA sequence database.

3.1.5. Analysis of *smtA* transcript abundance

Maximal induction of *smtA* transcripts has been observed after a 2 hour exposure of *Synechococcus* PCC 6301 cells to 2.5 μM Cd^{2+} (Huckle *et al.*, 1993). *smtA* transcripts were only detected in RNA isolated from Cd^{2+} exposed R2-PIM8, and not R2-PIM8(*smt*) (figure 3.1.8.), consistent with the *smt* mutant status of the latter.

Figure 3.1.1. Southern analysis of R2-PIM8 genomic DNA, probed with *smtA*, confirming the presence of *smtA* in a small plasmid-cured strain of *Synechococcus* PCC 7942

Total nucleic acid from R2-PIM8 was digested with *Sal*I (*lane 1*), *Hin*dIII (*lane 2*) and *Bam*HI (*lane 3*). The digested DNA was electrophoresed in a 0.8 % (w/v) agarose gel, transferred to a nylon filter, and probed with *smtA*.

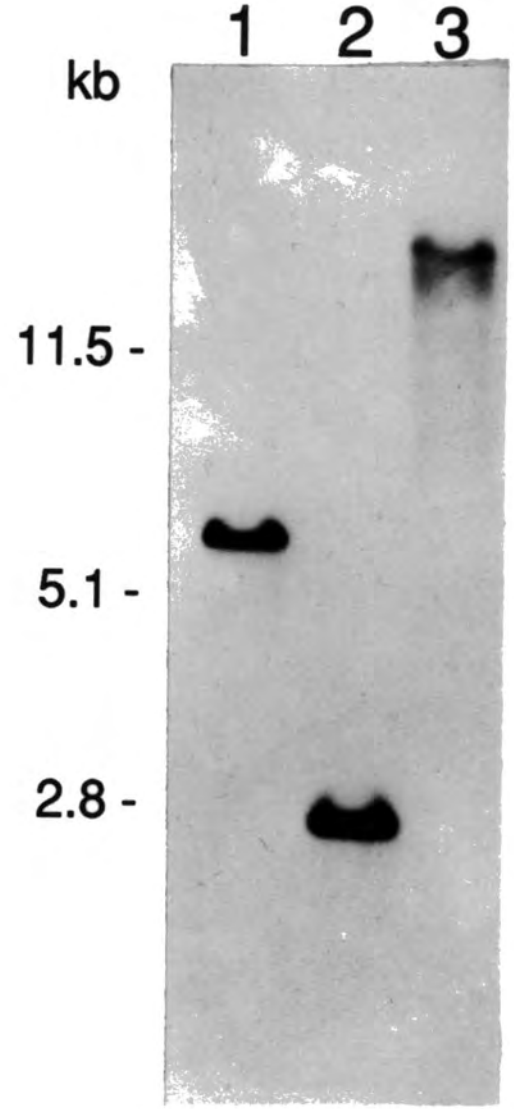

1
2
3
kb
11.5 -
5.1 -
2.8 -

Figure 3.1.2. Insertional inactivation of the *smt* divergon

Panel A: A 1,775 bp *Hin*dIII/*Sal*I fragment of *Synechococcus* PCC 7942 chromosomal DNA including the 168 bp protein coding region of *smtA* (*narrow diagonal shading*) and the 366 bp coding region of *smtB* (*wide diagonal shading*) was obtained from a size-fractionated *Sal*I/*Hin*dIII genomic library. A 1,064 bp 3' *smt* flank (*long arrow*) and a 340 bp 5' *smt* flank (*short arrow*) were cloned into pSU19 to generate pRECSU. The orientation of the cloned *smt* sequences resulted in the interruption by the vector DNA (including *cat*) of the *Synechococcus* PCC 7942 *smt* flanking sequences. The *smt* flanking sequences in pRECSU are separated by 371 bp in the *smt* divergon which includes the *smtA* and *smtB* transcription and translation start sites and operator-promoter sequences, which are therefore absent from pRECSU. *Xba*I was used to linearise pRECSU prior to transformation of R2-PIM8.

Panel B: The *upper diagram* represents the *smt* divergon of R2-PIM8. Transformation with linearised pRECSU should result in homologous recombination with *cat* directed into the *smt* divergon (*lower diagram*).

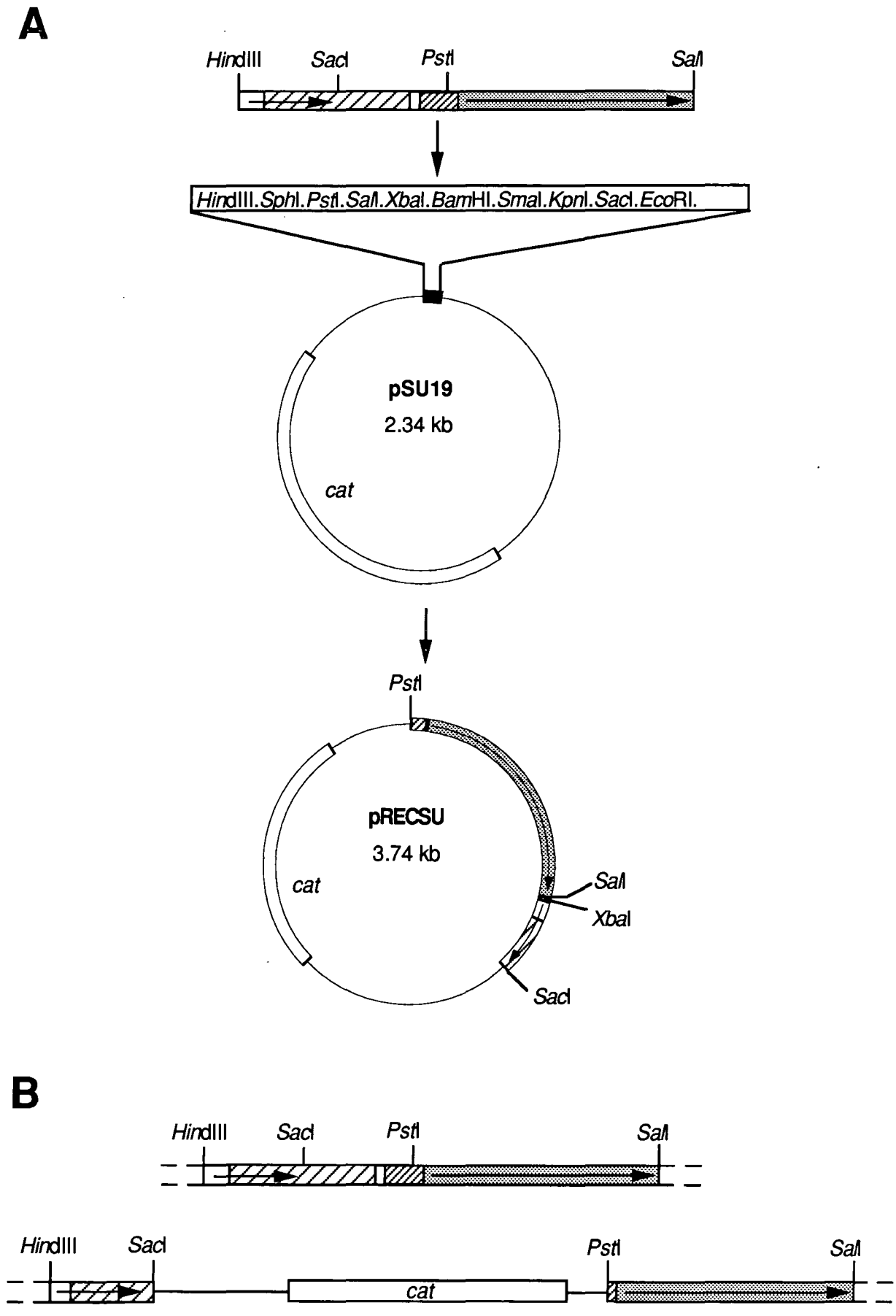

A
HindIII
SacI
PstI
SalI
HindIII.SphI.PstI.SalI.XbaI.BamHI.SmaI.KpnI.SacI.EcoRI.
pSU19
2.34 kb
cat
PstI
pRECSU
3.74 kb
cat
SalI
XbaI
SacI
B
HindIII
SacI
PstI
SalI
HindIII
SacI
PstI
SalI
cat

Figure 3.1.2b. Restriction map of the *smt* divergon

The protein coding regions of thedivergent genes *smtA* (*narrow diaganal shading*) and *smtB* (*wide diaganal shading*) are shown. The 100 bp operator-promoter region is expanded to show -10 sequences (underlined), Shine-Dalgarno (S.D.) sequences (underlined), determined transcript start sites (bent arrows) and inverted/direct repeats (under/over-lined).

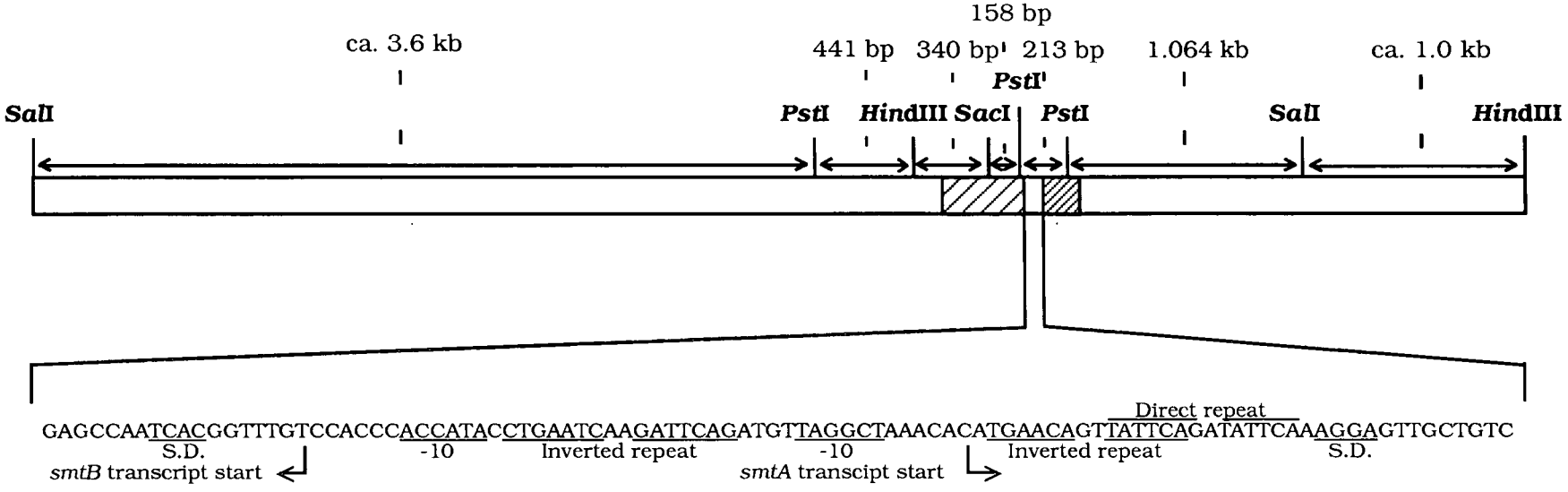
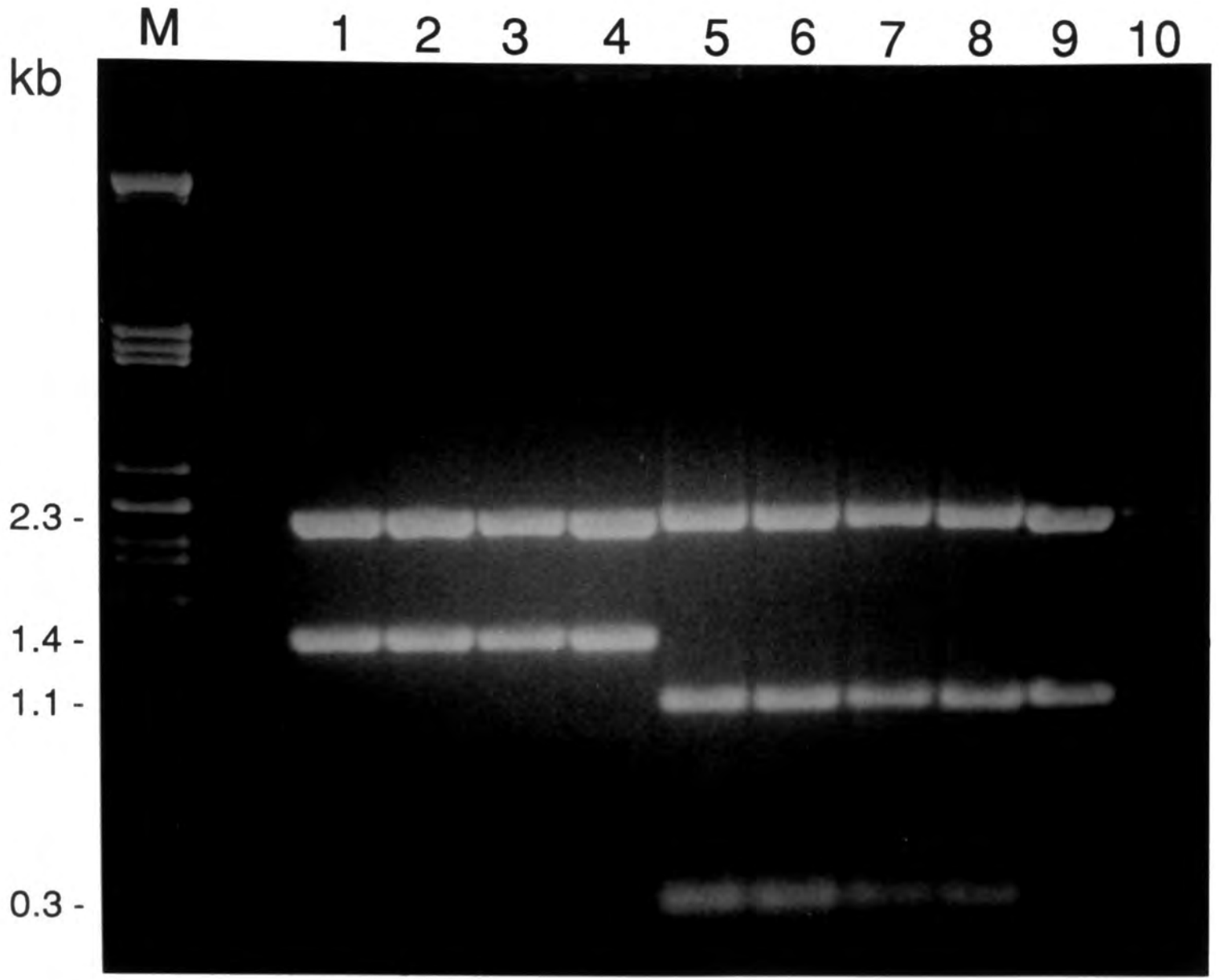

158 bp
ca. 3.6 kb
441 bp
340 bp
213 bp
1.064 kb
ca. 1.0 kb
PstI
SalI
PstI
HindIII
SacI
PstI
SalI
HindIII
Direct repeat
GAGCCAATCACGGTTTGTCCACCCACCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGATATTCAAAGGAGTTGCTGTC
S.D.
-10
Inverted repeat
-10
Inverted repeat
S.D.
smtB transcript start
smtA transcipt start

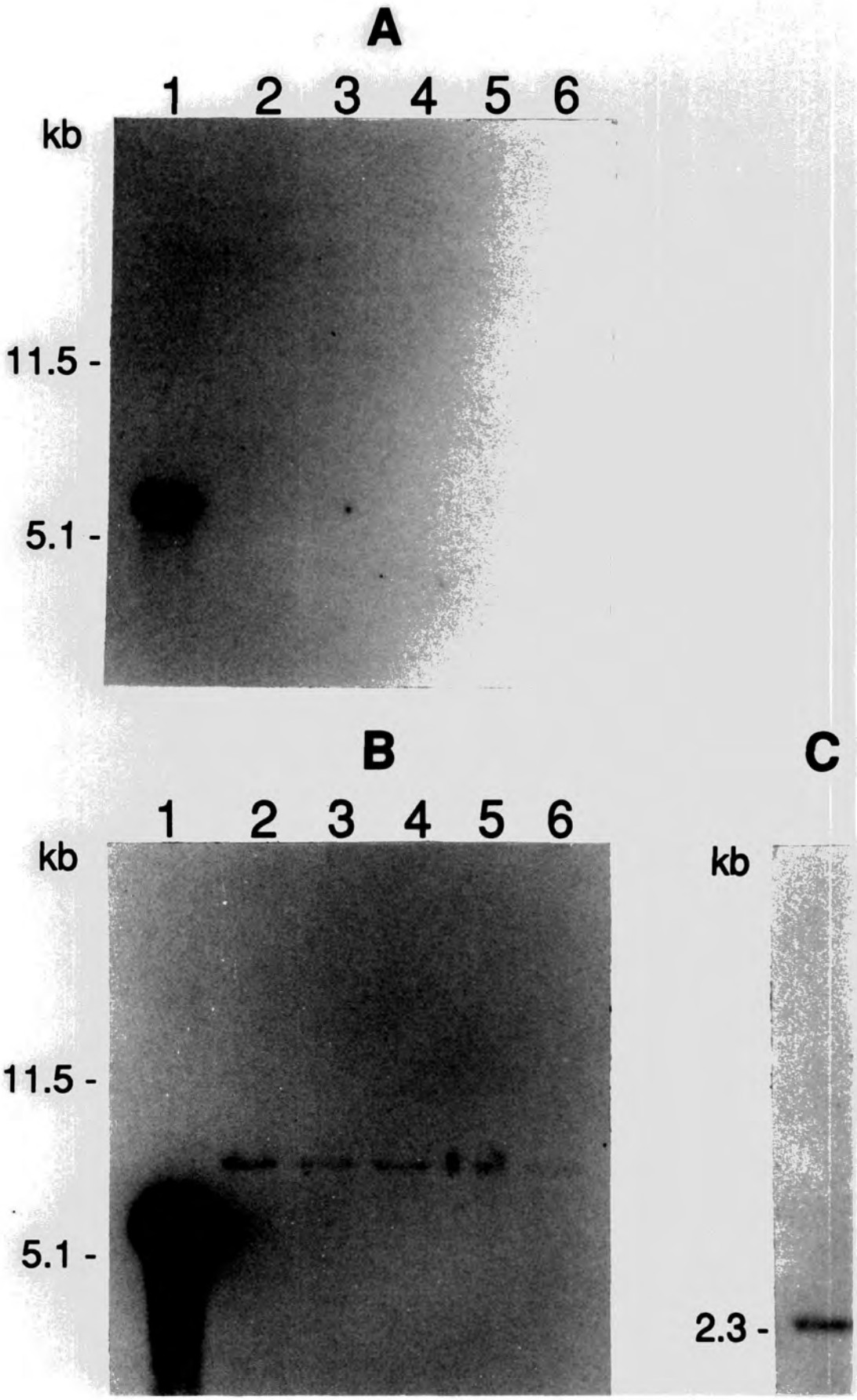
Figure 3.1.3. Diagnostic restriction analysis of pRECSU

Plasmid pRECSU consisting of *smt* 3' and 5' flanking sequences interrupted by *Escherichia coli* plasmid pSU19, isolated from four different clones, was digested with *Hin*dIII/*Sac*I (*lanes 1* to *4*) and *Hin*dIII/*Xba*I/*Sac*I (*lanes 5* to *8*). Plasmid pJSTNR3.1 consisting of pSU19 interrupted by *smt* 3' flanking sequences was digested with *Hin*dIII/*Sac*I (*lane 9*), and pSU19 was digested with *Sac*I (*lane 10*). *Lane M*, *Pst*I digested lambda DNA. Digested DNA was electrophoresed in a 0.8 % (w/v) agarose gel.


M
1
2
3
4
5
6
7
8
9
10
kb
2.3 -
1.4 -
1.1 -
0.3 -

3.1.4. Southern analysis of genomic DNA from R2-PIM8 and pRECSU transformants, R2-PIM8(*smt*) and R2-PIM8(*smt*).2 to .4, probed with part of a diagnostic-deletion region

Panels A and *B*: *Sal*I digested DNA from R2-PIM8 (*lane 1*), R2-PIM8(*smt*).2 to .4 (*lanes 2,3* and *4*), R2-PIM8(*smt*) (*lane 6*) and R2-PIM8(*smt*) cultured in the presence of 1.5 μM Cd^{2+} (*lane 5*). *Panel C*: Linearised pSU19 DNA. The digested DNA was electrophoresed in 0.8 % (w/v) agarose gels, transferred to nylon filters, and probed with part of a 371 bp diagnostic-deletion region. *Panel B* shows the probed filter in *panel A* after prolonged exposure to film.


A
1 2 3 4 5 6
kb
11.5 -
5.1 -
B
1 2 3 4 5 6
kb
11.5 -
5.1 -
C
kb
2.3 -

3.1.5. Southern analysis of genomic DNA from R2-PIM8, R2-PIM8(*smt*) and R2-PIM8(*smt*).2 to .4, probed with retained *smtB* sequences

*Pst*I digested DNA from R2-PIM8 (*lane 1*), R2-PIM8(*smt*).2 to .4 (*lanes 2,3* and *4*), R2-PIM8(*smt*) (*lane 6*) and R2-PIM8(*smt*) cultured in the presence of 1.5 μM Cd^{2+} (*lane 5*), was electrophoresed in a 0.8 % (w/v) agarose gel, transferred to a nylon filter, and probed with retained *smtB* sequences.

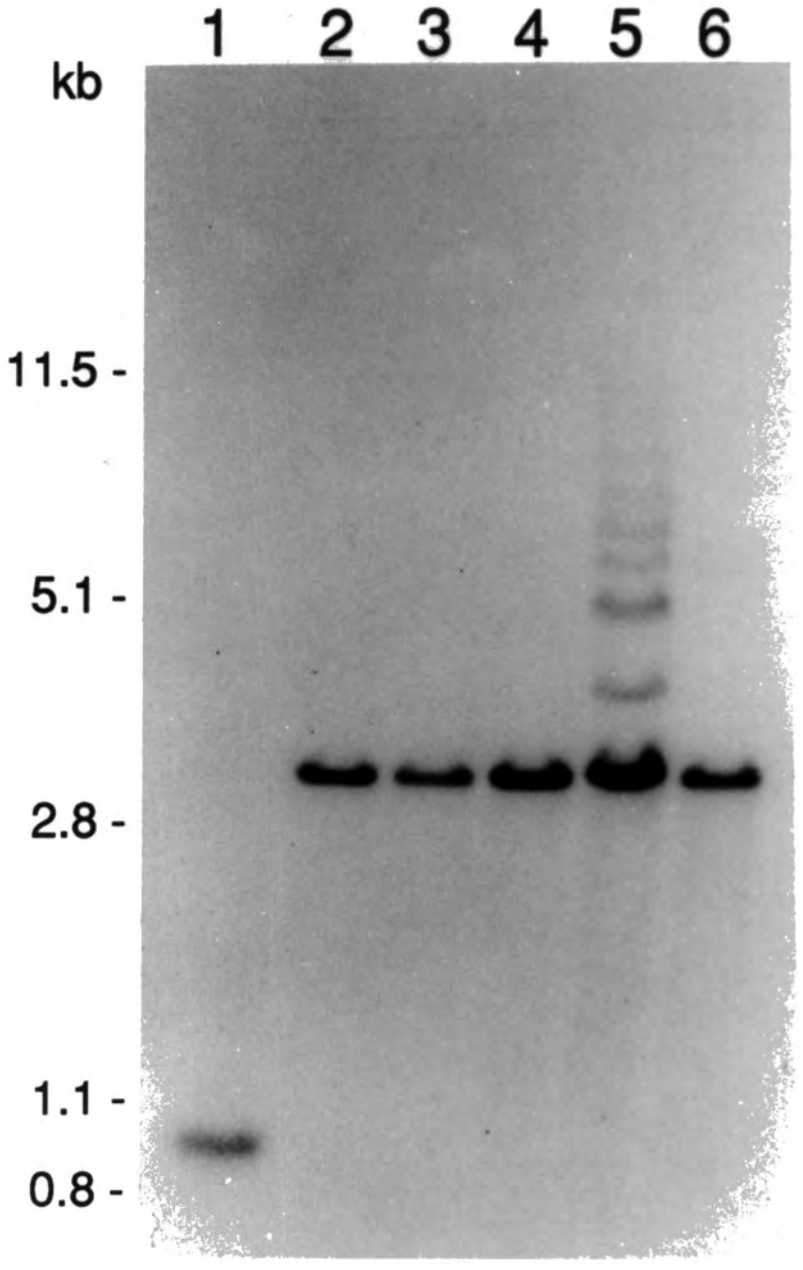

1
2
3
4
5
6
kb
11.5 -
5.1 -
2.8 -
1.1 -
0.8 -

Figure 3.1.6. Southern analyses of genomic DNA from R2-PIM8, R2-PIM8(*smt*) and R2-PIM8(*smt*).2 to .4, probed with pSU19

Panel A: *Sal*I digested R2-PIM8 DNA (*lane 1*), *Pst*I digested R2-PIM8(*smt*) DNA (*lane 2*), *Hin*dIII digested R2-PIM8(*smt*) DNA (*lane 3*) and *Sal*I digested R2-PIM8(*smt*) DNA (*lane 4*). *Panel B*: R2-PIM8(*smt*) DNA digested with *Pst*I (*lane 1*), *Sal*I/*Pst*I (*lane 2*), *Hin*dIII (*lane 3*) and *Sal*I/*Hin*dIII (*lane 4*). *Panel C*: DNA from R2-PIM8(*smt*).2 to .4 (*lanes 1* to *9*) and R2-PIM8(*smt*) cultured in the presence of 1.5 μM Cd^{2+} (*lanes 10* to *12*), digested with *Sal*I (*lanes 1*, *4*, *7* and *10*), *Hin*dIII (*lanes 2*, *5*, *8* and *11*) and *Pst*I (*lanes 3*, *6*, *9* and *12*). *Panel D*; R2-PIM8 DNA digested with *Sal*I (*lane 1*), *Hin*dIII (*lane 2*) and *Pst*I (*lane 3*). The digested DNA was electrophoresed in 0.8 % (w/v) agarose gels, transferred to nylon filters, and probed with pSU19.

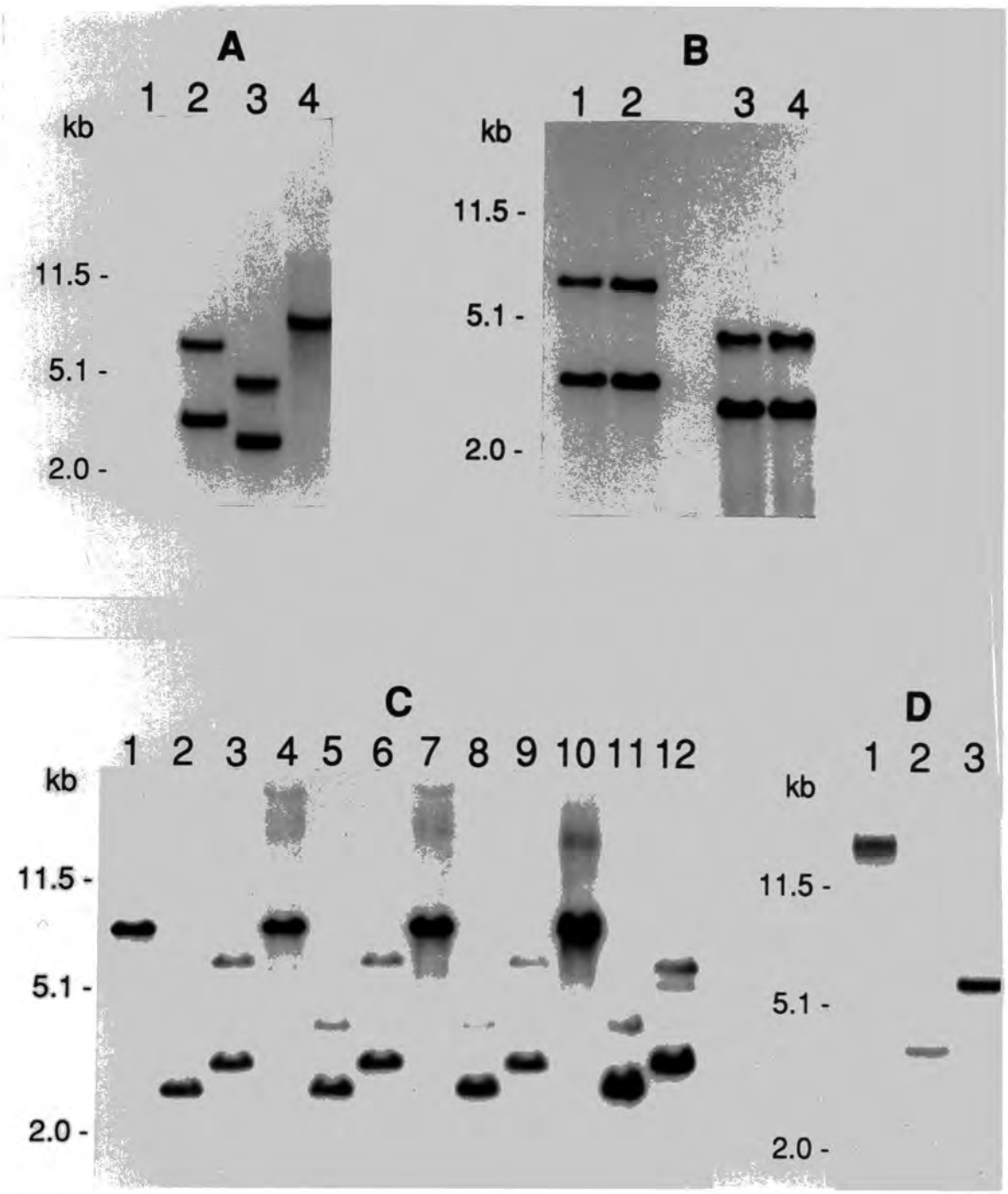

A
1 2 3 4
kb
11.5 -
5.1 -
2.0 -
B
1 2 3 4
kb
11.5 -
5.1 -
2.0 -
C
1 2 3 4 5 6 7 8 9 10 11 12
kb
11.5 -
5.1 -
2.0 -
D
1 2 3
kb
11.5 -
5.1 -
2.0 -

Figure 3.1.7. Analysis of plasmid pJSTNR4.1 recovered from R2-PIM8(*smt*)

Panel A: pJSTNR4.1, generated from a ca. 7.8 kb *Sal*I DNA fragment from R2-PIM8(*smt*) by plasmid recovery in *Escherichia coli*, was digested with *Sal*I (*lane 1*), *Hin*dIII (*lane 2*), *Pst*I (*lane 3*) and *Hin*dIII/*Pst*I (*lane 4*), and electrophoresed in a 1 % (w/v) agarose gel. *Panels B, C* and *D*: pJSTNR4.1 digested with *Sal*I (*lane 1*), *Hin*dIII (*lane 2*) and *Pst*I (*lane 3*) electrophoresed in a 1 % (w/v) agarose gel, transferred to a nylon filter, and probed with pSU19 (*panel B*), part of a 371 bp diagnostic deletion region (*panel C*) or retained *smtB* sequences (*panel D*).

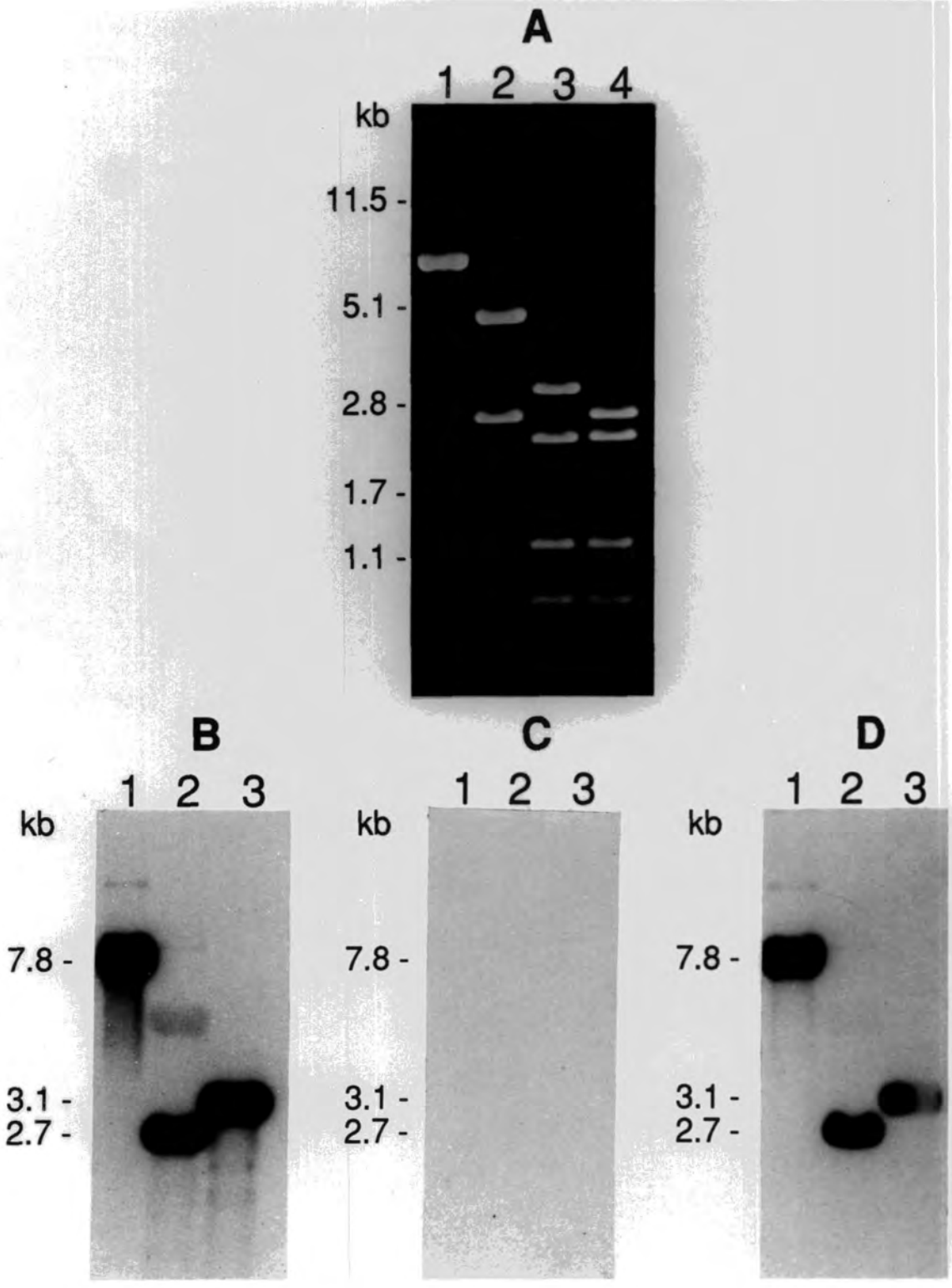
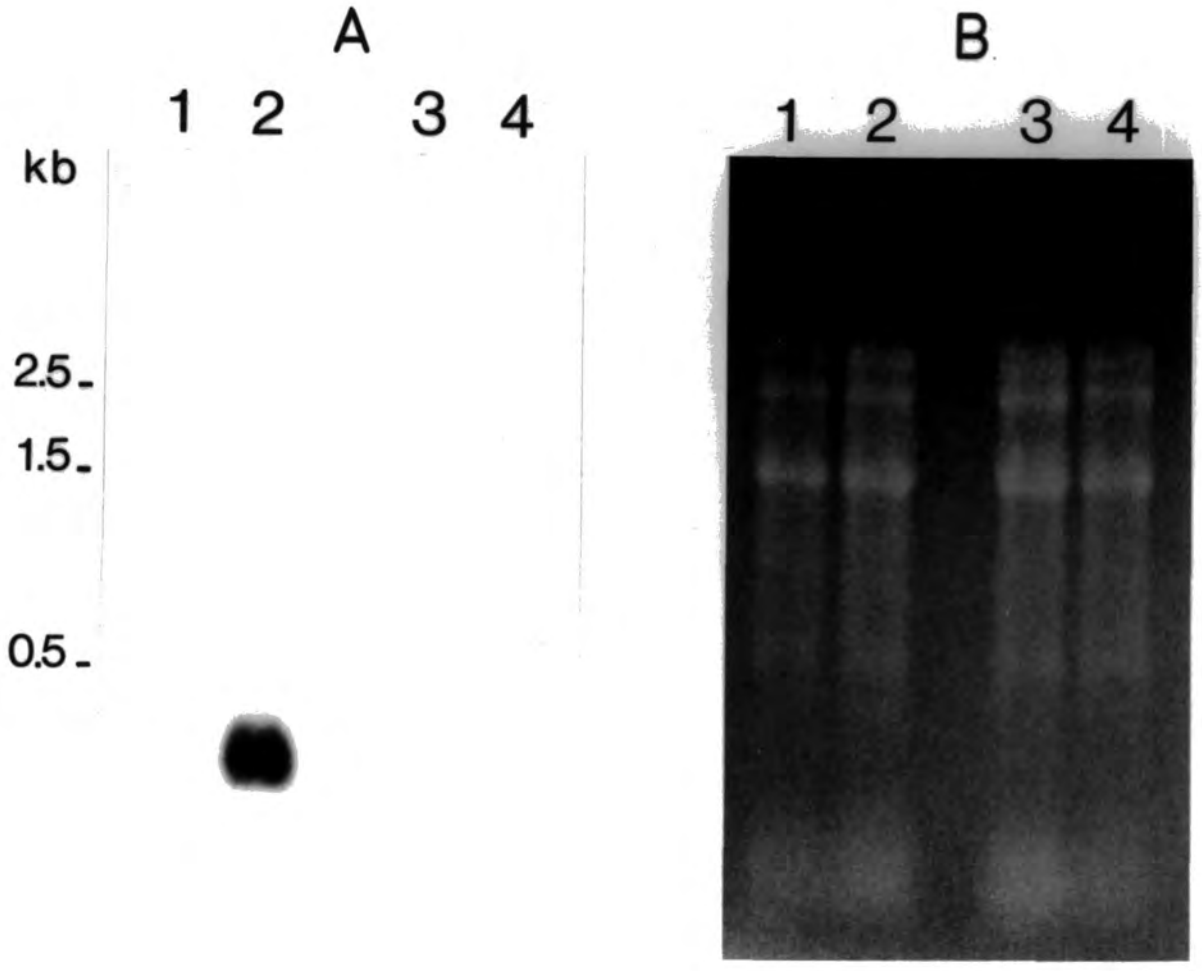

A
1 2 3 4
kb
11.5 -
5.1 -
2.8 -
1.7 -
1.1 -
B
1 2 3
kb
7.8 -
3.1 -
2.7 -
C
1 2 3
kb
7.8 -
3.1 -
2.7 -
D
1 2 3
kb
7.8 -
3.1 -
2.7 -

Figure 3.1.8. Northern analysis of nucleic acid from R2-PIM8 and R2-PIM8(*smt*)

panel A: Total nucleic acid isolated from R2-PIM8 (*lanes 1* and *2*) and R2-PIM8(*smt*) (*lanes 3* and *4*) incubated for 2 hours under standard growth conditions in the absence (*lanes 1* and *3*) or presence (*lanes 2* and *4*) of 2.5 μM Cd^{2+}, was electrophoresed in a 1.5 % (w/v) agarose gel containing formaldehyde, transferred to a nylon filter, and probed with *smtA*. *Panel B*: Visualisation of ethidium bromide stained nucleic acid, with intensity of rRNA bands indicating the quantity of RNA in each lane.


A
1 2 3 4
kb
2.5
1.5
0.5
B
1 2 3 4

3.2. PHENOTYPIC ANALYSIS OF R2-PIM8(*smt*)

Roles proposed for MTs include essential metal (Zn^{2+}) metabolism and the sequestration of excess amounts of certain metal ions. The phenotype of R2-PIM8(*smt*) was thus examined with respect to metal ion tolerance.

3.2.1. Survival and growth characteristics of R2-PIM8 and R2-PIM8(*smt*) in Allens medium supplemented with metal salts

The proportion of R2-PIM8 and R2-PIM8(*smt*) cultures growing in Allens medium supplemented with increasing levels of $ZnCl_2$, $CdCl_2$, $CuCl_2$, $Ag(NO_3)$ and $HgCl_2$ was monitored (figure 3.2.1.), and minimum inhibitory/maximum permissive concentrations of these metal salts were determined for both strains. Growth of R2-PIM8 and R2-PIM8(*smt*) was subsequently examined as a function of time in response to selected concentrations of $ZnCl_2$, $CdCl_2$ and $CuCl_2$ (figure 3.2.2.). R2-PIM8 survived in ca. 5 fold higher concentrations of Zn^{2+} than R2-PIM8(*smt*) (figure 3.2.1., *panel A*; figure 3.2.2., *panel A*). A higher tolerance to Cd^{2+} was observed for R2-PIM8 in comparison to R2-PIM8(*smt*), however this was only detected after a prolonged growth lag of > 148 hours (figure 3.2.1., *panel B*; figure 3.2.2., *panel B*). There was no significant difference in the minimum inhibitory concentration of Cu^{2+} (figure 3.2.1., *panel C*), and similar growth rates were observed for both R2-PIM8 and R2-PIM8(*smt*) at selected Cu^{2+} concentrations (figure 3.2.2, *panel C*). Observed growth of both strains at high Cu^{2+} concentrations was greatly inhibited (figure 3.2.2., *panel C*).

After 3 days incubation, a statistically significant higher proportion of R2-PIM8 cultures grew at 0.2 μM Ag^+ ($P_{1\%}$) and 0.25 μM Ag^+ ($P_{5\%}$) than R2-PIM8(*smt*) cultures (figure 3.2.1., *panel D*). However, growth at higher Ag^+ concentrations was observed following a prolonged lag. After 14 days incubation, survival of R2-PIM8 and R2-PIM8(*smt*) was observed up to the same Ag^+ concentration, and no significant difference in the number of surviving cultures was observed (figure 3.2.1., *panel D*). There was no significant difference in the minimum inhibitory concentration of Hg^{2+} for both strains,

growth at higher concentrations was observed following a prolonged lag (figure 3.2.1., *panel E*).

Gupta *et al.* (1992, 1993) observed amplification and specific rearrangement of the *smt* divergon in *Synechococcus* PCC 6301 selected for Cd^{2+} tolerance (refer to sections 2.3.4. and 4.3.2.). No rearrangement, of *smt*, in R2-PIM8 cultures growing at 4.0 μM Cd^{2+} and 4.5 μM Cd^{2+} after a prolonged growth lag (in this study) was detected by Southern analysis (figure 3.2.3.).

3.2.2. Transformation of R2-PIM8(*smt*) with the *smt* divergon

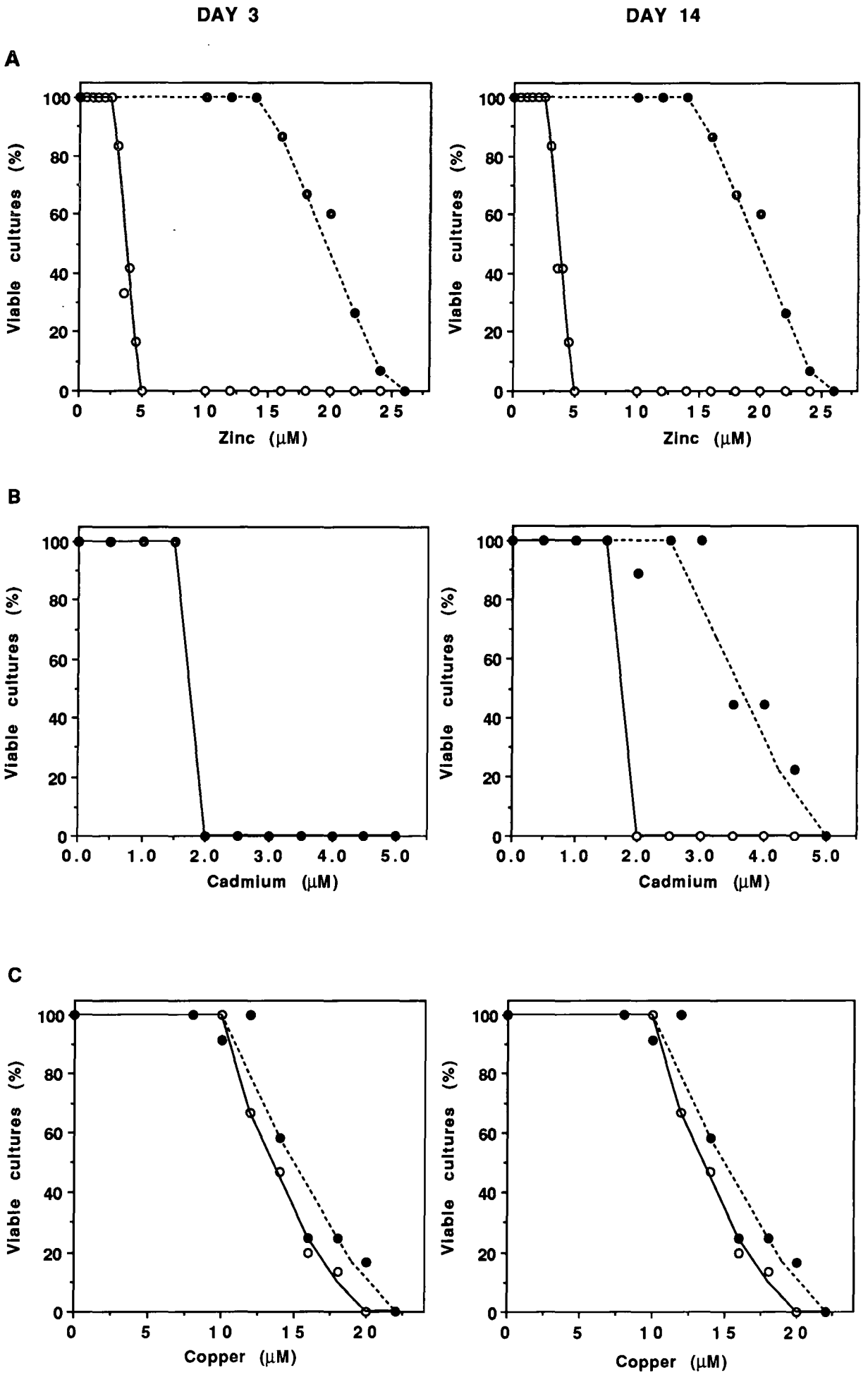
A 1,775 bp *Sal*I/*Hin*dIII *smt* fragment (figure 3.1.2., *panel A*), was used to transform Zn^{2+} hypersensitive R2-PIM8(*smt*) to normal Zn^{2+} tolerance and chloramphenicol sensitivity mediated by homologous recombination. Zn^{2+} tolerant colonies (> 100) were obtained, and after several rounds of streaking on Zn^{2+} supplemented (20 μM) plates to segregate mutants homozygous for an uninterrupted functional *smt* divergon, a number of colonies were grown in liquid culture and then plated to obtain single colonies. Resulting colonies were inoculated into liquid culture for analyses. Figure 3.2.4. depicts the tolerance of R2-PIM8 (*panel A*), R2-PIM8(*smt*) (*panel B*) and *smt*- restored R2-PIM8(*smt*) (*panel C*) on Allens agar plates supplemented with chloramphenicol (7.5 μg ml^{-1}) or Zn^{2+} (20 μM). For all colonies analysed restoration of Zn^{2+} resistance was coincident with loss of chloramphenicol resistance.

The structure and homozygosity (of the *smt* region) of *smt*- restored R2-PIM8(*smt*), hereafter referred to as R2-PIM8(*smt*- reintroduced), was confirmed by Southern analyses, using part of the 371 bp diagnostic deletion region and pSU19 as probes (figure 3.2.5.). The restriction patterns were as observed for R2-PIM8 (figure 3.1.1.; figure 3.1.6., *panel D*), confirming reintegration of a functional *smt* divergon with coincident loss of pSU19.

Observed tolerance of R2-PIM8(*smt*- reintroduced) to a range of concentrations of $ZnCl_2$ and $CdCl_2$ was identical to that observed for R2-PIM8. Growth of R2-PIM8 and R2-PIM8(*smt*- reintroduced) as a function of time in response to selected $ZnCl_2$ and $CdCl_2$ concentrations is shown (figure 3.2.6.).

Figure 3.2.1. Survival of R2-PIM8 and R2-PIM8(*smt*) in Allens medium supplemented with metal salts

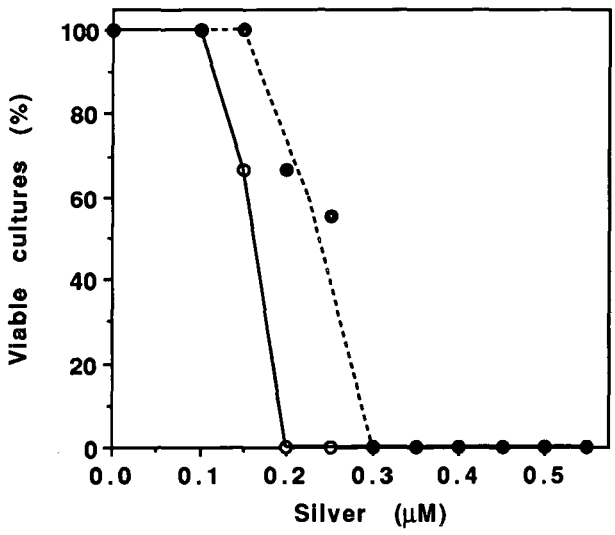
Survival of cultures of R2-PIM8 (*closed symbols*) and R2-PIM8(*smt*) (*open symbols*) as a function of Zn^{2+} (*panel A*), Cd^{2+} (*panel B*), Cu^{2+} (*panel C*), Ag^{+} (*panel D*) or Hg^{2+} (*panel E*) (*panels D* and *E* are shown overleaf). Cultures were inoculated at a density of 1×10^6 cells ml^{-1} in Allens medium supplemented with $ZnCl_2$, $CdCl_2$, $CuCl_2$, $Ag(NO_3)$ or $HgCl_2$ and monitored for growth daily. Viable cultures (estimated visually from culture colouration) on day 3 and day 14 are represented.

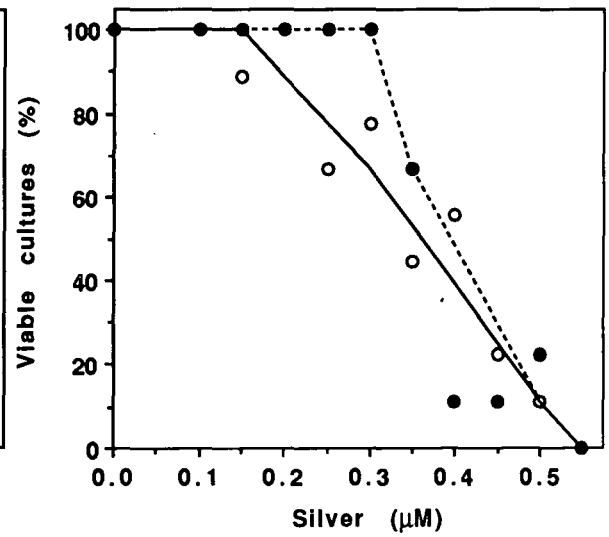

DAY 3
DAY 14
A
Viable cultures (%)
Zinc (μM)
B
Cadmium (μM)
C
Copper (μM)

DAY 3

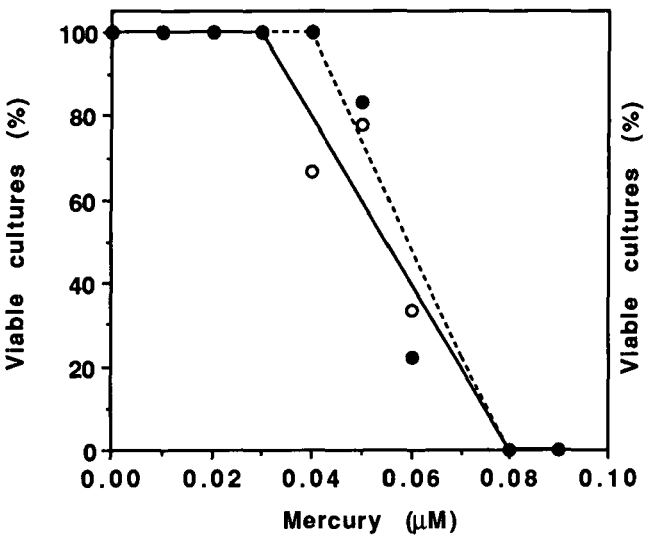
DAY 14

D





E



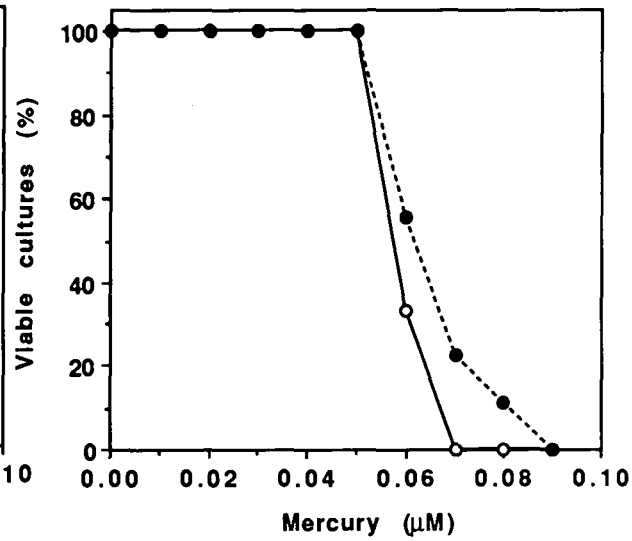
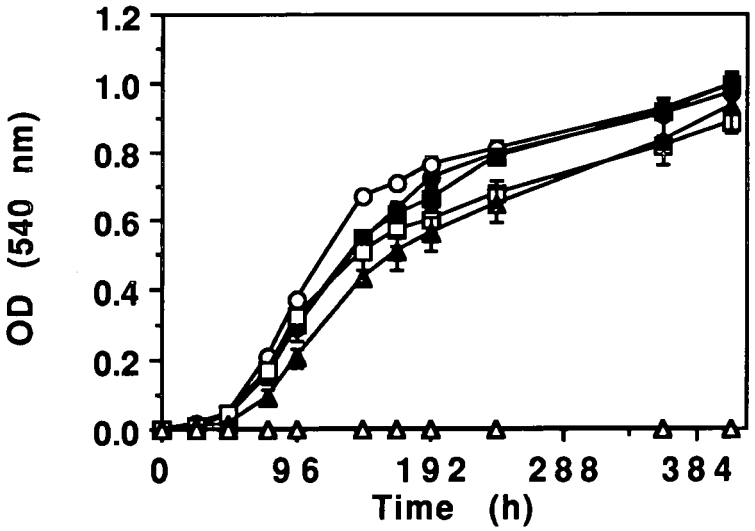


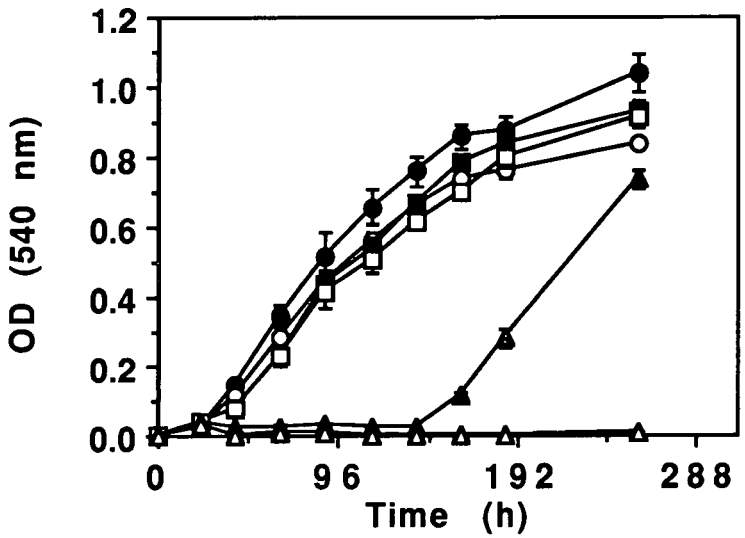
Figure 3.2.2. Growth of R2-PIM8 and R2-PIM8(*smt*) in Allens medium supplemented with $ZnCl_2$, $CdCl_2$ or $CuCl_2$

Growth of R2-PIM8 and R2-PIM8(*smt*) in Allens medium supplemented with $ZnCl_2$, $CdCl_2$ or $CuCl_2$ as a function of time. The data points represent the mean values estimated from three separate cultures, with standard deviation. R2-PIM8 (*closed symbols*) and R2-PIM8(*smt*) (*open symbols*) were inoculated at a cell density of 1×10^6 cells ml^{-1} with added: *Panel A*, 0 μM (*circles*), 2.5 μM (*squares*) and 14 μM (*triangles*) Zn^{2+}; *panel B*, 0 μM (*circles*), 1.5 μM (*squares*) and 3 μM (*triangles*) Cd^{2+}; *panel C*, 0 μM (*circles*), 8 μM (*squares*) and 12 μM (*triangles*) Cu^{2+}. Growth was estimated by measuring the optical density at 540 nm. Equivalent data were obtained in two further replicate experiments shown overleaf.

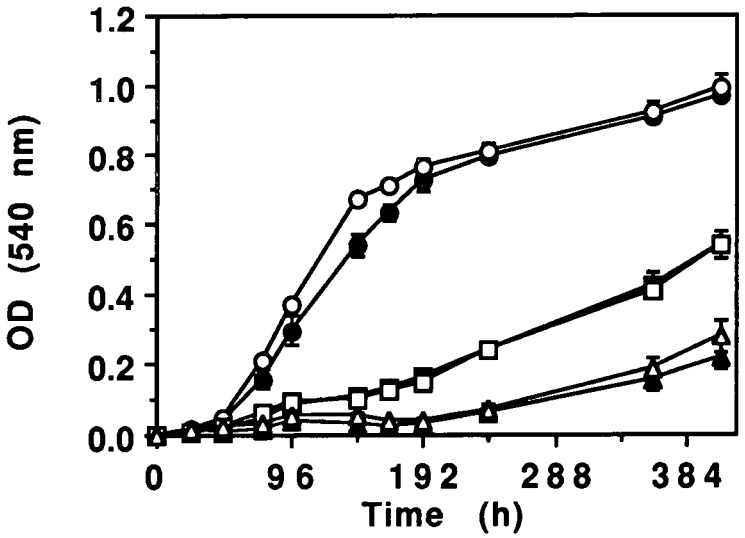
A


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (540 nm)
0
96
192
288
384
Time (h)

B


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (540 nm)
0
96
192
288
Time (h)

C


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (540 nm)
0
96
192
288
384
Time (h)

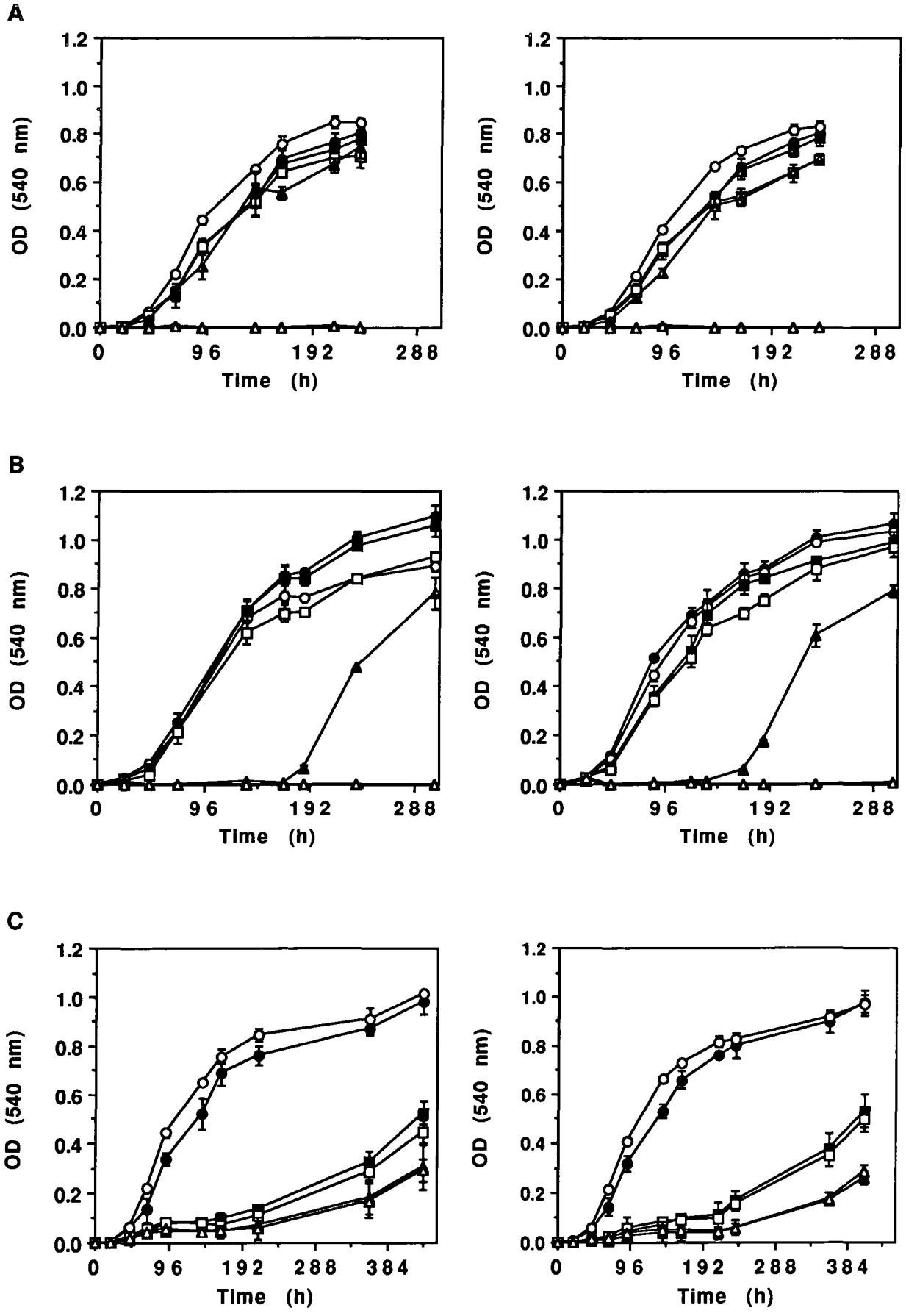

A
B
C
OD (540 nm)
Time (h)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
96
192
288
384

Figure 3.2.3. Southern analysis of genomic DNA isolated from R2-PIM8, cultured in the presence of Cd^{2+}

Total nucleic acid from R2-PIM8 cultured in the presence of 4.0 μM Cd^{2+} (*lanes 1* and *2*), 4.5 μM Cd^{2+} (*lanes 3* and *4*) or 0 μM Cd^{2+} (*lanes 5* and *6*), was digested with *Sal*I (*lanes 1*, *3* and *5*) and *Hin*dIII (*lanes 2*, *4* and *6*). Digested DNA was electrophoresed in a 0.8 % (w/v) gel, transferred to a nylon filter, and probed with a 213 bp *Pst*I *smt* fragment.

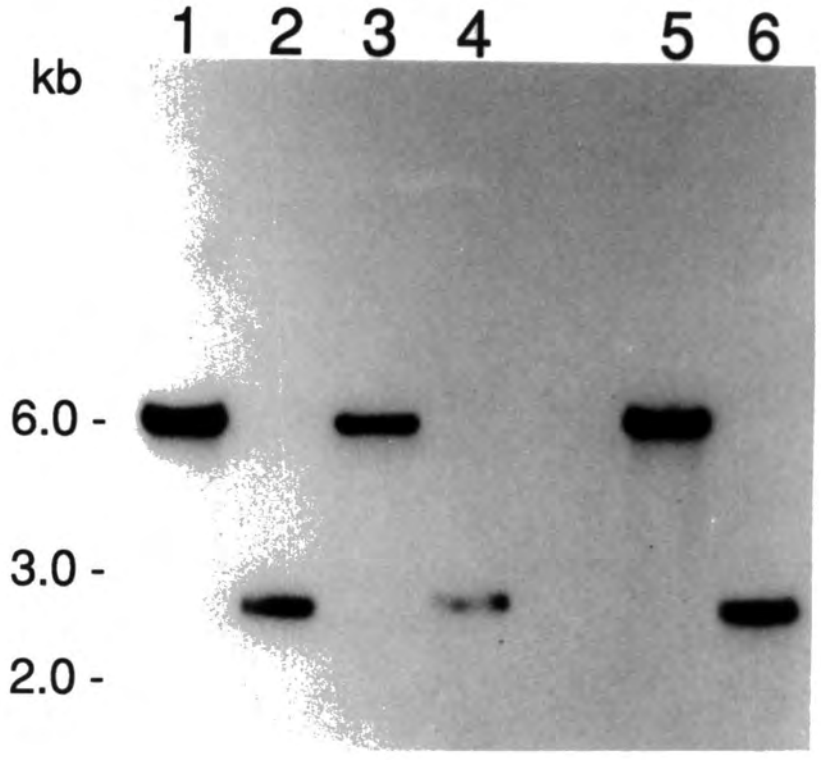
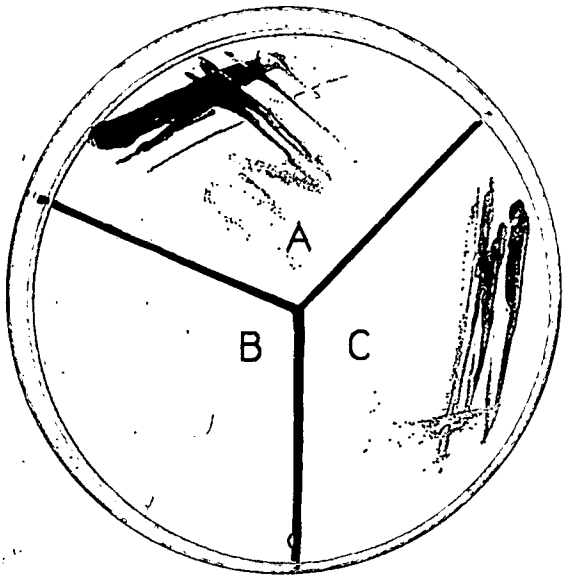

1 2 3 4 5 6
kb
6.0 -
3.0 -
2.0 -

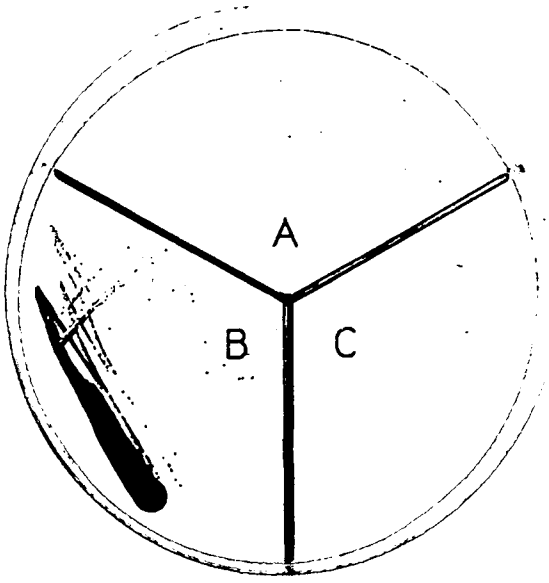
Figure 3.2.4. Growth of R2-PIM8, R2-PIM8(*smt*) and *smt*- restored R2-PIM8(*smt*) on Allens agar plates

R2-PIM8 (*panel A*), R2-PIM8(*smt*) (*panel B*) and *smt*- restored R2-PIM8(*smt*) (*panel C*) cells were streaked onto Allens agar plates (control) supplemented with chloramphenicol (7.5 μg ml^{-1}) or Zn^{2+} (20 μM).

ZINC



CHLORAMPHENICOL



CONTROL

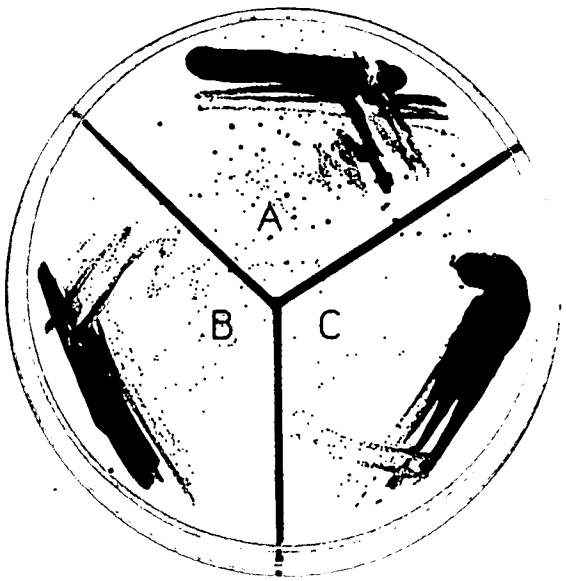


Figure 3.2.5. Southern analyses of genomic DNA from *smt*-restored R2-PIM8(*smt*), R2-PIM8(*smt*- reintroduced)

Total nucleic acid from R2-PIM8(*smt*- reintroduced) was digested with: *Panel A*, *Bam*HI (*lane 1*), *Sal*I (*lane 2*) and *Hin*dIII (*lane 3*); *Panel B*, *Sal*I (*lane 1*), *Hin*dIII (*lane 2*) and *Pst*I (*lane 3*). Digested DNA was electrophoresed in 0.8 % (w/v) agarose gels, transferred to nylon filters, and probed with part of the 371 bp diagnostic-deletion region (*panel A*) or pSU19 (*panel B*).

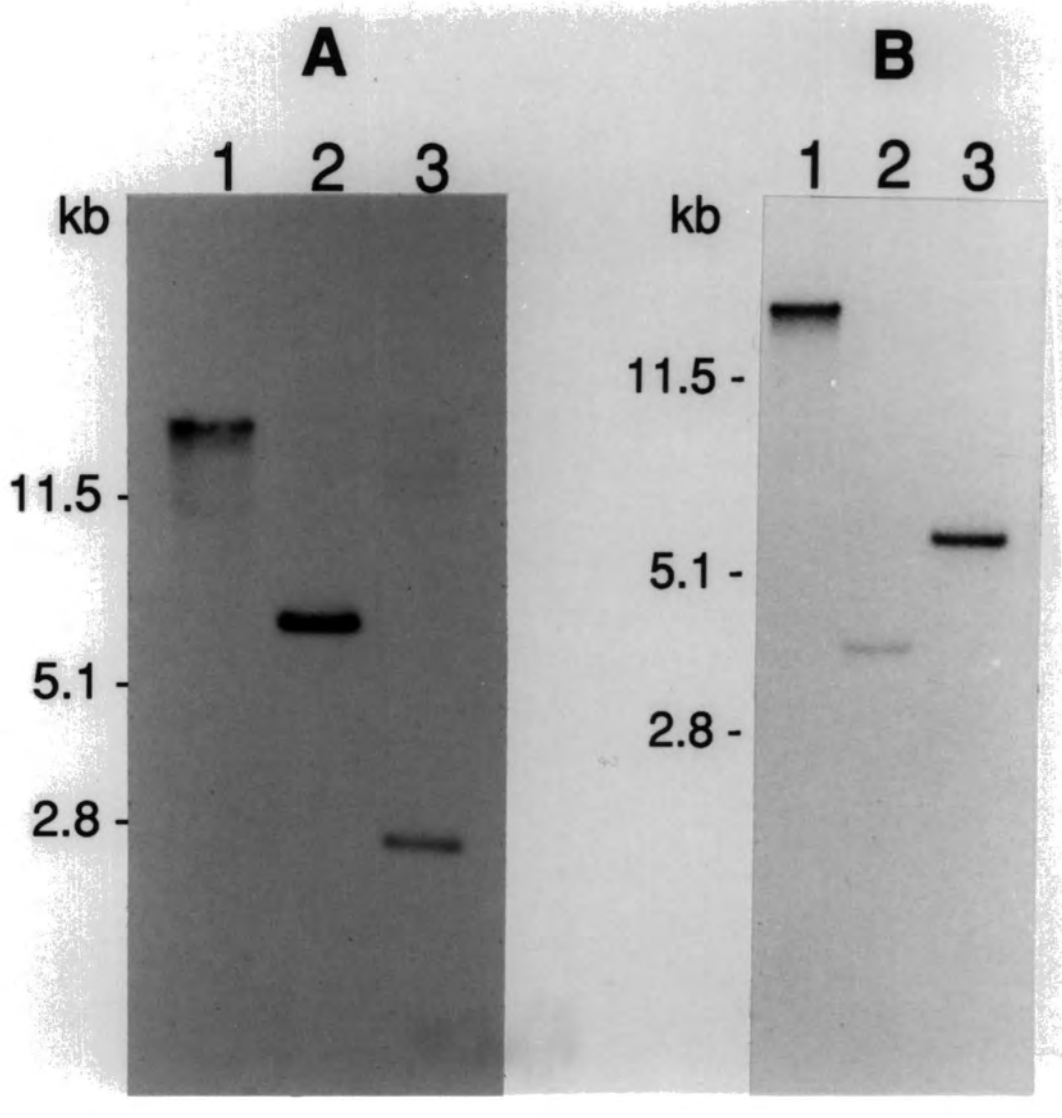
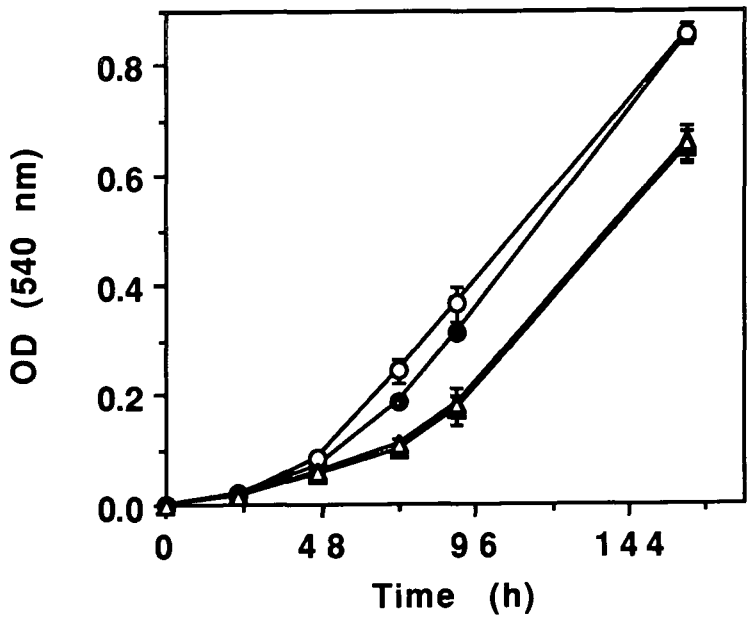

A
1 2 3
kb
11.5 -
5.1 -
2.8 -
B
1 2 3
kb
11.5 -
5.1 -
2.8 -

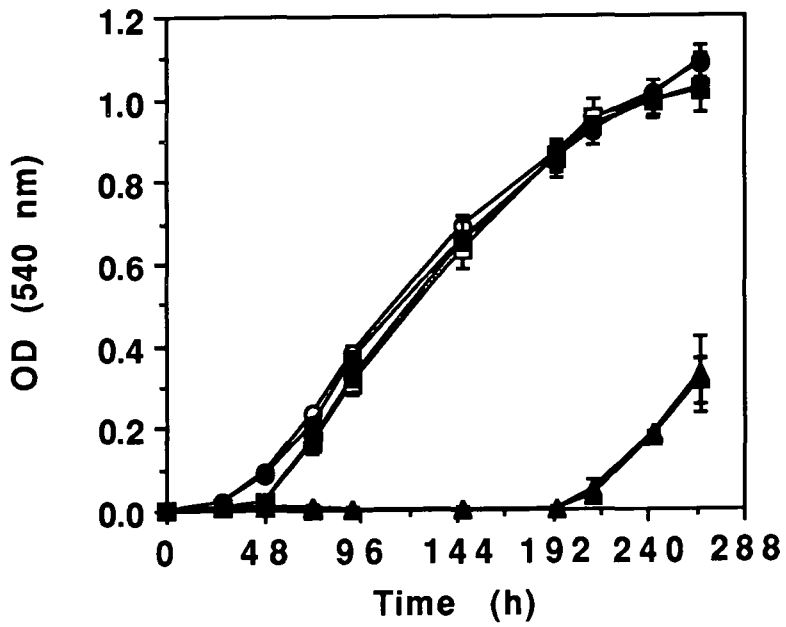
Figure 3.2.6. Growth of R2-PIM8 and R2-PIM8(*smt-* reintroduced) in Allens medium supplemented with $ZnCl_2$ or $CdCl_2$

Growth of R2-PIM8 and R2-PIM8(*smt-* reintroduced) in Allens medium supplemented with selected concentrations of $ZnCl_2$ or $CdCl_2$ as a function of time. The data points represent the mean values estimated from three separate cultures, with standard deviation. R2-PIM8 (*closed symbols*) and R2-PIM8(*smt-* reintroduced) (*open symbols*) were inoculated at a cell density of 1×10^6 cells ml^{-1} with added: *Panel A*, 0 μM (*circles*) and 12 μM (*triangles*) Zn^{2+}; *panel B*, 0 μM (*circles*), 1 μM (*squares*) and 2 μM (*triangles*) Cd^{2+}. Growth was estimated by measuring the optical density at 540 nm.

A


OD (540 nm)
0.0
0.2
0.4
0.6
0.8
0
48
96
144
Time (h)

B


OD (540 nm)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
48
96
144
192
240
288
Time (h)

3.3. GENERATION AND PHENOTYPIC ANALYSIS OF R2-PIM8 (*smtA+/B-*)

Mutants of R2-PIM8 were generated with functional *smtA* and non-functional *smtB*, and the individual roles of *smtA* and *smtB* were examined with respect to metal tolerance (refer to section 3.5.).

3.3.1. Generation of recombinants with functional *smtA* and non-functional *smtB*

Restoration of normal Zn^{2+} tolerance was used as a phenotypic selection for transformation of R2-PIM8(*smt*) with a 1,423 bp *SalI*/*Hin*dIII linear DNA fragment, containing functional *smtA* and non-functional *smtB* (refer to section 2.3.4.) (procedure performed by N.J. Robinson). Zn^{2+} tolerant transformants (three) were obtained, and after several rounds of streaking on Zn^{2+} supplemented plates (20 μM) to segregate homozygous mutants, two recombinants were grown in liquid culture and then plated to obtain single colonies. Resulting colonies were inoculated into liquid culture for analyses. Plating of Zn^{2+} tolerant recombinants on chloramphenicol supplemented plates (7.5 μg ml^{-1}) revealed one to be chloramphenicol sensitive (Cm^S), whilst the other remained Cm^r.

The structure (of the *smt* region) of the recombinants was confirmed by Southern analysis, using part of the 371 bp diagnostic deletion region as a probe (figure 3.3.1.). The 213 bp *PstI* *smt*-fragment (part of the diagnostic deletion region) was identified on *SalI* and *Hin*dIII fragments in both recombinants. These restriction fragments are of the size expected following integration of the 1,423 bp *smtA+/B-* DNA fragment into the R2-PIM8(*smt*) chromosome, downstream of pSU19 (integrated in the *smt* region). The recombinants, with reintegrated chromosomal *smtA*, lacking functional *smtB*, are hereafter referred to as R2-PIM8(*smtA+/B-*) (Cm^S transformant, used for subsequent analyses) and R2-PIM8(*smtA+/B-*).2 (Cm^r transformant).

The homologous 5' pSU19 flank in the 1,423 bp *smtA+/B-* linear DNA fragment was only 107 bp, and may have been too short to enable efficient replacement (by double reciprocal crossover or gene conversion) of pSU19 DNA by the integrating fragment. Integration therefore probably resulted from the less efficient single reciprocal crossover

event (refer to section 1.5.) to the 3' of pSU19 and is indicated by the hybridisation patterns of both recombinants. A smear (or band) was observed below the ca. 8.1 kb *Sal*I fragment of the Cm^S recombinant (R2-PIM8(*smtA+/B-*)), this may be a result of DNA degradation or recombination upon integration of the 1,423 bp DNA fragment. The loss of a functional *cat* in this recombinant may be due to mutation upon relaxation of selection for chloramphenicol resistance (although integrated *cat* has remained stable in R2-PIM8(*smt*) with no selection (data not shown)), or more likely recombination with 3' pSU19 sequences upon integration of the 1,423 bp fragment.

3.3.2. Survival and growth characteristics of R2-PIM8(*smt-* reintroduced) and R2-PIM8(*smtA+/B-*) in Allens medium supplemented with metal salts

R2-PIM8(*smt-*reintroduced), having an R2-PIM8 genotype (detectable by Southern analyses) and identical growth characteristics in media supplemented with $ZnCl_2$ and $CdCl_2$ as R2-PIM8, was used as a control for the phenotypic analysis of R2-PIM8(*smtA+/B-*). Both R2-PIM8(*smt-*reintroduced) and R2-PIM8(*smtA+/B-*) have been exposed to the same selection conditions (Allens agar plates supplemented with 20 μM Zn^{2+}) during their generation.

The proportion of R2-PIM8(*smt-*reintroduced) and R2-PIM8(*smtA+/B-*) cultures growing in Allens medium supplemented with a range of concentrations of $ZnCl_2$ and $CdCl_2$ was monitored (figure 3.3.2.), and minimum inhibitory/maximum permissive concentrations of these metal salts were determined for both strains. Growth of R2-PIM8(*smt-* reintroduced) and R2-PIM8(*smtA+/B-*) was subsequently examined as a function of time in response to selected concentrations of $ZnCl_2$ and $CdCl_2$ (figure 3.3.3.). Survival of R2-PIM8(*smt-*reintroduced) and R2-PIM8(*smtA+/B-*) cultures was observed up to the same Zn^{2+} concentration, and no significant difference in the numbers of cultures surviving at the higher Zn^{2+} levels was observed (figure 3.3.2., *panel A*). However, growth of R2-PIM8(*smt-*reintroduced) observed at high Zn^{2+} concentrations, was greatly inhibited in comparison to R2-PIM8(*smtA+/B-*) cultures (figure 3.3.3., *panel A*).

The prolonged growth lag of > 148 hours observed for R2-PIM8(*smt*- reintroduced) (and R2-PIM8) (figure 3.2.2, *panel B*; figure 3.2.6., *panel B*) growing at > 1.5 μM Cd^{2+} was not observed for R2-PIM8(*smtA+/B-*) (figure 3.3.2., *panel B*; figure 3.3.3., *panel B*). After 14 days incubation, survival of R2-PIM8(*smt*- reintroduced) and R2-PIM8(*smtA+/B-*) cultures was observed up to the same Cd^{2+} concentration, and there was no significant difference in the number of cultures surviving at the higher Cd^{2+} levels (figure 3.3.2, *panel B*).

It is noted that there was a slight reduction in the tolerance to Zn^{2+} and Cd^{2+} reported for R2-PIM8(*smt*- reintroduced) than was previously reported for R2-PIM8. Further analysis revealed that survival and growth characteristics of R2-PIM8(*smt*- reintroduced) and R2-PIM8 remained identical, and all cultures were displaying a proportional decreased tolerance. This slight decrease in metal tolerance was considered to result from uncontrolled variables (such as adjustment of growth facilities following servicing).

3.3.3. Growth of R2-PIM8(*smt*- reintroduced) and R2-PIM8(*smtA+/B-*) in Allens medium supplemented with $CdCl_2$, following pretreatment with $ZnCl_2$ or $CdCl_2$

Growth of R2-PIM8(*smt*- reintroduced) and R2-PIM8(*smtA+/B-*) were examined as a function of time in response to selected concentrations of $CdCl_2$, following 3 days pretreatment of cultures with no added metal, 8 μM Zn^{2+} or 1 μM Cd^{2+} (figure 3.3.4.). Growth assays, following culture pretreatment, were performed in parallel. R2-PIM8(*smtA+/B-*) displayed similar growth patterns with and without metal ion pretreatment, no prolonged growth lag was observed at 2 μM Cd^{2+} or 3 μM Cd^{2+} (figure 3.3.4., *panels A, B* and *C*). R2-PIM8(*smt*- reintroduced) pretreated with 8 μM Zn^{2+} no longer displayed a prolonged growth lag at 2 μM Cd^{2+}, and growth was observed at 3 μM Cd^{2+} from day 1 (figure 3.3.4., *panels A* and *B*). However, growth at 3 μM Cd^{2+} was greatly inhibited for > 192 hours (figure 3.3.4., *panel B*). R2-PIM8(*smt*- reintroduced) pretreated with 1 μM Cd^{2+}, displayed a growth lag of > 192 hours at 2 μM Cd^{2+} and 3 μM Cd^{2+} (figure 2.3.4., *panel C*). In this experiment no growth of R2-PIM8(*smt*-

reintroduced) was observed at 3 μM Cd^{2+} without metal pretreatment (figure 3.3.4., *panel A*).

3.3.4. Growth of R2-PIM8(*smt*- reintroduced) and R2-PIM8(*smtA+/B-*) in Allens medium supplemented with $ZnCl_2$, following pretreatment with $ZnCl_2$

Growth of R2-PIM8(*smt*- reintroduced) and R2-PIM8(*smtA+/B-*) were examined as a function of time in response to selected concentrations of $ZnCl_2$, following 3 days pretreatment of cultures with no added metal or 8 μM Zn^{2+} (figure 3.3.5.). Growth assays, following culture pretreatment, were performed in parallel. R2-PIM8(*smt*- reintroduced) and R2-PIM8(*smtA+/B-*) displayed similar growth patterns with and without Zn^{2+} pretreatment (figure 3.3.5., *panels A* and *B*). Growth of R2-PIM8(*smt*- reintroduced) in the presence of Zn^{2+} was inhibited in comparison to R2-PIM8(*smtA+/B-*) cultures.

Figure 3.3.1. Southern analysis of genomic DNA from R2-PIM8 and *smtA+/B-* transformants, R2-PIM8(*smtA+/B-*) and (R2-PIM8(*smtA+/B-*).2

*Sal*I (*lane 1*) and *Hin*dIII (*lane 2*) digested R2-PIM8 DNA, *Sal*I (*lane 3*) and *Hin*dIII (*lane 4*) digested R2-PIM8(*smtA+/B-*) (Cm^S recombinant) DNA, and *Sal*I (*lane 5*) and *Hin*dIII (*lane 6*) digested R2-PIM8(*smtA+/B-*).2 (Cm^r recombinant) DNA. Digested DNA was electrophoresed in a 0.8 % (w/v) agarose gel, transferred to a nylon filter, and probed with a 213 bp *smt* fragment (part of the diagnostic deletion region).

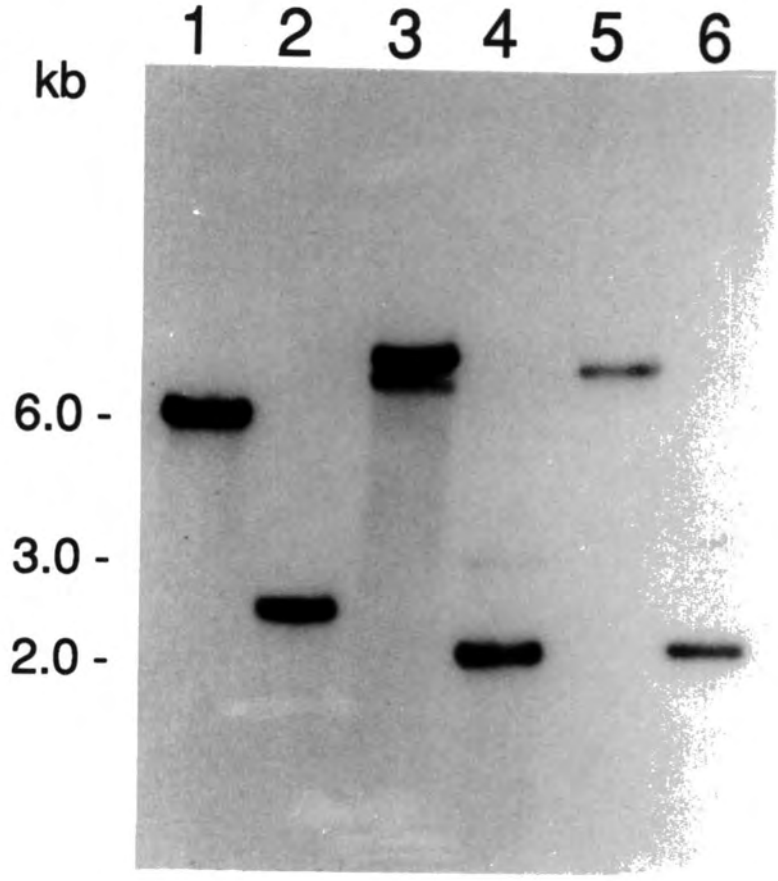

1 2 3 4 5 6
kb
6.0 -
3.0 -
2.0 -

Figure 3.3.2. Survival of R2-PIM8(*smt*- reintroduced) and R2-PIM8(*smtA+/B-*) in Allens medium supplemented with $ZnCl_2$ or $CdCl_2$

Survival of R2-PIM8(*smt*-reintroduced) (*closed symbols*) and R2-PIM8(*smtA+/B-*) (*open symbols*) as a function of Zn^{2+} (*panel A*) or Cd^{2+} (*panel B*). Cultures were inoculated at a density of 1 x 10^6 cells ml^{-1} in Allens medium supplemented with $ZnCl_2$ or $CdCl_2$ and monitored for growth daily. Viable cultures (estimated visually from culture colouration) on day 3 and day 14 are represented.

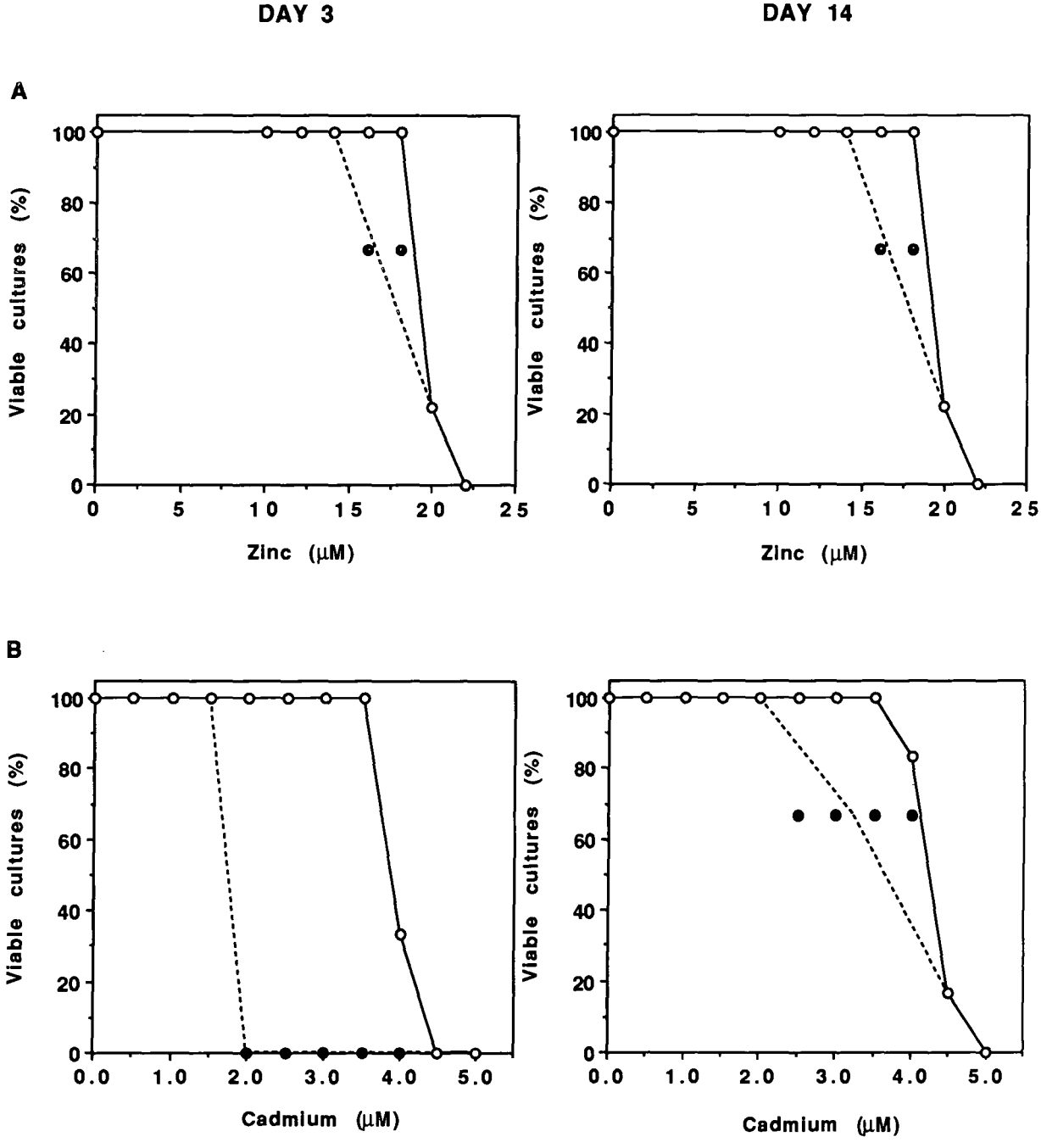
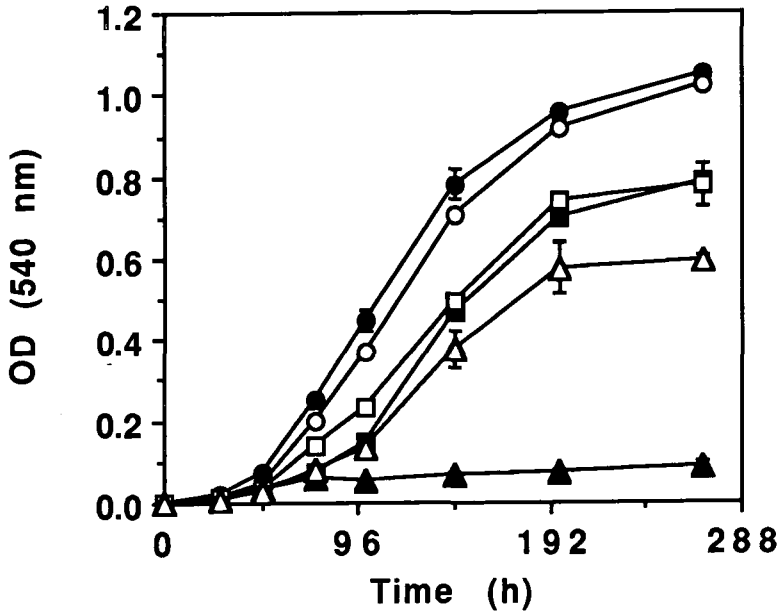

DAY 3
DAY 14
A
Viable cultures (%)
100
80
60
40
20
0
0
5
10
15
20
25
Zinc (μM)
B
Cadmium (μM)
0.0
1.0
2.0
3.0
4.0
5.0

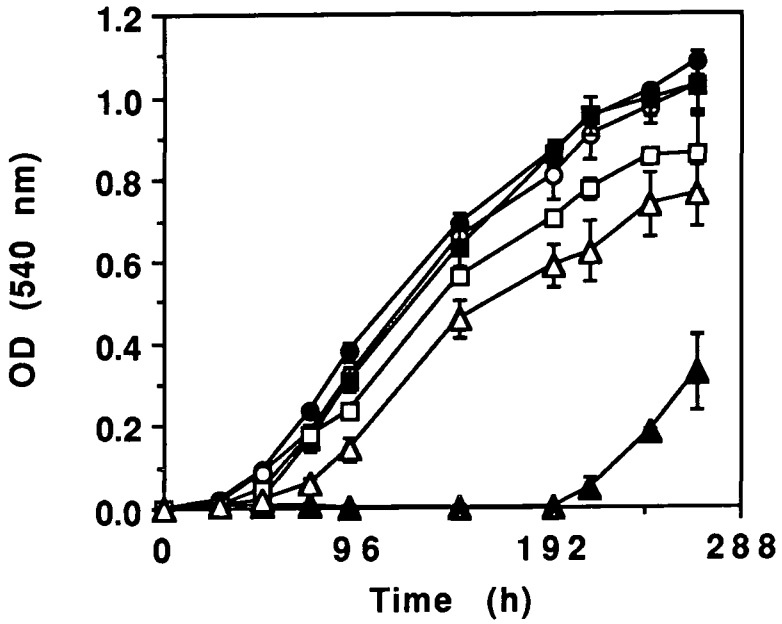
Figure 3.3.3. Growth of R2-PIM8 (*smt* reintroduced) and R2-PIM8(*smtA+/B-*) in Allens medium supplemented with $ZnCl_2$ or $CdCl_2$

Growth of R2-PIM8(*smt*-reintroduced) and R2-PIM8(*smtA+/B-*) in Allens medium supplemented with selected concentrations of $ZnCl_2$ or $CdCl_2$ as a function of time. The data points represent the mean values estimated from three separate cultures, with standard deviation. R2-PIM8(*smt*-reintroduced) (*closed symbols*) and R2-PIM8(*smtA+/B-*) (*open symbols*) were inoculated at a cell density of 1 x 10^6 cells ml^{-1} with added: *Panel A*, 0 μM (*circles*), 10 μM (*squares*) or 16 μM (*triangles*) Zn^{2+}; *panel B*, 0 μM (*circles*), 1 μM (*squares*) or 2 μM (*triangles*) Cd^{2+}. Growth was estimated by measuring the optical density at 540 nm. Equivalent data were obtained in a replicate experiment shown overleaf.

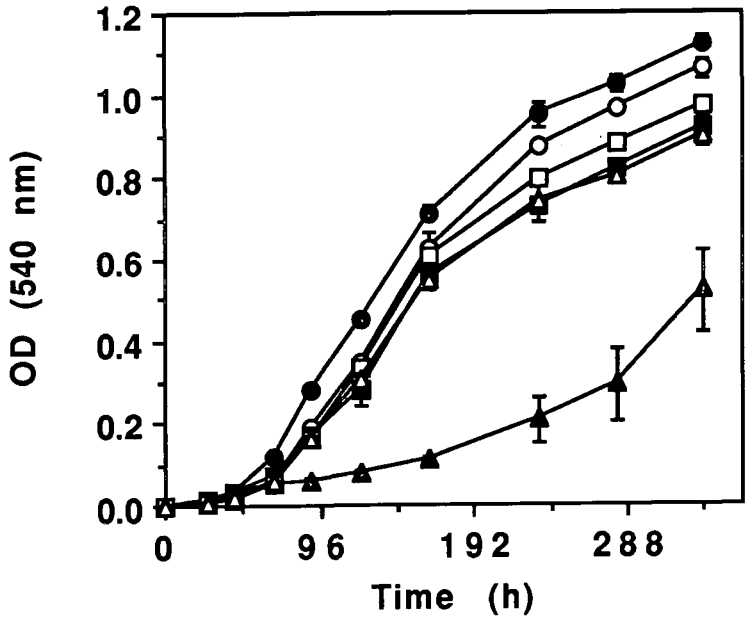
A


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (540 nm)
0
96
192
288
Time (h)

B


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (540 nm)
0
96
192
288
Time (h)

A


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (540 nm)
0
96
192
288
Time (h)

B

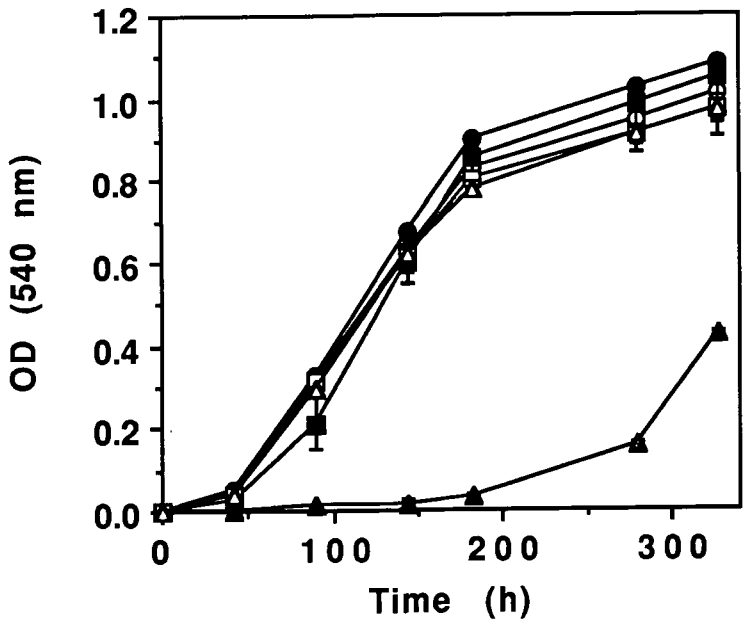
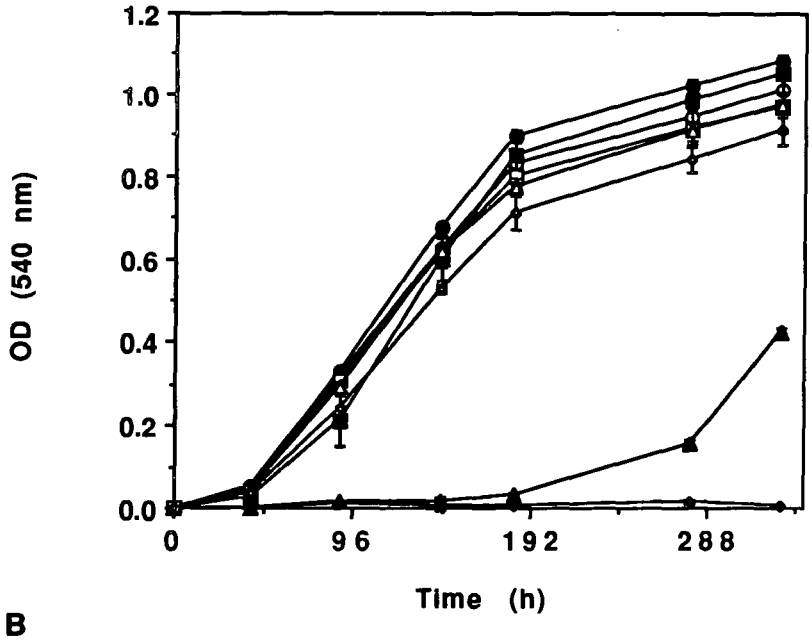

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (540 nm)
0
100
200
300
Time (h)

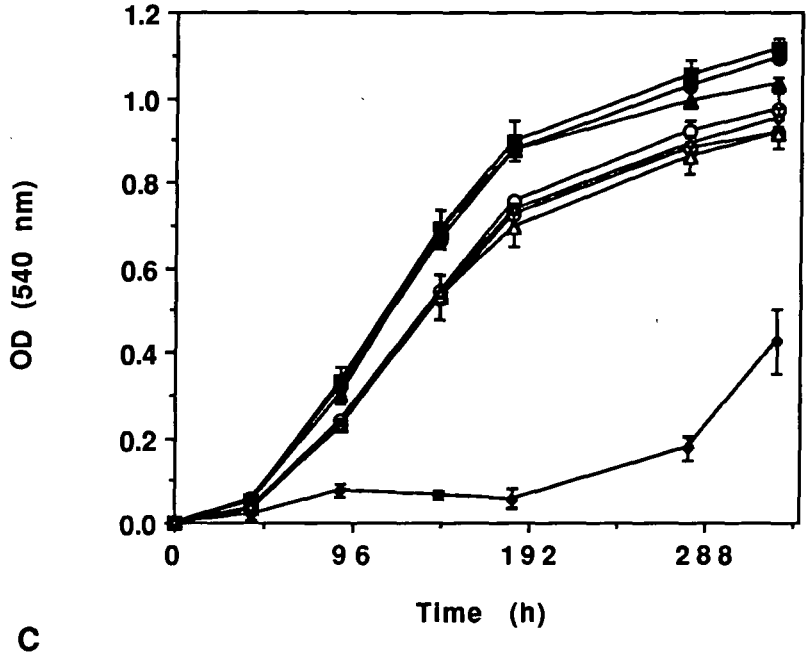
Figure 3.3.4. Growth of R2-PIM8(*smt-* reintroduced) and R2-PIM8(*smtA+/B-*) in Allens medium supplemented with $CdCl_2$, following pretreatment with $ZnCl_2$ or $CdCl_2$

Growth of R2-PIM8(*smt-* reintroduced) and R2-PIM8(*smtA+/B-*) in Allens medium supplemented with selected concentrations of $CdCl_2$ as a function of time. The data points represent the mean values estimated from three separate cultures, with standard deviation. R2-PIM8(*smt-* reintroduced) (*closed symbols*) and R2-PIM8(*smtA+/B-*) (*open symbols*) were pretreated for 3 days with: *Panel A*, no metal; *panel B*, 8 μM Zn^{2+}; *panel C*, 1 μM Cd^{2+}. Cultures were then inoculated at a cell density of 1 x 10^6 cells ml^{-1} with added 0 μM (*circles*), 1 μM (*squares*), 2 μM (*triangles*) or 3 μM (*diamonds*) Cd^{2+}. Growth was estimated by measuring the optical density at 540 nm. Assays (as depicted in *panels A, B* and *C*) were performed in parallel.

A


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (540 nm)
0
96
192
288
Time (h)

B


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (540 nm)
0
96
192
288
Time (h)

C

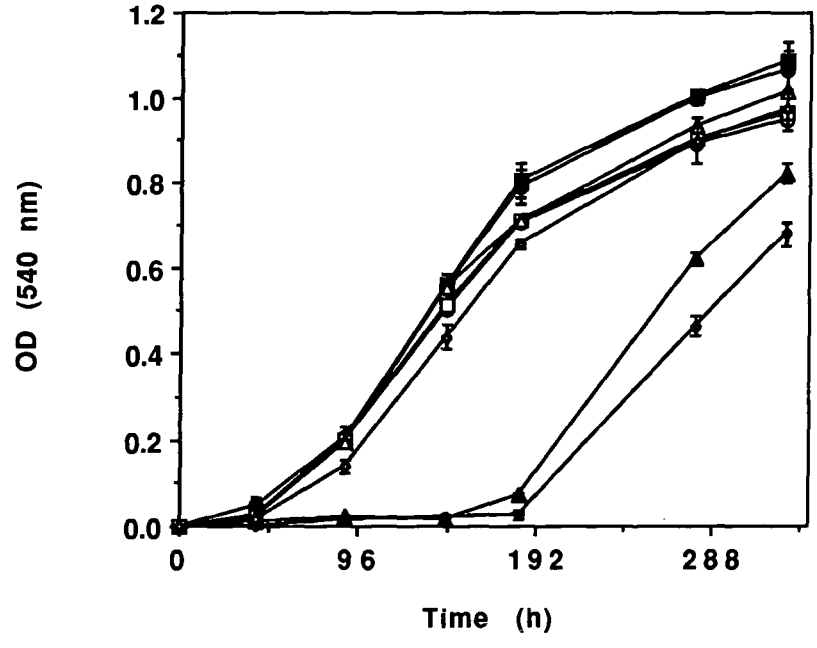
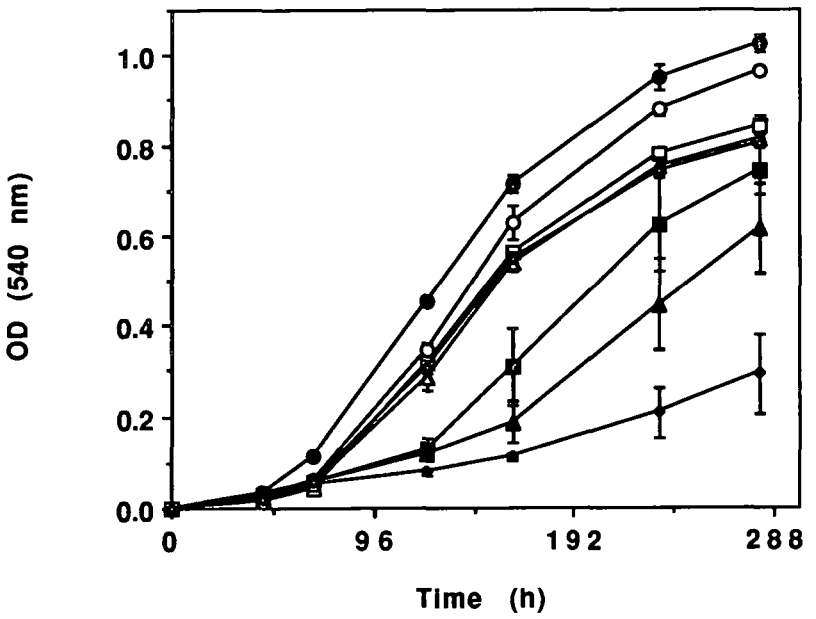

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (540 nm)
0
96
192
288
Time (h)

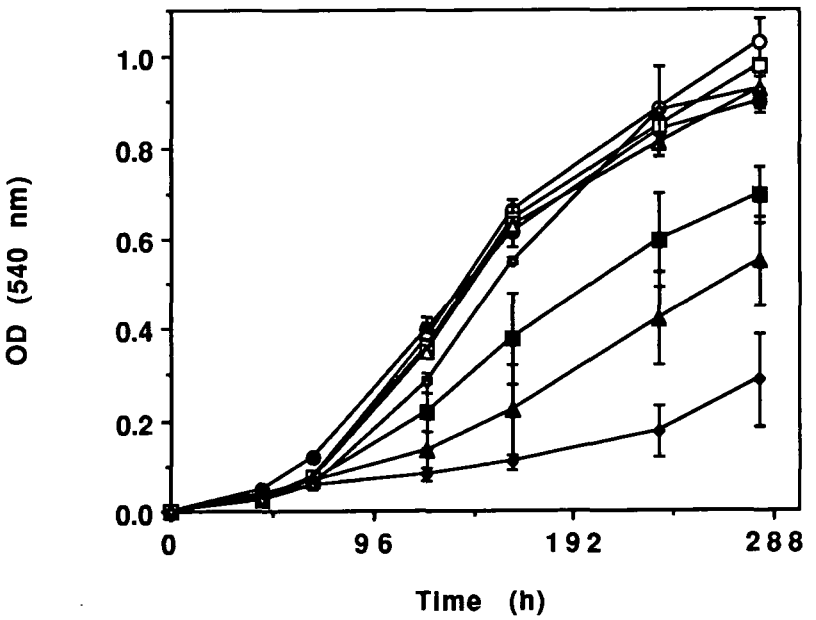
Figure 3.3.5. Growth of R2-PIM8(*smt*-reintroduced) and R2-PIM8(*smtA+/B-*) in Allens medium supplemented with $ZnCl_2$, following pretreatment with $ZnCl_2$

Growth of R2-PIM8(*smt*-reintroduced) and R2-PIM8(*smtA+/B-*) in Allens medium supplemented with selected concentrations of $ZnCl_2$ as a function of time. The data points represent the mean values estimated from three separate cultures, with standard deviation. R2-PIM8(*smt*-reintroduced) (*closed symbols*) and R2-PIM8(*smtA+/B-*) (*open symbols*) were pretreated for 3 days with: *Panel A*, no metal; *panel B*, 8 μM Zn^{2+}. Cultures were then inoculated at a cell density of 1 x 10^6 cells ml^{-1} with added 0 μM (*circles*), 12 μM (*squares*), 14 μM (*triangles*) or 16 μM (*diamonds*) Zn^{2+}. Growth was estimated by measuring the optical density at 540 nm. Assays (as depicted in *panels A* and *B*) were performed in parallel.

A


OD (540 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
96
192
288
Time (h)

B


OD (540 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
96
192
288
Time (h)

3.4. EXAMINATION OF ZINC ACCUMULATION BY *SYNECHOCOCCUS* CELLS

Zn^{2+} accumulation was examined in R2-PIM8, R2-PIM8(*smt*) and R2-PIM8(*smtA+/B-*) to determine whether the loss of functional *smtA* and/or functional *smtB* affected the total cellular Zn^{2+} content.

3.4.1. Examination of zinc accumulation by R2-PIM8 and R2-PIM8(*smt*)

Cultures were exposed to a level of Zn^{2+} that allowed growth of both R2-PIM8 and R2-PIM8(*smt*) (2.5 μM), and a level that was toxic to R2-PIM8(*smt*) but allowed growth of R2-PIM8 (14 μM). R2-PIM8 accumulated higher amounts of Zn^{2+} than R2-PIM8(*smt*) (following all permutations of Zn^{2+} exposure) in three separate experiments (figure 3.4.1.). It is proposed that differences in the absolute accumulation values obtained between individual experiments are attributable to uncontrolled variables (e.g. age of culture).

3.4.2. Examination of zinc accumulation by R2-PIM8, R2-PIM8(*smt*) and R2-PIM8(*smtA+/B-*)

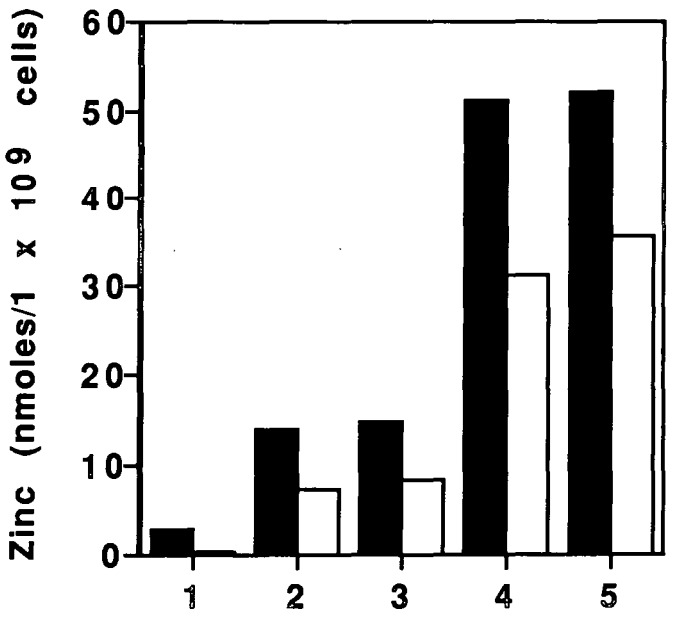
As observed previously, with cells washed in Tris.HCl (pH 7.8) only (section 3.4.1.), R2-PIM8 displays greater Zn^{2+} accumulation than R2-PIM8(*smt*) when cultures are exposed to 2.5 μM Zn^{2+} (figure 3.4.2.). Following exposure to 12 μM Zn^{2+} for 120 minutes, R2-PIM8(*smt*) displays greater Zn^{2+} accumulation than R2-PIM8, and this may reflect death of R2-PIM8(*smt*) cells following prolonged exposure to a toxic concentration of Zn^{2+}. R2-PIM8(*smtA+/B-*) displays greater Zn^{2+} accumulation than R2-PIM8(*smt*) following all permutations of Zn^{2+} exposure. Repetition of these experiments is required to determine their significance.

Following an EDTA wash, the results vary between the replicate experiments.

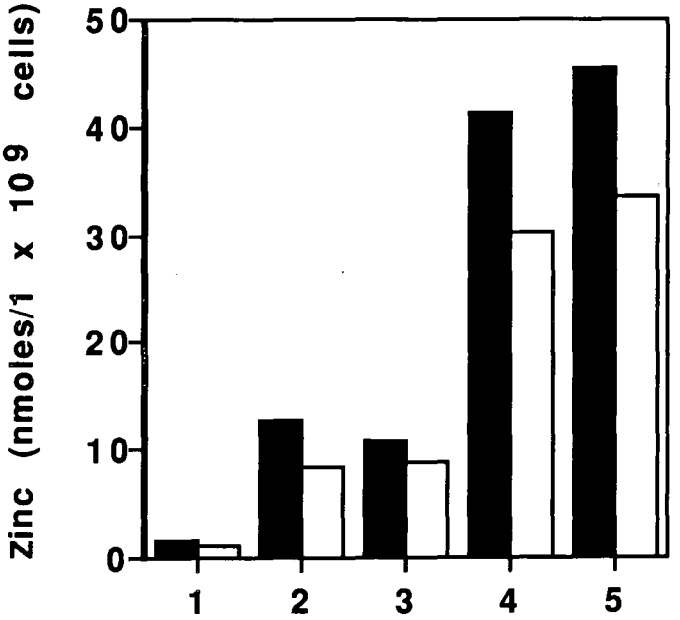
Figure 3.4.1. Accumulation of Zn^{2+} by R2-PIM8 and R2-PIM8(*smt*) exposed to $ZnCl_2$

Accumulation of Zn^{2+} by R2-PIM8 (*closed columns*) and R2-PIM8(*smt*) (*open columns*) exposed to: No metal (*1*); 2.5 μM Zn^{2+} for 30 minutes (*2*); 2.5 μM Zn^{2+} for 60 minutes (*3*); 14 μM Zn^{2+} for 30 minutes (*4*); or 14 μM Zn^{2+} for 60 minutes (*5*). Cells were washed in Tris.HCl (pH 7.8), and the Zn^{2+} content of 1 x 10^9 cells calculated. Three replica experiments are shown (*panels A, B* and *C*).

A


Zinc (nmoles/1 x 10 9 cells)
60
50
40
30
20
10
0
1
2
3
4
5

B


Zinc (nmoles/1 x 10 9 cells)
50
40
30
20
10
0
1
2
3
4
5

C

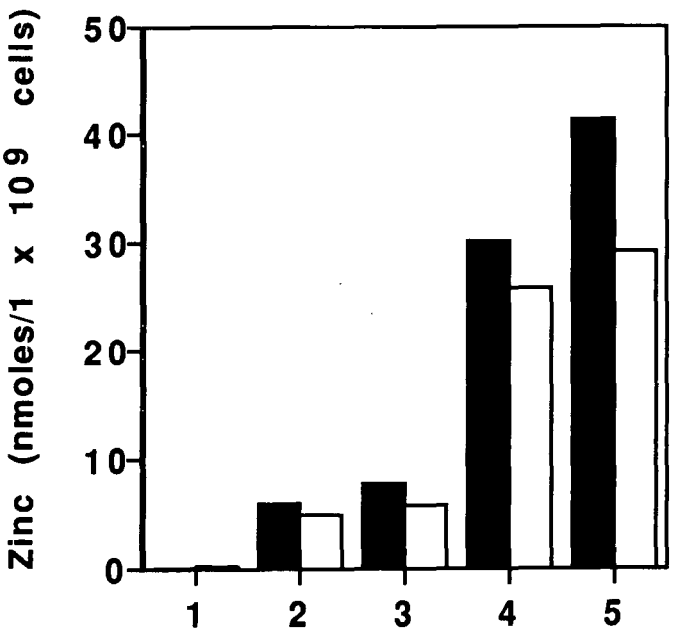
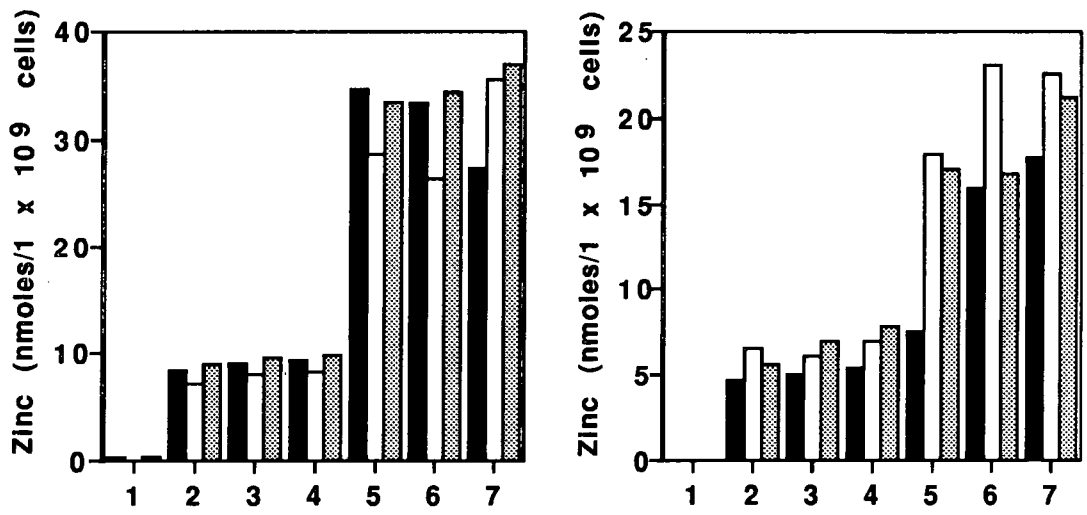

Zinc (nmoles/1 x 10 9 cells)
50
40
30
20
10
0
1
2
3
4
5

Figure 3.4.2. Accumulation of Zn^{2+} by R2-PIM8, R2-PIM8(*smt*) and R2-PIM8(*smtA+/B-*) exposed to $ZnCl_2$

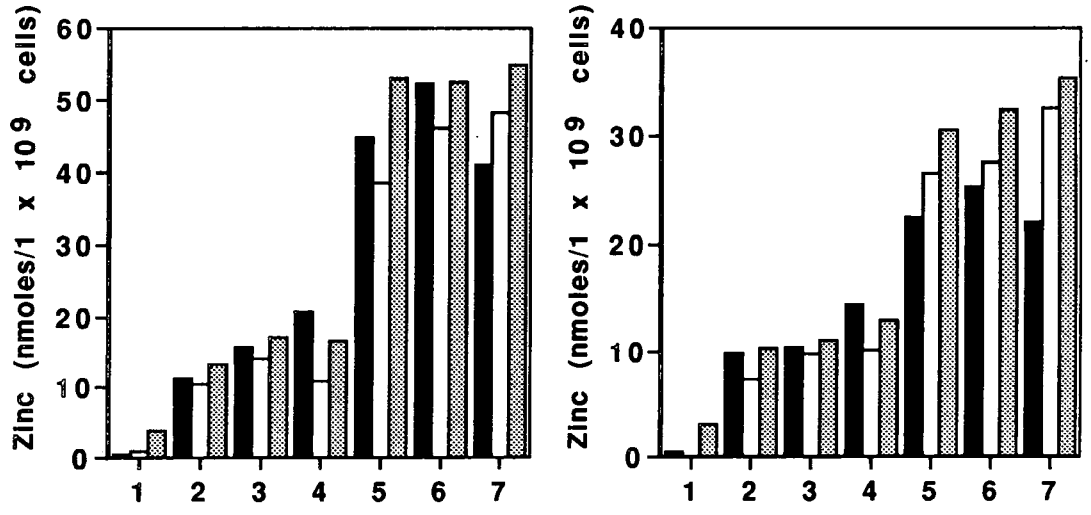
Accumulation of Zn^{2+} by R2-PIM8 (*closed columns*), R2-PIM8(*smt*) (*open columns*) and R2-PIM8(*smtA+/B-*) (*shaded columns*) exposed to: No metal (*1*); 2.5 μM Zn^{2+} for 30 minutes (*2*); 2.5 μM Zn^{2+} for 60 minutes (*3*); 2.5 μM Zn^{2+} for 120 minutes (*4*); 12 μM Zn^{2+} for 30 minutes (*5*); 12 μM Zn^{2+} for 60 minutes (*6*); or 12 μM Zn^{2+} for 120 minutes (*7*). Cells were washed in Tris.HCl (pH 7.8) (*left panels*), or Tris.HCl (pH 7.8) and 0.1 mM EDTA (*right panels*), and the Zn^{2+} content of 1 x 10^9 cells calculated. Two replica experiments are shown (*panels A* and *B*).

EDTA WASH

A


Zinc (nmoles/1 x 10 9 cells)
40
30
20
10
0
1 2 3 4 5 6 7
Zinc (nmoles/1 x 10 9 cells)
25
20
15
10
5
0
1 2 3 4 5 6 7

B


Zinc (nmoles/1 x 10 9 cells)
60
50
40
30
20
10
0
1 2 3 4 5 6 7
Zinc (nmoles/1 x 10 9 cells)
40
30
20
10
0
1 2 3 4 5 6 7

3.5. METALLOREGULATION FROM THE MT GENE, *smtA*, OPERATOR-PROMOTER

Metalloregulation from the *smtA* operator-promoter was examined by fusion of sequences upstream of *smtA*, to a promoterless *lacZ* reporter gene in the promoter probe shuttle vector pLACPB2 in R2-PIM8, R2-PIM8(*smt*) and R2-PIM8(*smtA+/B-*). This enabled analysis of the role of *smtB*.

3.5.1. Construction of *smt-lacZ* fusions

The construct pLACPB2(*smt*-5') (Huckle *et al.*, 1993), was used to generate a truncated derivative, pLACPB2(*smtB-*). pLACPB2(*smt*-5') contains ca. 600 bp of *smtA* 5' flanking region which includes *smtB* and the *smt* operator-promoter region. pLACPB2(*smtB-*) contains ca. 100 bp of *smtA* 5' flanking region which includes the *smt* operator-promoter region. pLACPB2(*smtB-*) lacks a functional *smtB*. In both cases the *smt* fragments were cloned into pLACPB2 using the *Bam*HI site to create a transcriptional fusion with *lacZ* (figure 3.5.1.).

R2-PIM8, R2-PIM8(*smt*) and R2-PIM8(*smtA+/B-*) were transformed to chloramphenicol and carbenicillin resistance with pLACPB2, pLACPB2(*smt*-5') and pLACPB2(*smtB-*). Several colonies were restreaked onto fresh Allens agar plates, grown in liquid culture, then plated to obtain single colonies. Resulting colonies were inoculated into liquid culture for analyses.

pLACPB2 constructs were isolated from transformants by plasmid recovery in *Escherichia coli*, and analysed by restriction digestion and sequencing. In each case, the isolated plasmid DNA was identical to the original construct.

3.5.2. β-galactosidase activity in R2-PIM8 and R2-PIM8(*smt*)

For the purpose of these assays 1.5 μM Cd^{2+} was considered to be the maximum permissive concentration for growth for both R2-PIM8 and R2-PIM8(*smt*) (figure 3.2.1., *panel B*), as cultures were exposed to metal ions for only 2 hours prior to assay and growth of R2-PIM8 (without Zn^{2+} pretreatment) above 1.5 μM Cd^{2+} was only observed following a lag of > 148 hours (figure 3.2.1., *panel B*; figure 3.2.2., *panel B*). 11 μM Zn^{2+}

and 2.5 μM Zn^{2+} were considered to be the maximum permissive concentrations for R2-PIM8 and R2-PIM8(*smt*), respectively. These were the levels of Zn^{2+} which allowed uninhibited growth of cultures during the period that assays were performed (figure 3.2.1., *panel A*; refer to section 3.3.2.).

β-galactosidase activity was initially assayed using the protocol described by Miller *et al.* (1972) (figure 3.5.2.). R2-PIM8 containing pLACPB2(*smt*-5') and pLACPB2(*smtB*-) showed metal-dependent β-galactosidase expression (figure 3.5.2., *panels A* and *B*, *2* and *3*). An elevated basal level of expression (in media containing no metal supplements) was observed with pLACPB2(*smtB*-) (with no plasmid borne *smtB*) (figure 3.5.2., *panels A* and *B*, *3*). At maximum permissive concentrations for growth, Zn^{2+} was the most potent elicitor (also observed previously following exposure of R2-PIM8 containing pLACPB2(*smt*-5') to a range of metal ions (section 1.3.; Huckle *et al.*, 1993)). Equivalent increases in β-galactosidase expression on exposure to metal ions were not detected in the control strain containing pLACPB2 alone (figure 3.5.2., *panels A* and *B*, *1*).

Maximum expression of pLACPB2(*smt*-5') occurs at lower concentrations of Zn^{2+} in R2-PIM8(*smt*) than in R2-PIM8 (figure 3.5.2., *panel A*, *2* and *4*). In both cases maximum expression occurs at the maximum permissive concentration of Zn^{2+} for growth. There was also elevated basal expression in R2-PIM8(*smt*) containing pLACPB2(*smt*-5') (figure 3.5.2., *panels A* and *B*, *4*) (although it is noted that elevated basal expression in R2-PIM8(*smt*) containing pLACPB2(*smt*-5') was less apparent in subsequent experiments using modified protocols, refer to sections 3.5.3. and 3.5.4.). Most significantly, there was highly elevated (> 20 fold) basal expression of β-galactosidase (which exceeds (by ca. 4 fold) maximal Zn^{2+} induced expression in R2-PIM8) and loss of metal dependency in R2-PIM8(*smt*) containing pLACPB2(*smtB*-) (figure 3.5.2., *panels A* and *B*, *5*). These cells are devoid of a functional plasmid or chromosomal *smtB*.

There were no detectable (using *in situ* hybridisation) changes in plasmid copy number in R2-PIM8 containing pLACPB2(*smt*-5'), upon exposure to $ZnCl_2$ or $CdCl_2$ (Huckle *et al.*, 1993). A replicate assay with $ZnCl_2$ is shown (figure 3.5.2.), as Zn^{2+} elicited the greatest response at the biologically significant levels.

3.5.3. β-galactosidase activity in R2-PIM8 and R2-PIM8(*smt*), assayed using a modified protocol

β-galactosidase activity in R2-PIM8 and R2-PIM8(*smt*), containing pLACPB2, pLACPB2(*smt*-5') or pLACPB2(*smtB*-), was assayed using the modified protocol described in section 2.3.16.2.. Cultures were induced with metal ions when their optical densities at 595 nm were ca. 0.2 (as previously), and the results (figure 3.5.3.) were similar to those observed previously using the protocol described by Miller *et al.* (1972) (figure 3.5.2.).

Zn^{2+} was the most potent elicitor (at maximum permissive concentrations for growth) of metal-dependent β-galactosidase expression. Maximum expression of pLACPB2(*smt*-5') occurred at lower concentrations of Zn^{2+} in R2-PIM8(*smt*) than in R2-PIM8 (figure 3.5.3., *panel A*, *2* and *4*), as observed previously (figure 3.5.2., *panel A*, *2* and *4*). The basal level of expression (in media with no metal supplements) of R2-PIM8 and R2-PIM8(*smt*) containing pLACPB2(*smt*-5') was similar (figure 3.5.3., *panels A* and *B*, *2* and *4*) (elevated basal expression in R2-PIM8(*smt*) containing pLACPB2(*smt*-5') was observed previously (figure 3.5.2., *panels A* and *B*, *4*)), and an elevated basal level of expression remained in R2-PIM8 containing pLACPB2(*smtB*-) (figure 3.5.3., *panels A* and *B*, *3*). High basal expression of β-galactosidase and loss of metal dependency was observed in R2-PIM8(*smt*) containing pLACPB2(*smtB*-) (figure 3.5.3., *panels A* and *B*, *5*).

3.5.4. β-galactosidase activity in R2-PIM8, R2-PIM8(*smt*) and R2-PIM8(*smtA+/B-*), assayed using a modified protocol

β-galactosidase activity in R2-PIM8, R2-PIM8(*smt*) and R2-PIM8(*smtA+/B-*), containing pLACPB2, pLACPB2(*smt*-5') or pLACPB2(*smtB*-), was assayed using the modified protocol, and cultures were induced with metal ions when their optical densities at 595 nm were ca. 0.08 (figure 3.5.4.). R2-PIM8 containing pLACPB2(*smt*-5') or pLACPB2(*smtB*-), R2-PIM8(*smt*) containing pLACPB2(*smt*-5'), and R2-PIM8(*smtA+/B-*) containing pLACPB2(*smt*-5') (these strains contain chromosomal and/or plasmid encoded *smtB*), showed metal-dependent β-galactosidase expression from the *smt*

operator-promoter (figure 3.5.4., *panels A* and *B*, *2*, *5*, *6* and *8*). Equivalent increases in β-galactosidase expression on exposure to metal ions were not detected in the control strains containing pLACPB2 alone (figure 3.5.4., *panels A* and *B*, *1*, *4* and *7*). R2-PIM8 containing pLACPB2(*smtB-*) showed elevated basal expression (figure 3.5.4., *panels A and B*, *6*).

With Zn^{2+}, maximum metal-dependent β-galactosidase expression was observed in R2-PIM8 containing pLACPB2(*smt*-5') (figure 3.5.4., *panel A*, *5*). Maximal metal-dependent expression was less in the other strains (i.e. R2-PIM8 containing pLACPB2(*smtB-*) (figure 3.5.4., *panel A*, *6*), R2-PIM8(*smt*) containing pLACPB2(*smt*-5') (figure 3.5.4., *panel A*, *8*) and R2-PIM8(*smtA+/B-*) containing pLACPB2(*smt*-5') (figure 3.5.4., *panel A*, *2*)). This phenomenon (observed with Zn^{2+}) was previously undetected when more dense cultures were used. As observed previously, at maximum permissive concentrations for growth, Zn^{2+} was the most potent inducer of metal-dependent expression from the *smt* operator-promoter (figure 3.5.4., *panels A* and *B*, *2*, *5*, *6* and *8*).

Highly elevated basal expression of β-galactosidase and loss of metal dependency was observed in R2-PIM8(*smt*) containing pLACPB2(*smtB-*) (figure 3.5.4., *panels A and B*, *9*). Elevated basal expression and loss of metal dependency was also observed in R2-PIM8(*smtA+/B-*) containing pLACPB2(*smtB-*) (figure 3.5.4., *panels A and B*, *3*). Both of these strains are devoid of a functional *smtB*. R2-PIM8(*smtA+/B-*) had lower (ca. 2 fold) β-galactosidase activity in comparison to R2-PIM8(*smt*) containing pLACPB2(*smtB-*) coinciding with the presence of *smtA* in the former, but not the latter.

Two replicate assays with $ZnCl_2$ are shown (not Cd^{2+}) as Zn^{2+} elicited the greatest response at the biologically significant levels, and demonstrated more subtle effects at these low culture densities.

Figure 3.5.1. Nucleotide sequence of the *smt* operator-promoter fused to *lacZ*

smtA 5' flanking sequences were cloned into the promoter probe vector pLACPB2, using the *Bam*HI site to create a transcriptional fusion with *lacZ*, and the constructs pLACPB2(*smt*-5') and pLACPB2(*smtB*-) were generated. The nucleotide sequence of the *smt* operator-promoter region fused to *lacZ* is shown, and the *smt* sequences of pLACPB2(*smtB*-), which lacks a functional *smtB*, are indicated (*bold type*) (pLACPB2(*smt*-5') contains a complete *smtB*). *smtA* and *smtB* -10 promoter sequences are underlined, and partial amino acid sequences for *smtB* and *lacZ* are shown.

The *smtA* 5' flanking sequences of pLACPB2(*smt*-5) continue 5' as far as the *Hin*dIII site (refer to figure 3.1.2b.).

CTG CAG CAC TGG TTT TGT CAT GAGCCAATCACGGTTTGTCCACCC

Q L V P K T M

smtB

ACCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGT

-10 (*smtB*) -10 (*smtA*)

*Bam*HI

TATTCAGATATTCAGGATCCCCGGGAATTCATCGAGCAACATATTAATGA

GCCAGAGAAATGCTGGCGGCACTGAAAGTTTTTGTACAAGCCGATGAAAG

CGGCGACGCGCAGTTAATCCCACAGCCGCCAGTTCCGCTGGCGGCATTTTA

ACTTTCTTTATCACACAGGAAACAGCT ATG ACC ATG ATT ACG GAT TCA

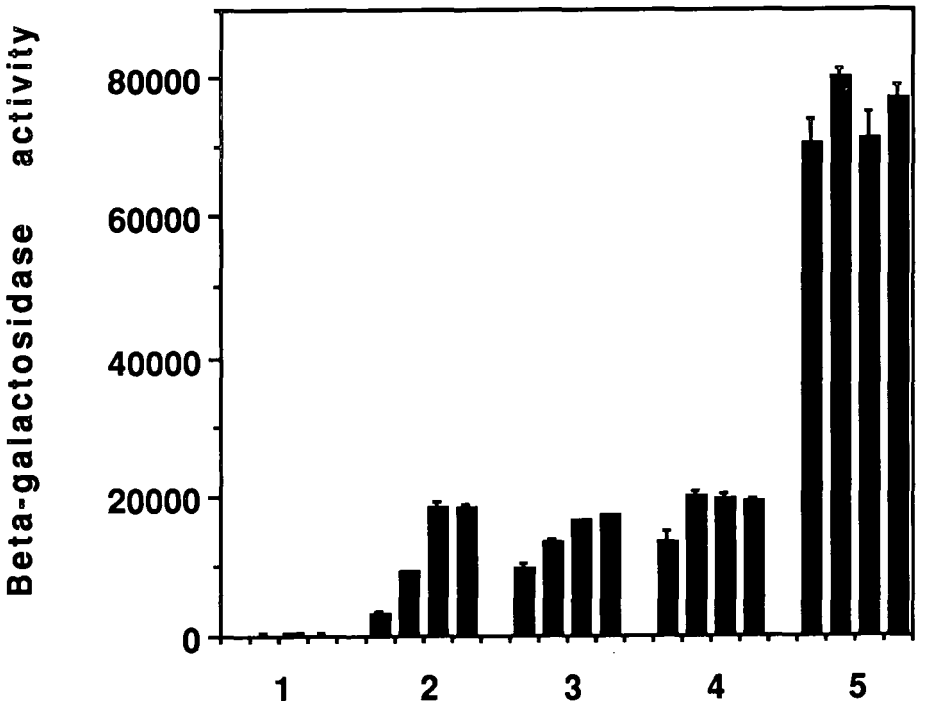
M T M I T D S

lacZ

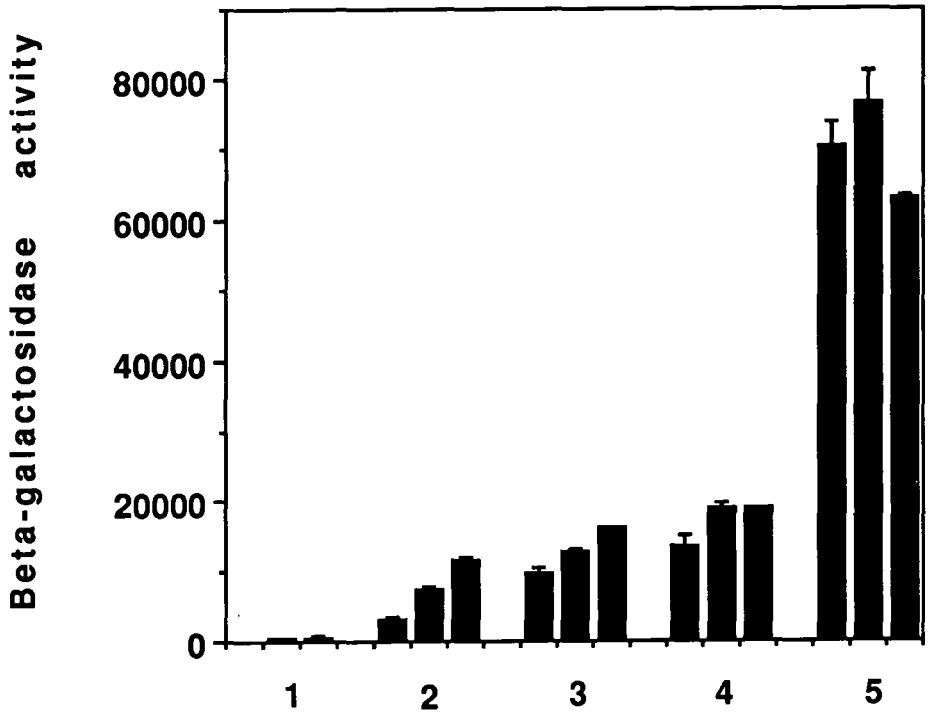
Figure 3.5.2. Beta-galactosidase activity in R2-PIM8 and R2-PIM8(*smt*)

Beta-galactosidase activity (assayed using the protocol described by Miller *et al.* (1972)) of: *1*, R2-PIM8 containing pLACPB2 only as a control; *2*, R2-PIM8 containing pLACPB2(*smt*-5'), which contains plasmid encoded, and chromosomal, *smtB*; *3*, R2-PIM8 containing pLACPB2(*smtB*-), which lacks plasmid encoded, but contains chromosomal, *smtB*; *4*, R2-PIM8(*smt*) containing pLACPB2(*smt*-5'), which only contains plasmid encoded *smtB*; *5*, R2-PIM8(*smt*) containing pLACPB2(*smtB*-), devoid of *smtB*. *Panel A*, each strain was exposed to increasing concentrations (0 μM, 2.5 μM, 11 μM and 12 μM) of $ZnCl_2$ (left to right) prior to assay. *Panel B*, each strain was exposed to increasing concentrations (0 μM, 1.5 μM and 5 μM) of $CdCl_2$ prior to assay. Cultures were induced with metal ions when their optical densities at 595 nm were ca. 0.2, and assays with $ZnCl_2$ and $CdCl_2$ were performed in parallel. The data represent the mean values estimated from three separate assays, with standard deviation. Equivalent data were obtained in a replicate experiment with $ZnCl_2$, shown overleaf.

A


Beta-galactosidase activity
80000
60000
40000
20000
0
1
2
3
4
5

B


Beta-galactosidase activity
80000
60000
40000
20000
0
1
2
3
4
5

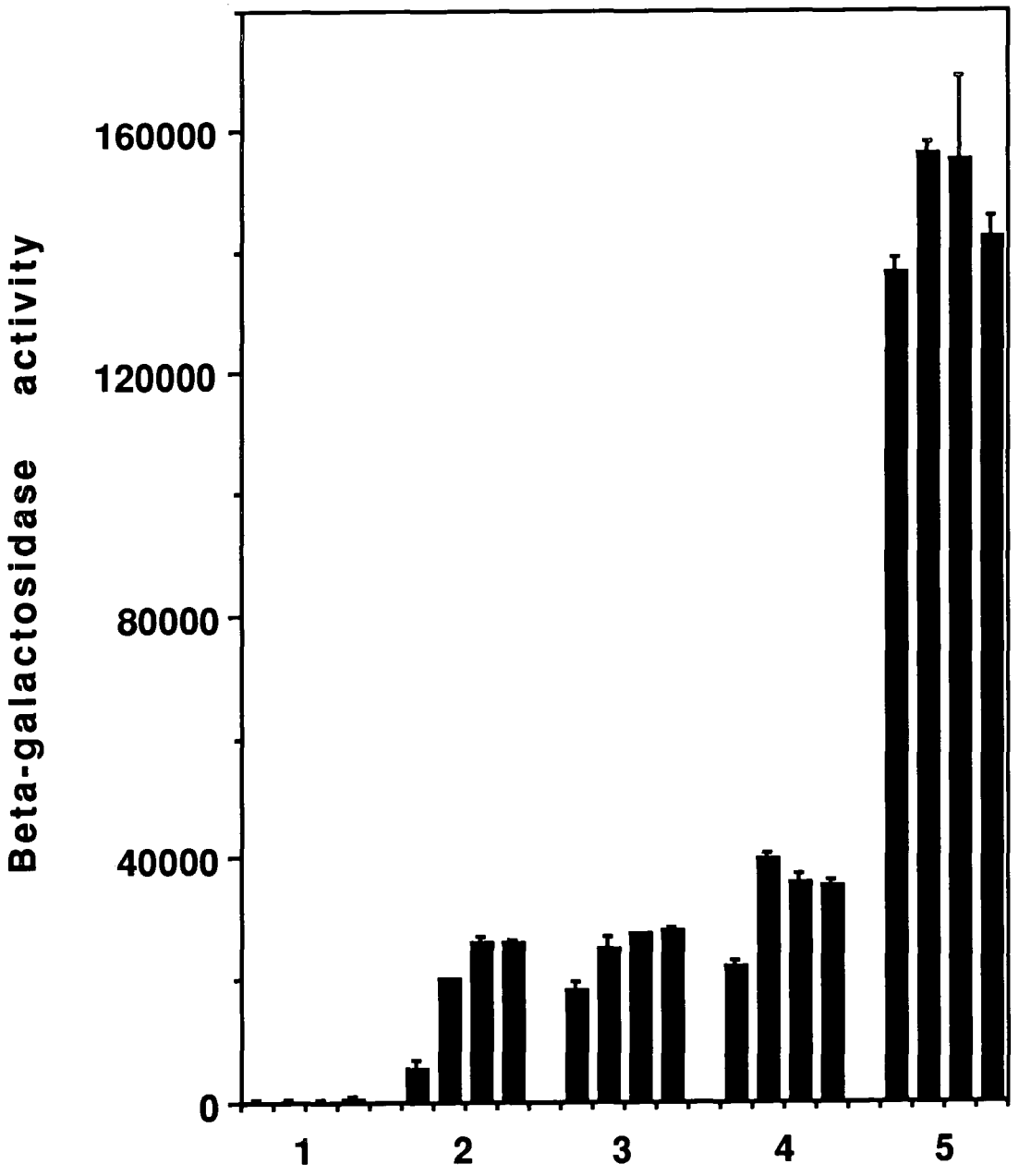
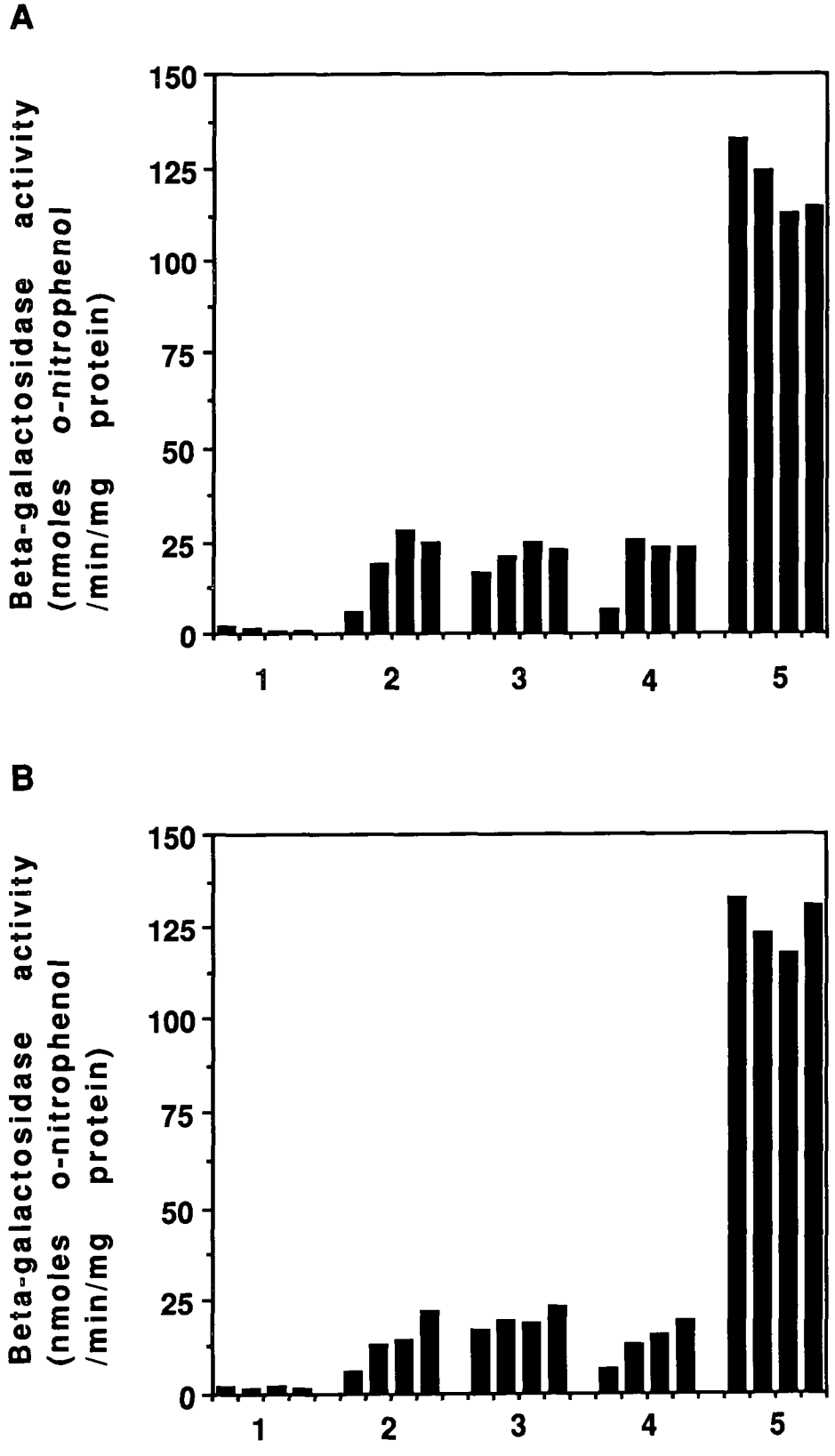
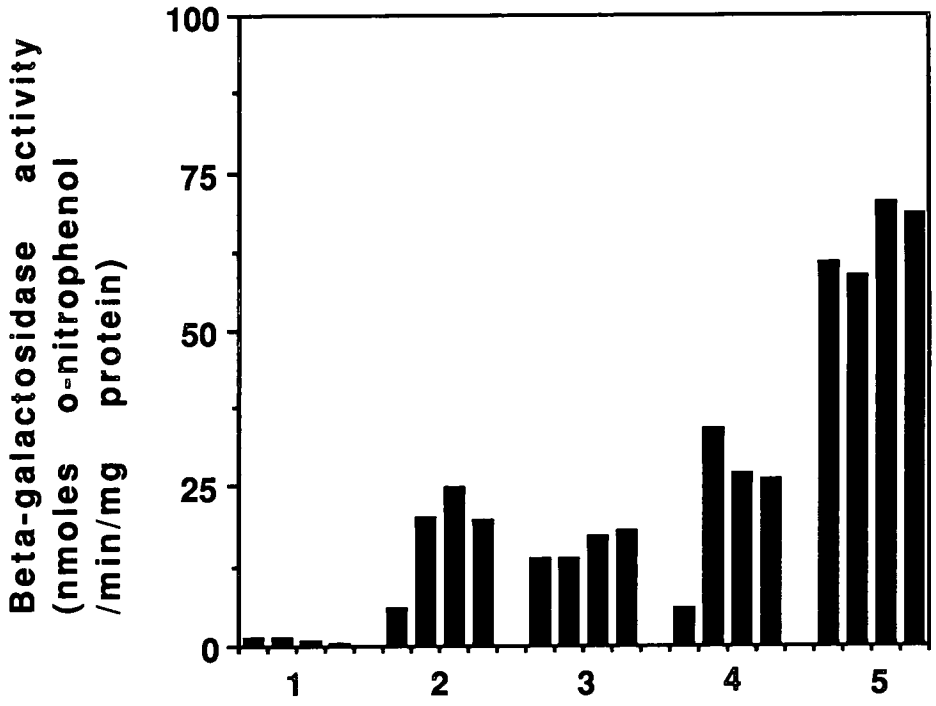

Beta-galactosidase activity
160000
120000
80000
40000
0
1
2
3
4
5

Figure 3.5.3. Beta-galactosidase activity in R2-PIM8 and R2-PIM8(*smt*), assayed using modified protocols

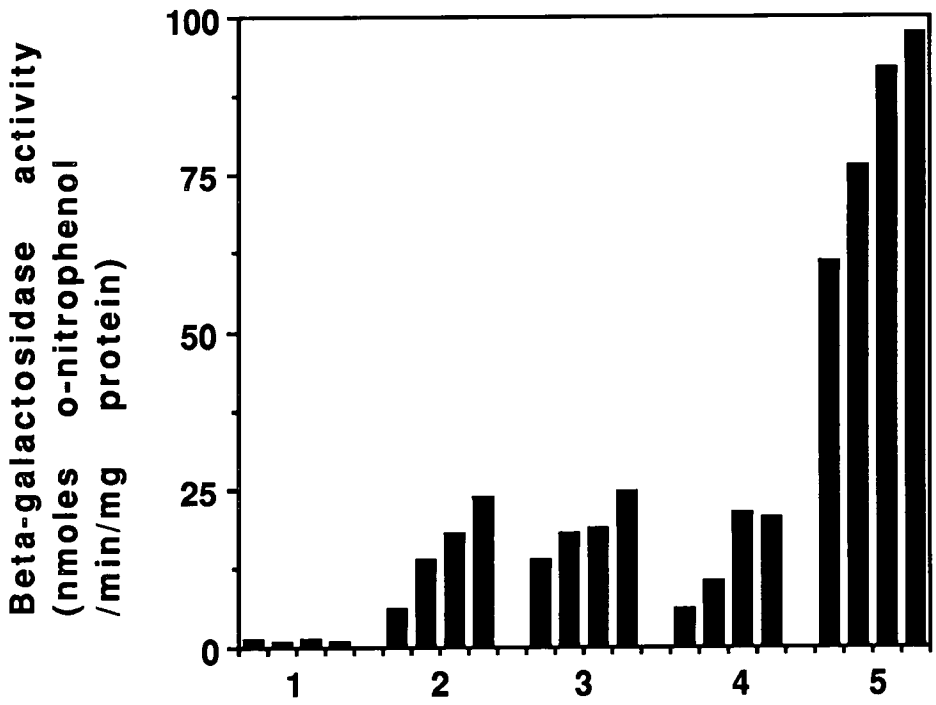
Beta-galactosidase activity of: *1*, R2-PIM8 containing pLACPB2 only, as a control; *2*, R2-PIM8 containing pLACPB2(*smt*-5'), which contains plasmid encoded, and chromosomal, *smtB*; *3*, R2-PIM8 containing pLACPB2(*smtB*-), which lacks plasmid encoded, but contains chromosomal, *smtB*; *4*, R2-PIM8(*smt*) containing pLACPB2(*smt*-5'), which only contains plasmid encoded *smtB*; 5, R2-PIM8(*smt*) containing pLACPB2(*smtB*-), devoid of *smtB*. *Panel A*, each strain was exposed to increasing concentrations (0 μM, 2.5 μM, 11 μM and 12 μM) of $ZnCl_2$ (left to right) prior to assay. *Panel B*, each strain was exposed to increasing concentrations (0 μM, 1.5 μM, 2 μM and 5 μM) of $CdCl_2$ prior to assay. Cultures were induced with metal ions when their optical densities at 595 nm were ca. 0.2, and assays with $ZnCl_2$ and $CdCl_2$ were performed in parallel. Equivalent data were obtained in two further replicate experiments shown overleaf.


A
Beta-galactosidase activity
(nmoles o-nitrophenol
/min/mg protein)
150
125
100
75
50
25
0
1
2
3
4
5
B
Beta-galactosidase activity
(nmoles o-nitrophenol
/min/mg protein)
150
125
100
75
50
25
0
1
2
3
4
5

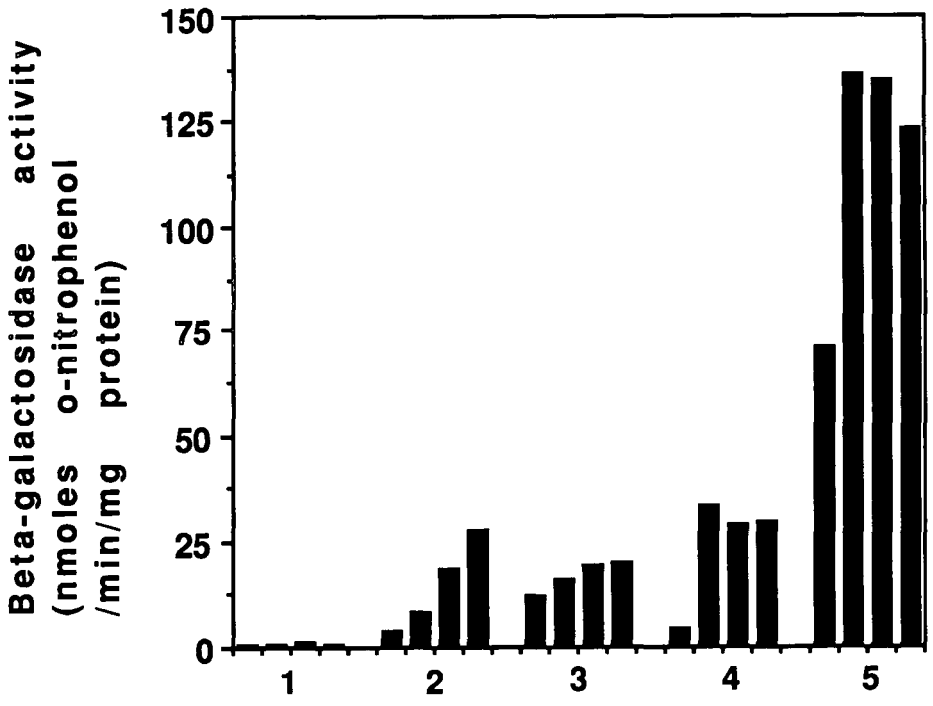
A


Beta-galactosidase activity
(nmoles o-nitrophenol
/min/mg protein)
100
75
50
25
0
1
2
3
4
5

B


Beta-galactosidase activity
(nmoles o-nitrophenol
/min/mg protein)
100
75
50
25
0
1
2
3
4
5

A


Beta-galactosidase activity
(nmoles o-nitrophenol
/min/mg protein)
150
125
100
75
50
25
0
1
2
3
4
5

B

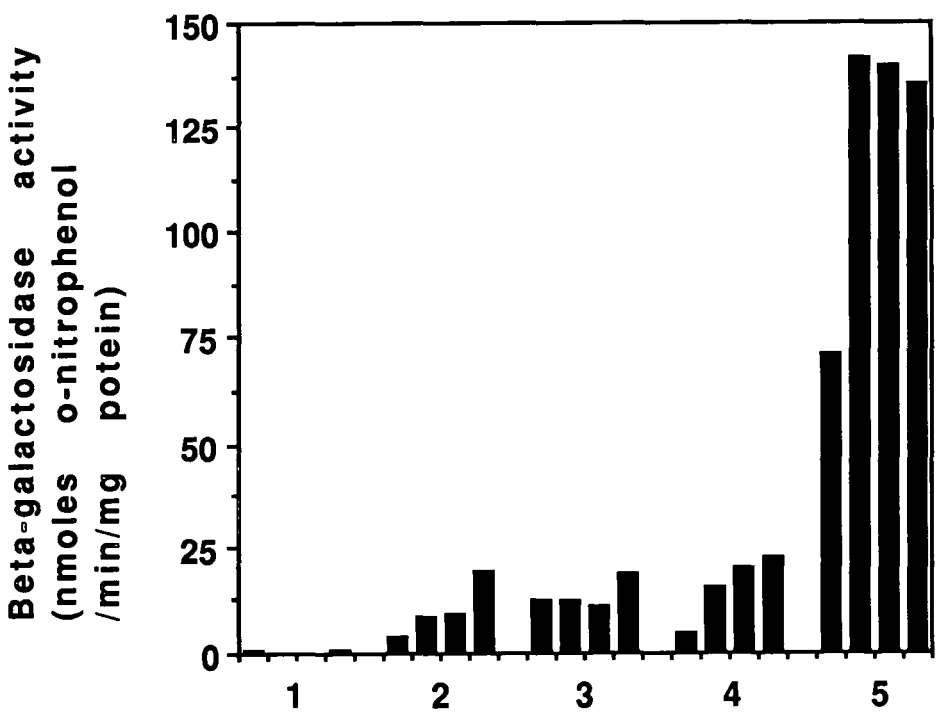
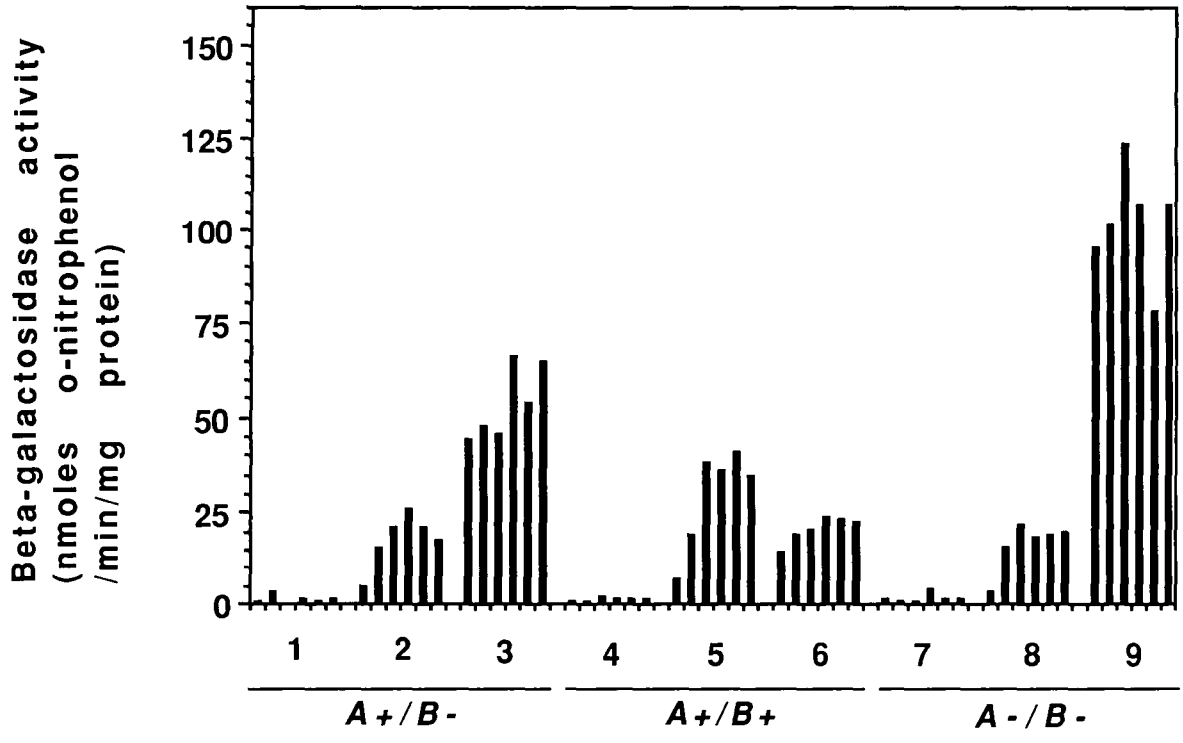

Beta-galactosidase activity
(nmoles o-nitrophenol
/min/mg potein)
150
125
100
75
50
25
0
1
2
3
4
5

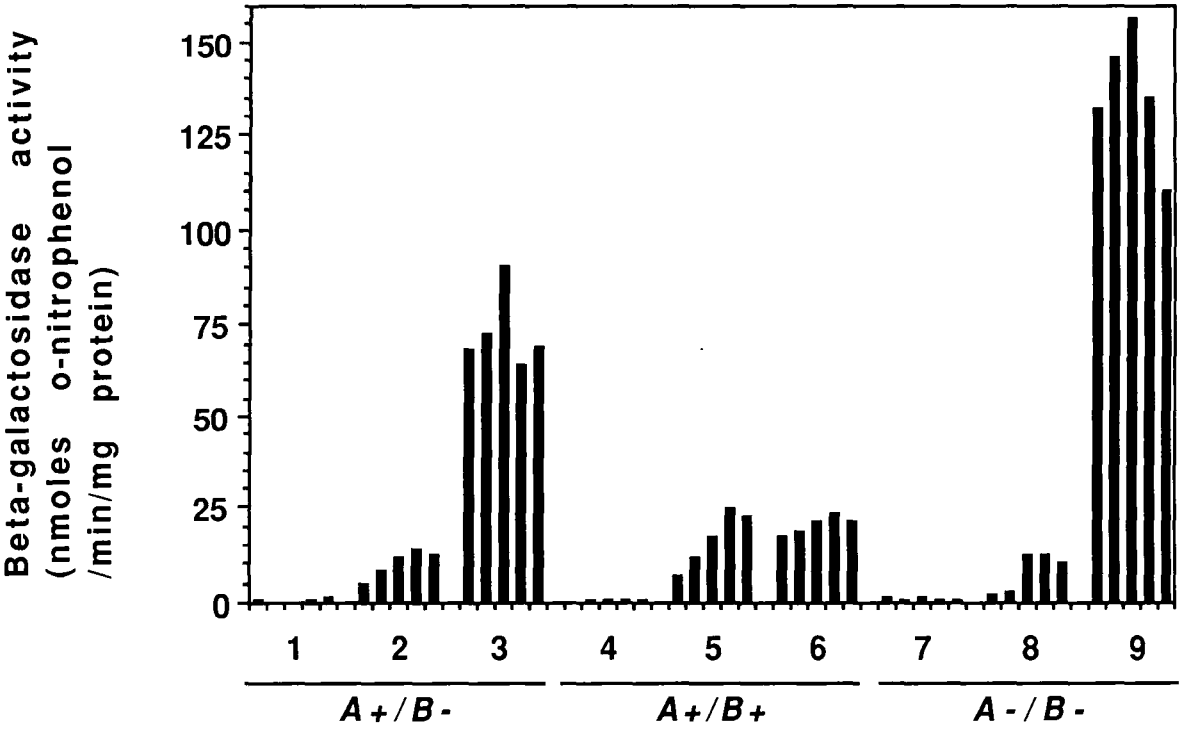
Figure 3.5.4. Beta-galactosidase activity in R2-PIM8, R2-PIM8(*smt*) and R2-PIM8(*smtA+/B-*), assayed using modified protocols

Beta-galactosidase activity of: *1*, R2-PIM8(*smtA+/B-*) containing pLACPB2 only; *2*, R2-PIM8(*smtA+/B-*) containing pLACPB2(*smt*-5'), which only contains plasmid encoded *smtB*; *3*, R2-PIM8(*smtA+/B-*) containing pLACPB2(*smtB-*), devoid of *smtB*; *4*, R2-PIM8 containing pLACPB2 only; *5*, R2-PIM8 containing pLACPB2(*smt*-5'), which contains plasmid encoded, and chromosomal, *smtB*; *6*, R2-PIM8 containing pLACPB2(*smtB-*), which lacks plasmid encoded, but contains chromosomal, *smtB*; *7*, R2-PIM8(*smt*) containing pLACPB2 only; *8*, R2-PIM8(*smt*) containing pLACPB2(*smt*-5'), which only contains plasmid encoded *smtB*; *9*, R2-PIM8(*smt*) containing pLACPB2(*smtB-*), devoid of *smtB*. In each case the presence/absence of chromosomal *smtA* and *smtB* is shown (i.e. *A+/B-*, *A+/B+* or *A-/B-*). *Panel A*, each strain was exposed to increasing concentrations (0 μM, 2.5 μM, 11 μM, 12 μM, 14 μM and 16 μM) of $ZnCl_2$ (left to right) prior to assay. *Panel B*, each strain was exposed to increasing concentrations (0 μM, 1.5 μM, 3 μM, 5 μM and 10 μM) of $CdCl_2$ prior to assay. Cultures were induced with metal when their optical densities at 595 nm were ca. 0.08, and assays with $ZnCl_2$ and $CdCl_2$ were performed in parallel. Equivalent data were obtained with $ZnCl_2$ in two further replicate experiments shown overleaf.

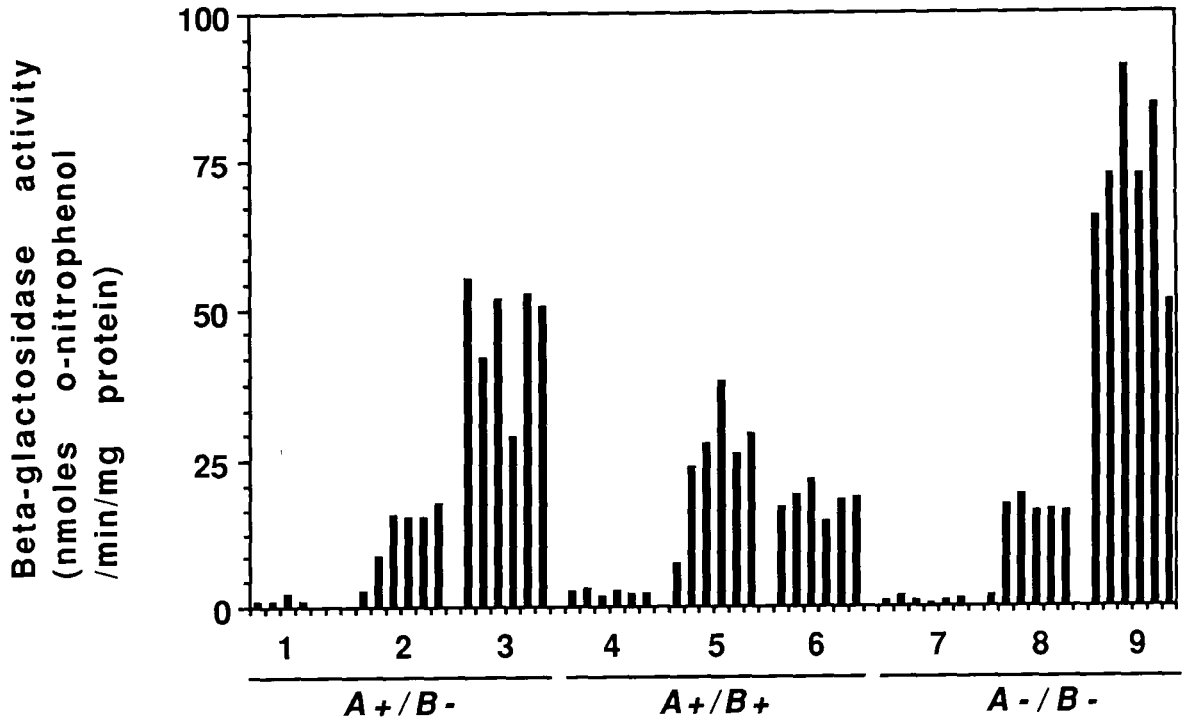
A Zinc



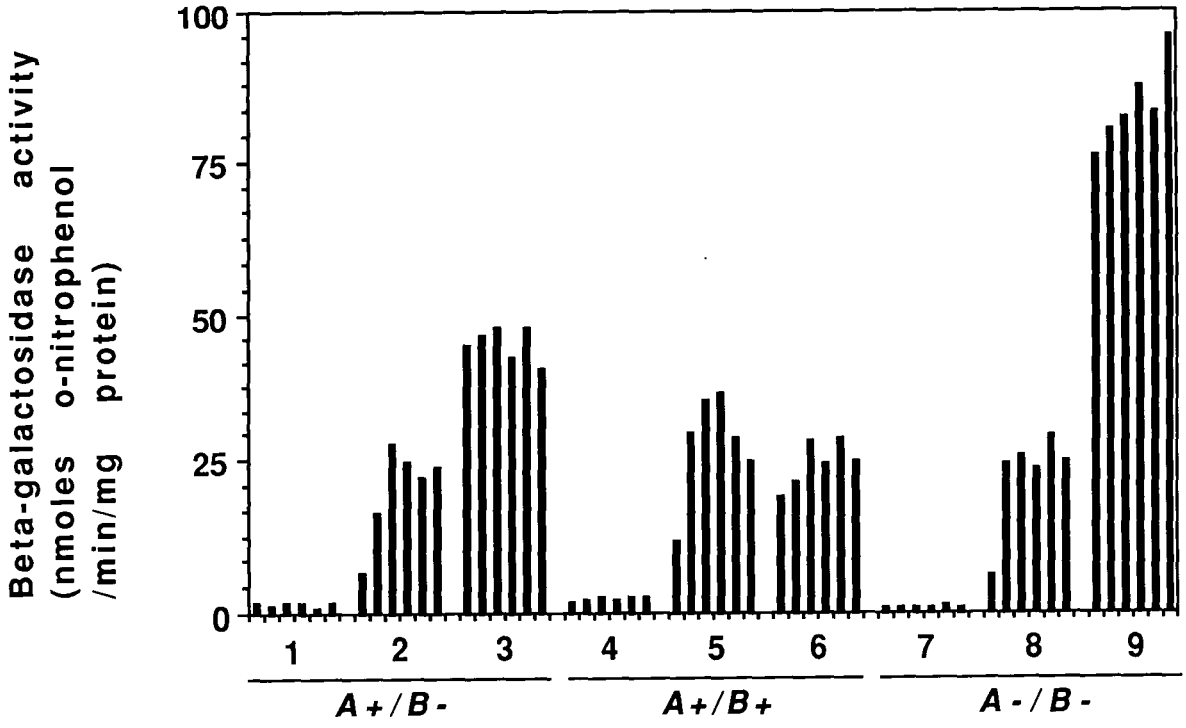
B Cadmium



Zinc


Beta-glactosidase activity
(nmoles o-nitrophenol
/min/mg protein)
100
75
50
25
0
1
2
3
4
5
6
7
8
9
A+/B-
A+/B+
A-/B-

Zinc


Beta-galactosidase activity
(nmoles o-nitrophenol
/min/mg protein)
100
75
50
25
0
1
2
3
4
5
6
7
8
9
A+/B-
A+/B+
A-/B-

CHAPTER 4

DISCUSSION

The production of mutants of *Synechococcus* PCC 7942 (strain R2-PIM8) which lack a functional *smt* divergon is described. These mutants (designated R2-PIM8(*smt*)) have enabled analysis of the function of the two genes, *smtA* and *smtB*. The function of SmtA with respect to a role in essential metal metabolism/homoeostasis and the sequestration of excess amounts of certain metal ions is discussed in this chapter. Furthermore, examination of the regulation of SmtA expression by the divergently transcribed SmtB, has revealed the latter to be a *trans*-acting repressor of *smtA* transcription. SmtB is thus one of the first characterised cyanobacterial transcription factors. Its role in metallo-regulation of *smtA* expression is discussed.

4.1. INTERRUPTION OF THE *smt* DIVERGON

4.1.1. Localisation of *smt*

Metal resistance determinants are mostly plasmid borne in Gram-negative and Gram-positive bacteria (refer to section 1.2.). *Synechococcus* PCC 7942 has two indigenous plasmids (there is also some suggestion of the existence of a megaplasmid in this strain (refer to section 3.1.1.)) which could potentially harbour *smt*. However, despite the recent report of an involvement of the large plasmid in the acclimation of *Synechococcus* PCC 7942 to sulphur stress (Nicholson and Laudenbach, 1993), no plasmid encoded functions have previously been identified in cyanobacteria (Ciferri *et al.*, 1989). The chromosomal localisation of *smt* has now been confirmed by Southern analyses (although, this is with the reservation that this designation could be changed if further evidence of a megaplasmid is reported for *Synechococcus* PCC 7942) (section 3.1.1.). Furthermore, the complete nucleotide sequence (lacking *smt*) of the *Synechococcus* PCC 7942 small plasmid, pUH24, has now been reported (Van der Plas *et al.*, 1992).

4.1.2. Confirmation of the *smt* deficient status of R2-PIM8(*smt*)

R2-PIM8(*smt*) mutants which lack a functional *smt* divergon were generated via insertional inactivation/partial gene deletion mediated by homologous recombination. This involved transformation of R2-PIM8 with plasmid pRECSU (figure 3.1.2.) consisting of *smt* flanking sequences interrupted by the *cat* containing *Escherichia coli* plasmid pSU19. The structure and homozygosity (of the *smt* region) of the Cm^r mutants were confirmed by Southern analyses and plasmid recovery in *E. coli* (involving the generation of a ca. 7.8 kb plasmid from *Sal*I digested R2-PIM8(*smt*) DNA) (sections 3.1.3. and 3.1.4.). A 371 bp region (of the *smt* divergon), including the *smtA* and *smtB* transcription/translation start sites and operator-promoter sequences, was replaced by pSU19 in R2-PIM8(*smt*). Furthermore, while *smtA* transcripts were readily detected in RNA from R2-PIM8 exposed to Cd^{2+}, they were not detected in R2-PIM8(*smt*) RNA (figure 3.1.8.).

4.2. PHENOTYPIC ANALYSIS OF R2-PIM8(*smt*) and R2-PIM8(*smtA+/B-*)

4.2.1. Analysis of tolerance of R2-PIM8(*smt*) to trace metal ions

R2-PIM8(*smt*) shows reduced (ca. 5 fold) tolerance to Zn^{2+} in comparison to R2-PIM8 (figure 3.2.1., *panel A*; figure 3.2.2., *panel A*). Furthermore, *smt* mediated restoration of Zn^{2+} tolerance can be used as a phenotypic selection for transformation of R2-PIM8(*smt*). Zn^{2+} hypersensitive R2-PIM8(*smt*) was transformed to normal Zn^{2+} tolerance with a linear DNA fragment containing the complete *smt* divergon. All of the resulting Zn^{2+} resistant (Zn^r) colonies exhibited restored chloramphenicol sensitivity (indicating *smt* replacement of pSU19). Growth curves indicated Zn^{2+} and Cd^{2+} tolerance characteristics, of R2-PIM8(*smt*-reintroduced), identical to R2-PIM8 (figure 3.2.6.), and Southern analyses confirmed reintegration of the functional *smt* divergon with concomitant loss of pSU19 (figure 3.2.5.; section 3.2.2.). Thus, the Zn^{2+} and Cd^{2+} (see below) hypersensitive phenotype of R2-PIM8(*smt*) was solely due to loss of the *smt* divergon. *smt* therefore has a role in providing "normal" tolerance to Zn^{2+} and Cd^{2+}.

Greater tolerance of R2-PIM8, compared to R2-PIM8(*smt*), to Cd^{2+} was detected upon prolonged (> 148 hour) exposure. Olafson *et al.* (1980) noted a marked growth lag of ca.

6 days in *Synechococcus* sp. exposed to Cd^{2+}. The onset of growth coincided with accumulation of MT, and extrachromosomal MT gene amplification was proposed. However, *smt* is now confirmed to be chromosomal (with the reservation that there may be a megaplasmid in *Synechococcus* PCC 7942 as noted in section 4.1.1.). Gupta *et al.* (1992; 1993) observed amplification and highly iterated palindrome mediated rearrangement, involving deletion of a 352 bp region, of *smt* in *Synechococcus* PCC 6301 selected for Cd^{2+} resistance (refer to section 4.3.2.). A highly iterated octameric palindrome, designated HIP1, was present at the borders of the observed deletion (Gupta *et al.*, 1993). No rearrangement, of *smt*, was detected (by Southern analysis) in R2-PIM8 cultures growing at 4.0 μM Cd^{2+} and 4.5 μM Cd^{2+} in this study. However, an increased lag phase is an effect of trace metal ions on unicellular organisms which is often observed (e.g. Foster, 1977; Nies, 1992b; Rouch *et al.*, 1985; Mitra *et al.*, 1975; Higham *et al.*, 1984). This may reflect the period for a resistance mechanism to be expressed and reduce the intracellular concentration of a metal ion and/or the repair of metal ion initiated cellular damage antecedent to resumption of growth. Alternatively, the lag may reflect the time for growth from a few viable cells at Cd^{2+} concentrations toxic to the majority of cells, to be detected (cellular death and concomitant binding of Cd^{2+} may reduce the concentration of biologically available Cd^{2+}).

The prolonged growth lag prior to the detection of greater R2-PIM8, compared to R2-PIM8(*smt*), Cd^{2+} tolerance, was not observed prior to the detection of (ca. 5 fold) greater tolerance of R2-PIM8 to Zn^{2+} (figure 3.2.2.). This probably reflects Zn^{2+} being a more potent inducer (at maximum permissive concentrations for growth) of expression from the *smtA* operator-promoter (refer to section 4.4.2.).

There was no marked difference in Cu^{2+} tolerance of R2-PIM8 and R2-PIM8(*smt*). Olafson (1986), observed a protracted growth lag in cells exposed to elevated Cu^{2+}, but no MT synthesis coincident with the onset of growth. Energy-dependent copper efflux has been proposed as an alternative mechanism of Cu^{2+} resistance in *Synechococcus* sp. (Olafson, 1986) and in another cyanobacterium *Nostoc calcicola* (Verma and Singh, 1991). The sequence of a *Synechococcus* PCC 6301 P-type ATPase, started by Cozens and Walker (1987), has recently been completed (cited in Silver *et al.*, 1993). A 10-fold

increase in copper resistance resulted from disruption of the P-type ATPase gene (cited in Silver *et al.*, 1993), suggesting that this ATPase is involved in copper influx.

Initially, R2-PIM8(*smt*) cultures showed slightly reduced resistance to Ag^+ toxicity, however following a protracted lag period R2-PIM8(*smt*) and R2-PIM8 displayed no significant difference in resistance (figure 3.2.1., *panel D*). *smt* may therefore provide slight early resistance to Ag^+, and an alternative mechanism of Ag^+ resistance may exist in *Synechococcus* PCC 7942 (providing resistance upon prolonged exposure).

No significant difference in resistance to Hg^{2+} was observed for R2-PIM8 and R2-PIM8(*smt*) cultures. Growth of both R2-PIM8 and R2-PIM8(*smt*) cultures was observed at higher Hg^{2+} concentrations following a protracted lag (figure 3.2.1., *panel E*).

4.2.2. Analysis of tolerance of R2-PIM8(*smtA+/B-*) to trace metal ions

Restoration of Zn^{2+} tolerance was also used as a phenotypic selection for transformation of R2-PIM8(*smt*) with a rearranged *smt* divergon, containing functional *smtA* and non-functional *smtB*. Growth curves indicated (early) increased tolerance of isolated recombinants, designated R2-PIM8(*smtA+/B-*), to Zn^{2+} and Cd^{2+}, as compared to R2-PIM8(*smt*-reintroduced) (and hence R2-PIM8) (figure 3.3.3.). Southern analysis confirmed reintegration of *smtA* (section 3.3.1.), thus the loss of *smtA* (not *smtB*) was responsible for the Zn^{2+} and Cd^{2+} hypersensitive phenotype of R2-PIM8(*smt*).

smtB is shown (section 4.4.) to encode a repressor of *smtA* transcription and R2-PIM8(*smtA+/B-*) has constitutive derepressed expression from the *smtA* operator-promoter (refer to section 4.4.2.). The prolonged growth lag observed with R2-PIM8(*smt*-reintroduced) (and R2-PIM8) in higher Cd^{2+} concentrations (> 1.5 μM) was not observed with R2-PIM8(*smtA+/B-*) (figure 3.3.3., *panel B*). Growth of R2-PIM8(*smt*-reintroduced) observed at high Zn^{2+} concentrations, was greatly inhibited in comparison to R2-PIM8(*smtA+/B-*) (figure 3.3.3., *panel A*). Functional deletion of *smtB* therefore brings about an (early) increase in metal ion (Zn^{2+}/Cd^{2+}) tolerance in cells containing functional *smtA*.

4.2.3. Analysis of tolerance of R2-PIM8(*smt*- reintroduced) and R2-PIM8(*smtA+/B-*) to trace metal ions, following metal pretreatment

R2-PIM8(*smt*- reintroduced) pretreated with a non-toxic concentration of Zn^{2+} (to induce *smtA* expression) exhibited the phenotype observed for R2-PIM8(*smtA+/B-*) at 2 μM Cd^{2+}. However, growth of the former observed at 3 μM Cd^{2+} remained greatly inhibited for > 192 hours (figure 3.3.4., *panel B*). R2-PIM8(*smtA+/B-*) displayed similar growth patterns with and without metal pretreatment, and no prolonged growth lag was observed at 2 μM Cd^{2+} and 3 μM Cd^{2+} (figure 3.3.4.). Cells containing *smtB* showed metal-dependent expression from the *smtA* operator-promoter (determined via β-galactosidase assays), and high basal, metal-independent, expression was observed for cells devoid of *smtB* (figure 3.5.2.; refer to section 4.4.2.). Phenotypic differences observed at 3 μM Cd^{2+} for Zn^{2+} pretreated R2-PIM8(*smt*-reintroduced) and R2-PIM8(*smtA+/B-*) may reflect a difference in the level of *smtA* expression prior to growth of cultures in Cd^{2+}.

A decrease in a metal induced lag following metal pretreatment of bacteria has previously been observed. For example, when metal resistant *Alcaligenes eutrophus* strains (harbouring the *czc* determinant) were preincubated with non-toxic concentrations of metal cations (e.g. Zn^{2+} or Cd^{2+}), the pretreated cells displayed a decreased lag phase of growth (as compared to non-pretreated cells) in the presence of high concentrations of a metal cation (e.g. Zn^{2+} or Cd^{2+}) (Nies, 1992b); furthermore, a lag phase observed with *Escherichia coli* (carrying the *pco* determinant) upon addition of Cu^{2+} to the growth medium could be reduced by preinduction with $CuSO_4$ (Rouch *et al.*, 1985).

A prolonged growth lag was observed at 2 μM Cd^{2+} and 3 μM Cd^{2+} for R2-PIM8(*smt*-reintroduced) pretreated with a non-toxic concentration of Cd^{2+} (figure 3.3.4., *panel C*). Furthermore, growth patterns of R2-PIM8(*smt*-reintroduced) and R2-PIM8(*smtA+/B-*), in Zn^{2+} supplemented medium, remained unaltered for both strains pretreated with a non-toxic concentration of Zn^{2+} (figure 3.3.5.). In these cases, metal pretreatment may not have affected subsequent growth characteristics in the presence of metal ions, due to the *smt* system being optimised to deal with Zn^{2+}. At biologically

significant (maximum permissive) concentrations, β-galactosidase assays have revealed that Zn^{2+} elicits greatest (metal-dependent) expression from the *smtA* operator-promoter (refer to section 4.4.2.), maximal Cd^{2+} induced expression occurs above maximum permissive concentrations of Cd^{2+} for growth. Cultures treated with Zn^{2+} may therefore express greater SmtA levels than cultures treated with Cd^{2+} (cells devoid of *smtB* expressing a higher level of MT with/without metal), resulting in an early higher level of tolerance. Moreover, Zn^{2+} pretreatment may not induce a greater level of SmtA than is normally induced upon growth of cells in elevated Zn^{2+}, with the result that metal pretreatment does not affect Zn^{2+} tolerance. Furthermore, the affinity of Cd^{2+} for the binding sites in thiolate clusters is greater than that of Zn^{2+} (Vasák, 1991), Cd^{2+} may therefore replace MT bound Zn^{2+} in Zn^{2+} pretreated cells. The released Zn^{2+}, in this case, could be a good inducer of *de novo* synthesis.

It is noted that the phenotypic analysis of cultures, with respect to growth characteristics, in metal supplemented media following metal pretreatment, requires greater repetition.

4.2.4. Analysis of metal accumulation

In three separate experiments, R2-PIM8(*smt*) (deficient in the metal binding protein SmtA) showed reduced accumulation of Zn^{2+}, compared to R2-PIM8, following exposure to a non-inhibitory and a toxic concentration of Zn^{2+} (non-toxic to R2-PIM8) for up to 60 minutes (figure 3.4.1.). This is consistent with Zn^{2+} sequestration by SmtA in the latter. Moreover, Shi *et al.* (1992) reported that *Escherichia coli* expressing SmtA as a recombinant fusion protein showed enhanced (ca. 3 fold) accumulation of Zn^{2+} following growth in low Zn^{2+} concentrations, although no enhanced accumulation was observed in cells grown in inhibitory concentrations of Zn^{2+}. It is noted that, in *E. coli*, *smtA* expression was not coupled to metal ion concentration and this could significantly affect the influence of SmtA on metal accumulation.

Upon prolonged exposure (120 minutes) to a concentration of Zn^{2+} that was toxic to R2-PIM8(*smt*), but not to R2-PIM8, greater Zn^{2+} accumulation was observed in the former (greater experimental repetition is required to determine whether this is

significant). This may reflect R2-PIM8(*smt*) necrosis concomitant with increased Zn^{2+} uptake. R2-PIM8(*smtA+/B-*) showed greater accumulation than R2-PIM8(*smt*) probably due to the constitutive derepressed expression of SmtA in this strain.

4.3. FUNCTION OF SMTA

4.3.1. Role in essential metal homoeostasis

Circumstantial evidence suggesting a role for *smtA* in Zn^{2+} homoeostasis includes; an observed high Zn^{2+} affinity (relative to equine MT) of a recombinant GST-SmtA fusion protein following expression in *Escherichia coli* (Shi *et al.*, 1992); MT induction by, and association with, Zn^{2+} in *Synechococcus* sp. (Olafson *et al.*, 1980); at biologically significant (maximum permissive) concentrations Zn^{2+} is the most potent inducer of metal-dependent expression from the *smtA* operator-promoter (refer to section 4.4.; Huckle *et al.*, 1993); and a ca. 5-fold detectable hypersensitivity of R2-PIM8(*smt*) to Zn^{2+} (section 4.2.).

In vitro Zn^{2+} transfer between transcription factors and higher eukaryotic apo-MT has implicated the latter in Zn^{2+} homoeostasis as it relates to the regulation of gene expression (Zeng *et al.*, 1991a; 1991b). Zn^{2+} requiring transcription factors are not well characterised in prokaryotes, and it has been proposed that prokaryotes may have avoided the "hidden costs" of the precise Zn^{2+} homoeostasis required for maintaining Zn^{2+} binding transcription factors (Luisi, 1992). However, intracellular "Zn^{2+} buffering" remains a requirement of these organisms, and reduced tolerance of R2-PIM8(*smt*) to elevated Zn^{2+} reveals such a function for SmtA. Viability of R2-PIM8(*smt*) confirms no essential role (under these culture conditions) for SmtA in the donation, or removal, of Zn^{2+} to, or from, apo-proteins. Although SmtA is not vital under these "optimal" growth conditions, it can not be precluded that such a role, in essential metal metabolism, exists for SmtA, which may be vital in a natural (less static or more metal limited) environment. Moreover, other proteins could substitute for SmtA in its absence.

To further investigate the role of *smt* in Zn^{2+} homoeostasis, attempts were made to compare growth characteristics of R2-PIM8 and R2-PIM8(*smt*) in Zn^{2+} deficient media.

No reduction of growth rate was observed in either strain in the absence of Zn^{2+} supplements, and is most probably due to the inability to remove sufficient Zn^{2+} from the media.

4.3.2. Role in metal detoxification

A role proposed for all MTs is detoxification of the metal ions sequestered by the protein. R2-PIM8(*smt*) has reduced tolerance to Zn^{2+} and Cd^{2+}. Furthermore, slight, short term, reduced resistance to Ag^{+} was detected. No marked difference in resistance of R2-PIM8 and R2-PIM8(*smt*) to Cu^{2+} and Hg^{2+} was detected, indicating independence of Cu^{2+} and Hg^{2+} resistance to *smt* mediated metal tolerance.

Olafson *et al.* (1980) reported an increase in the amount of MT in *Synechococcus* sp. following supplementation of the growth medium with either Zn^{2+} or Cd^{2+}, but not Cu^{2+}. The native protein was isolated associated predominantly with either Zn^{2+} or Cd^{2+}, corresponding to the metal administered to the cells (refer to section 1.3.). Following expression of SmtA as a recombinant fusion protein in *Escherichia coli*, Zn^{2+}, Cd^{2+}, copper ions and Hg^{2+} (all metals examined) were indicated to bind to SmtA (Shi *et al.*, 1992). Metal displacement curves indicated SmtA to have a high affinity for Zn^{2+} in comparison to equine MT and a lower relative (to equine MT) affinity for Cd^{2+} and copper ions. Furthermore, preliminary metal accumulation data indicate Zn^{2+} sequestration by SmtA.

It has previously been demonstrated that the abundance of *smtA* transcripts increases in response to elevated concentrations of a range of metal ions (including Zn^{2+}, Cd^{2+}, Cu^{2+} and Hg^{2+}) (Huckle *et al.*, 1993). Furthermore, maximum permissive concentrations for growth, of a range of metal ions (including Zn^{2+}, Cd^{2+} and Cu^{2+} (not Hg^{2+})) induced β-galactosidase activity in R2-PIM8 containing sequences upstream of *smtA* (including *smtB* and the *smt* operator-promoter region), fused to a promoterless *lacZ* gene. However, R2-PIM8(*smt*) showed decreased tolerance to only a subset of these inducers (Zn^{2+} and Cd^{2+}). It is therefore suggested that not all metals which induce *smtA* mRNA are detoxified by SmtA. Durnam and Palmiter (1984) observed that although Zn^{2+}, Cu^{2+}, Hg^{2+}, Ag^{+}, Co^{2+}, Ni^{2+} and Bi^{3+} induced MT mRNA accumulation

in Cd^r and Cd^{2+} sensitive animal cells, Cd^r cells (which overproduce MT due to gene amplification) showed increased resistance to only a subset of these metals (Zn^{2+}, Cu^{2+}, Hg^{2+} and Bi^{3+}). Ag^+, Co^{2+} and Ni^{2+} were therefore considered to be gratuitous inducers.

Amplification (Gupta *et al.*, 1992) and specific rearrangement (Gupta *et al.*, 1993) of *smt* has been reported in *Synechococcus* PCC 6301 cells selected for Cd^{2+} resistance by stepwise adaptation. MT gene amplification has previously been observed in mammalian cells selected for Cd^{2+} resistance, and yeast cells selected for Cu^{2+} resistance (refer to section 1.1.3.). Characterisation of *smt* from a Cd^{2+} tolerant cell line (C3.2) revealed the functional deletion of *smtB* (Gupta *et al.*, 1993), conferring constitutive derepressed expression from the *smtA* operator-promoter (refer to section 4.4.2.). It was considered that the functional deletion of *smtB*, encoding a *trans*-acting repressor of *smtA* transcription (refer to section 4.4.), may confer a selective advantage for continuously metal challenged cells (Gupta *et al.*, 1993). R2-PIM8(*smtA+/B-*) (R2-PIM8(*smt*) transformed with the rearranged *smt* divergon), exhibited (early) increased tolerance to Zn^{2+} and Cd^{2+} as compared to R2-PIM8 (with/without metal pretreatment) (section 4.2.2.). It is therefore confirmed that Cd^{2+} tolerance in C3.2 is (at least in part) due to the functional deletion of *smtB*. HIP1 mediated rearrangement of *smt* in this cell line therefore conferred a selective advantage for such continuously metal challenged cells. This (early) increase in metal tolerance could be of importance in a "natural" metal-polluted environment. Spontaneous mutations increasing the level of resistance to Zn^{2+} have been found in *Alcaligenes eutrophus* CH34 selected for Zn^{2+} resistance (Collard *et al.*, 1993). In the Zn^r mutants, the resistance to Zn^{2+} was constitutively expressed, and it was suggested that the mutations may alter a *trans*-acting repressor.

Although R2-PIM8(*smt*) displayed reduced tolerance to Zn^{2+} and Cd^{2+}, a level of residual tolerance remained. This residual tolerance may however be less in non-culture conditions where cell growth is not "optimal". Several observations (discussed above) indicate that SmtA has a role in Zn^{2+}/Cd^{2+} detoxification (and providing slight early tolerance to Ag^+). However, it must be considered whether these metal ions are at high, or sufficiently fluctuating, concentrations in most natural environments to require the

existence of such a protein (the residual tolerance of R2-PIM8(*smt*) being sufficient for survival). SmtA mediated metal tolerance could be a detectable phenotype resulting from the metal binding properties of this protein, although the gene may have been selected to perform an alternative role (such as Zn^{2+} metabolism/storage).

4.3.3. Role in scavenging oxygen free radicals

Copper can catalyse the formation of hydroxyl radicals (refer to section 1.2.4.; Halliwell and Gutteridge, 1984). A role for MT in protection against the cytotoxic effects of oxygen free radicals has been proposed (refer to section 1.1.3.). R2-PIM8(*smt*) did not show hypersensitivity to Cu^{2+}, suggesting that SmtA does not perform such a role. This does not however preclude the possibility that SmtA confers protection against oxidative damage to a specific cellular target, such as DNA. Chubatsu and Meneghini (1993), reported that neither overexpression nor lower expression of MT in chinese hamster cells resulted in differential resistance to the killing action of H_2O_2, however the high concentration of MT and its hydroxyl scavenging properties conferred protection to DNA from hydroxyl radical attack.

4.4. ANALYSIS OF THE FUNCTION OF SMTB

4.4.1. Gene architecture and protein sequence

Previous observations suggesting a role for SmtB as a transcriptional regulator include; the divergent organisation of *smtA* and *smtB*; similarity of the deduced SmtB polypeptide to known bacterial transcriptional regulators; and the presence of a putative DNA binding motif. These observations are discussed.

The *smtA* and *smtB* genes are arranged in a divergent orientation about the 100 bp operator-promoter region, containing the divergent *smtA* and *smtB* promoters (21 bp apart (refer to figure 3.5.1.)). The divergent organisation of genes with closely spaced promoters has precedent in numerous prokaryotic systems, and represents a general type of gene organisation (Beck and Warren, 1988). Promoters of divergently transcribed genes have been found in three arrangements (cited in Beck and Warren, 1988): They

can be back to back, with intervening DNA between the promoters (the DNA between the promoters may contain binding sites for various regulatory proteins); they can overlap; or they can be face to face (the 5' termini for the transcripts may or may not overlap). Advantages of divergent transcription units include (cited in Beck and Warren, 1988): Closely spaced RNA polymerase binding sites providing the opportunity for the interaction of different RNA polymerase molecules on the same site of DNA, either directly by protein-protein interactions or indirectly by alterations of the topology of a neighbouring promoter; the regulation for both genes may be provided by the binding of regulatory proteins to regions within the divergent promoters; tightly controlled coordinate expression of the two genes; the prevention of gene activation by transcriptional read through from neighbouring genes; a possible greater stability of these autonomous DNA regions than other units of genes in the course of recombination and translocation of genetic information. Many of the prokaryotic divergons are regulatory units in which the product of the regulatory gene regulates expression of the divergent structural gene. This means that the site of action of the product of the regulatory gene is adjacent to its encoding gene. In bacteria, regulatory proteins and RNAs can act more efficiently in *cis* (McFall, 1986) (co-translation does not occur in eukaryotes since the mRNA has to be translocated to the cytosol for translation, and there is hence no *cis-trans* effect), thereby allowing effective control of gene expression by low concentrations of the regulatory molecule. Similar promoter architecture has been characterised in prokaryotic metal resistance determinants, where a divergent gene encodes the regulator of the system (refer to section 1.2.).

The deduced SmtB polypeptide shows similarity at the amino acid level to; the ArsR *trans*-acting repressor proteins of the *Escherichia coli* and *Staphylococcus ars* operons (described in section 1.2.1.) (Huckle *et al.*, 1993); to the CadC protein, of unknown function that is essential for high level Cd^{2+} resistance in *Staphylococcus aureus* (described in section 1.2.2.1.), and to CadC of *Bacillus firmus* OF4, proposed to have a role in sodium/proton antiport (Mack Ivey *et al.*, 1992), (Huckle *et al.*, 1993); to MerR of *Streptomyces lividans*, the proposed regulator of the *mer* operon (described in section 1.2.3.) (Morby *et al.*, 1993); and to NolR, a regulator of *nod* gene expression in *Rhizobium*

meliloti (Morby *et al.*, 1993). Similarity scores show that SmtB is most similar to the ArsR proteins of the *Staphylococcus xylosus* and *Staphylococcus aureus ars* operons (Huckle *et al.*, 1993).

Huckle *et al.* (1993) reported a 21 amino acid region within the SmtB polypeptide sequence, which scores highly (5.5) on a Dodd and Egan helix-turn-helix distinction matrix (Dodd and Egan, 1990). X-ray crystallography of several proteins and protein-DNA complexes have elucidated these DNA binding motifs in detail (Struhl, 1989). The crucial structure in this class of proteins consists of two α-helices separated by a β-turn. The structural motif, consisting of the two helices, related by the two fold symmetry of the dimeric protein, is at the correct orientation (34 Angstroms apart, equivalent to one turn of duplex DNA) to mediate protein-DNA interaction (Freemont *et al.*, 1991). Amino acids within one of the helices (the recognition helix) directly contact bases exposed in the major groove of the target DNA. The other α helix lies across the major groove and makes non-specific contacts to DNA. The amino-termini of both helices point towards the phosphate backbone, using the positive helix-dipole for the correct positioning of the recognition helix. The amino acid side chains of the recognition helix are presumed to make sequence-specific interactions with exposed functional groups in the major groove of the DNA. The prokaryotic helix-turn-helix proteins bind as dimers to DNA sequences that have dyad symmetrical character (Schleif, 1988). The *smt* operator-promoter region contains 3 repeat sequences that are potential sites for interaction with the helix-turn-helix motif of SmtB (refer to section 1.3.).

4.4.2. Metalloregulation of expression from the *smtA* operator-promoter

Sequences upstream of *smtA* can confer metal-dependent expression upon a promoterless *lacZ* gene (refer to sections 3.5.2., 3.5.3. and 3.5.4.). Metal-dependent β-galactosidase expression was observed for R2-PIM8 containing pLACPB2(*smt*-5') or pLACPB2(*smtB*-), R2-PIM8(*smt*) containing pLACPB2(*smt*-5'), and R2-PIM8(*smtA*+/*B*-) containing pLACPB2(*smt*-5') (these strains contain chromosomal and/or plasmid encoded *smtB*). At maximum permissive concentrations for growth, β-galactosidase assays (performed using both protocols) revealed Zn^{2+} to be a more potent elicitor of

metal-dependent expression from the *smtA* operator-promoter than Cd^{2+} (figures 3.5.2., 3.5.3. and 3.5.4.). Concordant with this, Huckle *et al.* (1993) observed that Zn^{2+} elicited the greatest induction of β-galactosidase expression, following exposure of R2-PIM8 containing pLACPB2(*smt*-5') to maximum permissive concentrations of a range of trace metal ions.

R2-PIM8(*smt*) containing pLACPB2(*smtB*-) (devoid of *smtB*) showed extraordinarily high elevated basal expression of β-galactosidase and loss of metal dependency (figures 3.5.2. (*5*), 3.5.3. (*5*) and 3.5.4. (*9*)). Overexpression was complemented in R2-PIM8(*smt*) by plasmid borne *smtB* (in the construct pLACPB2(*smt*-5')) (figures 3.5.2. (*4*), 3.5.3. (*4*) and 3.5.4. (*8*)), demonstrating *smtB* to be a repressor of *smtA* transcription. Furthermore, lack of overexpression in R2-PIM8 containing pLACPB2(*smtB*-) (which lacks a functional plasmid borne *smtB*) revealed that SmtB can act in *trans* (figures 3.5.2. (*3*), 3.5.3. (*3*) and 3.5.4. (*6*)).

Using the method described by Miller (1972), R2-PIM8 containing pLACPB2(*smtB*-) and R2-PIM8(*smt*) containing pLACPB2(*smt*-5'), had an elevated basal level of β-galactosidase expression (in media containing no metal supplements) (figure 3.5.2. (*3* and *4*)). However, using the modified protocol, elevated basal expression was only observed for the former (figures 3.5.3. (*3*) and 3.5.4. (*6*)) and was proposed to be due to an inequity in the number of *smtB* copies and the SmtB target site (the *smt* operator-promoter), resulting in titration of the repressor, and/or due to SmtB acting more efficiently in *cis* (refer to section 4.4.1.).

R2-PIM8(*smtA+/B-*) containing pLACPB2(*smt*-5') showed metal-dependent reporter gene activity, and no high basal expression (figure 3.5.4. (*2*)). There is an inequity in the number of *smtB* copies and the SmtB target site in this strain, indicating that the elevated basal expression observed for R2-PIM8 containing pLACPB2(*smtB*-) was most likely to be due to SmtB acting more efficiently in *cis*. Loss of the plasmid borne *smtB* in R2-PIM8(*smtA+/B-*) revealed loss of metal dependency and high basal β-galactosidase expression (figure 3.5.4. (*3*)), consistent with *smtB* encoding a transcriptional repressor. The level of expression from the *smtA* operator-promoter in these cells was diminished (ca. 2 fold) in comparison to that observed for R2-PIM8(*smt*) containing pLACPB2(*smtB*-)

(figure 3.5.4. (*9*)). This indicates the involvement of an activatory element for maximal expression from the *smtA* operator-promoter. Zn^{2+} may act as the effector of the activatory element, and lack of *smtB* in R2-PIM8(*smtA+/B-*) results in increased *smtA* expression (R2-PIM8(*smt*) is devoid of *smtA*) and hence reduced available endogenous Zn^{2+}. Further evidence to support the involvement of a positive regulatory element for maximal *smtA* expression has been reported by Morby *et al.* (1993). R2-PIM8(*smt*) containing truncated derivatives of pLACPB2(*smtB-*) (truncated *smt* operator-promoter region) showed elevated metal-independent reporter gene expression, which was lower (ca. 2 fold) than that observed for R2-PIM8(*smt*) containing pLACPB2(*smtB-*). The region lost in the truncated derivative corresponded to a protein binding site (MAC3, refer to section 4.4.3.) and represents a *cis*-acting activatory region (Morby *et al.*, 1993). Furthermore, *smtA* has no region corresponding to an *Escherichia coli* -35 consensus sequence (Huckle *et al.*, 1993) and may therefore be activated by positively acting proteins substituting for the -35 element (refer to section 1.4.1.). The "extended -10" sequence (5'-TGN-3'), which may compensate for the absence of a -35 consensus region, is present in the *smtA* promoter (J.W. Huckle, unpublished observations).

When β-galactosidase assays were performed using the modified protocol with cultures of a low cell density (optical density at 595 nm of ca. 0.08), more subtle effects were observed (figure 3.5.4.). Maximal Zn^{2+} induced expression was observed for R2-PIM8 containing pLACPB2(*smt*-5'), and there was a marked loss of reporter gene activity in strains containing either a plasmid or a chromosomal *smtB* (not both). This may be explained by one or more of the following; loss of "remote" *cis*-acting activatory elements essential for maximal expression; inequity in the number of *smtB* copies and the SmtB target site; *smtB* acting more efficiently in *cis* (the latter two situations might result in increased basal *smtA* expression and hence reduced available endogenous Zn^{2+} which may in turn affect the degree of activation from "remote" *cis*-acting activatory elements and/or the MAC3 complex); and/or further regulatory elements may be required for "normal" *smt* expression, which could be involved in the regulation of *smtB*. The 7-2-7 hyphenated inverted repeat of the *smt* operator-promoter (refer to section 1.3.) corresponds to the MAC2 (refer to section 4.4.3.) binding site. This was proposed to play

a role in the regulation of *smtB*, and/or slightly modify *smtA* expression (Morby *et al.*, 1993). Furthermore, it must be noted that in the absence of SmtA, the level of available endogenous Zn^{2+} may be affected by an adjustment in the activity of (other) mechanisms involved in Zn^{2+} homoeostasis.

4.4.3. Summary of the functional analysis of SmtB

Several observations (refer to section 4.4.1.) suggested a role for SmtB as a transcriptional repressor. Sequences upstream of *smtA* (including *smtB* and/or the *smt* operator-promoter), fused to a promoterless *lacZ*, conferred metal dependant β-galactosidase expression in R2-PIM8 and indicated that the *smtA* operator-promoter is maximally induced by Zn^{2+} in comparison to other metal ions. Equivalent experiments in R2-PIM8(*smt*) and R2-PIM8(*smtA+/B-*) revealed that *smtB* encodes a repressor of *smtA*. Furthermore, SmtB can act in *trans*. It is proposed that Zn^{2+} is the most potent (metal ion) inducer of SmtB mediated derepression of *smtA* transcription.

The specific HIP1 mediated rearrangement observed in a Cd^{2+} tolerant cell line (Gupta *et al.*, 1993) shown to involve functional deletion of *smtB*, is now confirmed to represent the loss of a repressor of *smtA* transcription. Reconstruction of this genotype (in R2-PIM8(*smtA+/B-*)) confirms that this alone can give a metal resistant phenotype. Derepressed expression from the *smtA* operator-promoter provides increased (early) resistance to Zn^{2+} and Cd^{2+} (refer to sections 3.3.2., 3.3.3.).

Specific interactions between proteins extracted from R2-PIM8 and defined regions surrounding the *smtA* operator-promoter have most recently been detected by electrophoretic mobility shift assays (Morby *et al.*, 1993). Three *smt* operator-promoter associated complexes (MAC1, MAC2 and MAC3) were identified. MAC1 showed Zn^{2+} dependant dissociation and involved a region of DNA immediately upstream of *smtA*. Treatment with Zn^{2+} chelators facilitated reassociation of MAC1 *in vitro*. MAC1 was only observed in extracts of cells containing *smtB* (MAC2 and MAC3 were retained when using extracts from R2-PIM8(*smt*)). SmtB was therefore required for the formation of a Zn^{2+} responsive complex with the *smt* operator-promoter. Furthermore, direct interaction of MAC1 with metal ions was indicated (Morby *et al.*, 1993). Based upon the

predicted structure of SmtB, direct SmtB-DNA interaction exerting metal ion inducible negative control was proposed.

The mode of action of SmtB is therefore proposed to be similar to that of the *trans*-acting inducer-responsive negative regulator of the *ars* operon, ArsR (of the *E. coli* plasmid R773). In the absence of inducer, constitutively produced ArsR binds as a dimer to an operator site proximal to the *ars* promoter and represses transcription (refer to section 1.2.1.). Addition of inducer releases the repressor from the DNA and permits transcription of the *ars* operon.

The characterised metal responsive transcription factors, involved in the regulation of eukaryotic MT genes, are activated to bind specific DNA promoter sequences and induce MT transcription by binding metal ions (refer to section 1.1.4.). In contrast it is proposed that SmtB metal ion binding mediates dissociation of SmtB from specific *smt* operator-promoter sequences, resulting in induction of MT transcription. SmtB acts as an inducible negative regulator of MT transcription, whereas inducible positive regulation occurs with eukaryotic MT genes.

4.5. OTHER CYANOBACTERIAL REGULATORY GENES

The *ntcA* gene of *Synechococcus* PCC 7942 is required for full expression of proteins subject to ammonium repression, and is proposed to encode a transcriptional activator required for the expression of a number of genes involved in nitrogen assimilation (Vega-Palas *et al.*, 1990; 1992). The predicted NtcA protein shows similarity to CysR, also from *Synechococcus* PCC 7942. *cysR* has a role in sulphate transport in *Synechococcus* PCC 7942 (Laudenbach and Grossman, 1991). In addition to similarity to each other, both NtcA and CysR show similarity to the family of bacterial DNA binding regulatory proteins that includes Fnr (refer to sections 1.4.1. and 1.4.2.) and Crp (regulator of catabolite-sensitive genes, refer to section 1.4.1.) from *Escherichia coli*, and FixK (regulator of genes involved in nitrogen fixation) from *Rhizobium meliloti*.

The *sphS* and *SphR* genes of *Synechococcus* PCC 7942 encoding the "sensory kinase" and "response regulator" respectively of a two-component regulatory system are

proposed to be involved in the signal-transduction mechanism underlying regulation of the phosphate regulon (Aiba *et al.*, 1993; refer to section 1.4.3.).

In addition, other ORFs have most recently been identified in cyanobacteria that are predicted to encode regulatory proteins. These however remain uncharacterised.

4.6. CONCLUDING REMARKS

Viability of R2-PIM8(*smt*), lacking a functional *smt* divergon, confirms that *smt* performs no vital role (under these growth conditions) required for growth in non-metal supplemented media. SmtA is involved in Zn^{2+} and Cd^{2+} detoxification, and may provide slight early tolerance to Ag^{+} (although an alternative mechanism for Ag^{+} resistance probably exists in *Synechococcus* PCC 7942). Cu^{2+} and Hg^{2+} resistances are indicated to be independent from *smt* mediated metal tolerance. Several observations indicate that the *smt* divergon has primarily evolved to deal with Zn^{2+}. Furthermore, residual Zn^{2+}/Cd^{2+} tolerance of R2-PIM8(*smt*) could be indicative of a more fundamental role for SmtA than metal detoxification (metal detoxification being a consequence of the metal binding properties of SmtA). A role for SmtA in Zn^{2+} homoeostasis/metabolism is proposed.

Metal ion induced expression of *smtA* is directed by an operator-promoter under the control of metal responsive factors. The divergently transcribed gene, *smtB*, encodes a *trans*-acting inducer- (metal ion) responsive negative regulator of *smtA* transcription, and high basal, metal-independent, expression occurs from the *smtA* operator-promoter in the absence of *smtB*. In addition to SmtB, other regulatory elements (including a transcriptional activator) are proposed to be involved in the regulation of expression from the *smt* operator-promoter. SmtB is thus a member of a small group of known cyanobacterial regulators, and the regulation of gene expression at *smt* (which is proposed to involve multiple regulatory factors) is seemingly analogous to that described for other bacterial systems (e.g. the *ars* operon of the *Escherichia coli* plasmid R773 (refer to section 1.2.1.)).

R2-PIM8(*smtA+/B-*) mutants, containing functional *smtA* and non-functional *smtB*, have been generated. These mutants show elevated constitutive expression of a reporter gene, fused to the *smtA* operator-promoter, and show (early) increased tolerance to Zn^{2+} and Cd^{2+}. Specific rearrangement of *smt* in a Cd^{2+} tolerant *Synechococcus* PCC 6301 cell line (C3.2) (Gupta *et al.*, 1993) is now confirmed to be the result of functional deletion of the repressor gene, *smtB*. Loss of SmtB is shown to result in (early) increased tolerance to Zn^{2+} and Cd^{2+}, and therefore appears to provide a selective advantage to continuously metal challenged cells.

The mechanism of action of SmtA in Zn^{2+} homoeostasis is unknown. By analogy to eukaryotic MTs, SmtA may serve as an intracellular "sink" for excess metal. The possibility that SmtA may be part of a more dynamic mechanism of metal homoeostasis (e.g. involving metal efflux) cannot be eliminated.

4.7. FUTURE WORK

It is necessary to determine whether SmtB and the protein component of MAC1, which is indicated to interact directly with metal ions (refer to section 3.4.3.), are synonymous and to identify the putative metal ion and DNA binding domains.

The deduced SmtB polypeptide contains 3 cysteine residues, none of which are arranged in cysteine-Xaa-cysteine, cysteine-Xaa-Xaa-cysteine or cysteine-cysteine motifs. To assign function to critical residues of SmtB, and determine whether SmtB interacts directly with metal ions, attempts were made (data not shown) to convert the SmtB cysteine residues to serines. The method employed was oligonucleotide site-directed mutagenesis by the gapped duplex method using the pMa/c phasmid vectors (Stanssens *et al.*, 1989). Although site-directed mutations were obtained, other point mutations were observed in the *smtB* coding region (on several occasions). Further work to determine critical metal binding residues of SmtB is therefore required. Three mutants of *arsR* (of the *Escherichia coli* plasmid R337) have been isolated using hydroxylamine mutagenesis (Rosen *et al.*, 1993). These mutations resulted in reducing expression of the *ars* operon in the presence of inducers, and mobility shift assays

showed that the presence of inducers had less effect on the binding of these mutated ArsR proteins to the *ars* operator. It was therefore proposed that the mutated ArsR proteins, shown to be mutated at cysteine residues (cysteine$_{34}$ to tyrosine, cysteine$_{32}$ to tyrosine and cysteine$_{32}$ to phenylalanine), were defective in the inducer interaction region, and cysteine$_{32}$ and cysteine$_{34}$ had a role in the interaction with inducers. Random/site-directed mutations of the Tn*21*, Tn*501* and *Bacillus* MerR proteins have revealed that three cysteine residues are required for specific Hg^{2+} binding and efficient activation of transcription by the bound complex and the RNA polymerase (cited in Misra, 1992; Helmann *et al.*, 1990). Most recently, subsequent to the work described herein on site-directed mutagenesis of SmtB (and work described elsewhere on mutagenesis of ArsR), a comparison of the protein sequences of SmtB, ArsR from *E. coli* and *Staphylococcus ars* operons, and CadC from *Staphylococcus aureus* and *Bacillus firmus* OF4 was made (Bairoch, 1993), assuming these proteins to be a family of transcriptional regulators (such a role has not been shown for CadC). Conserved cysteine and histidine residues were identified, and proposed to be involved in metal binding.

The isolation of genes (other than *smtB*) which encode *trans*-acting regulatory factors (the protein components of MAC2 and MAC3), which interact with the *smt* operator-promoter region (refer to section 4.4.3.), would be of interest. Furthermore, the precise MAC1, MAC2 and MAC3 binding sites require determination.

Structural studies on SmtB via biophysical and spectroscopic techniques could answer key questions regarding the precise metal ion/DNA binding domains of SmtB, and identify conformational changes conferred in the presence/absence of metal ions. If direct interaction of SmtB with metal ions is confirmed, it would also be of interest to determine the pathway by which metal ions reach SmtB.

Finally, an important extension of the work described in this thesis would be to apply metal (Zn^{2+}/Cd^{2+}) flux analysis to examine the mechanism of action of SmtA and the role(s) of the *smt* divergon in metal accumulation.

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Isolation of a prokaryotic metallothionein locus and analysis of transcriptional control by trace metal ions

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Summary

In eukaryotes, metallothioneins (MTs) are involved in cellular responses to elevated concentrations of certain metal ions. We report the isolation and analysis of a prokaryotic MT locus from *Synechococcus* PCC 7942. The MT locus (*smt*) includes *smtA*, which encodes a class II MT, and a divergently transcribed gene, *smtB*. The sites of transcription initiation of both genes have been mapped and features within the *smt* operator–promoter region identified. Elevated concentrations of the ionic species of Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn elicited an increase in the abundance of *smtA* transcripts. There was no detectable effect of elevated metal (Cd) on *smtA* transcript stability. Sequences upstream of *smtA*, fused to a promoterless *lacZ* gene, conferred metal-dependent β-galactosidase activity in *Synechococcus* PCC 7942 (strain R2-PIM8). At maximum permissive concentrations, Zn was the most potent elicitor *in vivo*, followed by Cu and Cd with slight induction by Co and Ni. The deduced SmtB polypeptide has similarity to the ArsR and CadC proteins involved in resistance to arsenate/arsenite/antimonite and to Cd, contains a predicted helix-turn-helix DNA-binding motif and is shown to be a repressor of transcription from the *smtA* operator–promoter.

Introduction

The isolation and properties of metallothioneins (MTs) are the subjects of an extensive literature (refer to Riordan and Vallee, 1991). Proposed functions vary for the structurally distinct MTs in different organisms and include detoxification of Cd and certain other metals, regulation of Zn and Cu metabolism and provision of Zn for newly synthesized enzymes. Following the structural characterization of Zn-fingers, Zn-twists and Zn-clusters in DNA-binding proteins, animal MT has also been implicated in Zn-homoeostasis as it relates to the regulation of gene expression (Vallee, 1991; Zeng *et al.*, 1991).

In mammalian cells, inducers of MT genes include exposure to ionic species of Ag, Au, Bi, Cd, Co, Cu, Hg, Ni or Zn, numerous hormones and second messengers, growth factors, inflammatory agents and cytokines, many cytotoxic agents, and stress-producing conditions (for a review see Kägi, 1991). *cis*-acting metal-regulatory elements of eukaryotic MT genes are known (for reviews see Hamer, 1986; Palmiter, 1987) and *trans*-acting metal-responsive factors have been identified in animals (Labbé *et al.*, 1991 and citations therein) and characterized in yeasts (Dameron *et al.*, 1991, and citations therein). In prokaryotes, a number of systems exhibit metal-responsive gene expression including both metal-resistance determinants and genes involved in the metabolism of essential trace metals (for reviews see Bagg and Neilands, 1987; Silver and Misra, 1988; Hennecke, 1990; Brown *et al.*, 1992). The biochemical mechanisms which regulate some of these systems are understood (for examples see Lund and Brown, 1989; Rouch *et al.*, 1989; San Francisco *et al.*, 1990; Ansari *et al.*, 1992; Ji and Silver, 1992; Brown *et al.*, 1992).

Reports have indicated the presence of low-molecular-weight, cysteine-rich, metal ligands, analogous to MTs, in several prokaryotes (cited in Silver and Misra, 1988). However, there is only one published amino acid sequence of a prokaryotic MT purified from the cyanobacterium *Synechococcus* sp. (Olafson *et al.*, 1988). This protein has little amino acid sequence similarity to any other MT and is thus defined as a class II MT (for nomenclature refer to Kojima, 1991). Based upon this sequence, polymerase chain reaction (PCR) primers were synthesized and the resulting PCR products used as probes to identify increases in the abundance of the corresponding transcripts following exposure of *Synechococcus* PCC 6301 to Zn, Cd and Cu (Robinson *et al.*, 1990). The encoded protein (expressed in *Escherichia coli*) was shown to have high affinities for trace metals (especially Zn) comparable to those of known MTs (Shi *et al.*, 1992).

This paper describes the isolation and structural characterization of a genomic fragment carrying the MT gene from *Synechococcus* PCC 7942 and an analysis of its regulation by metal ions.

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AAGCTTTACTACAACGAGCGCCGCTATCTACAGCAACTCGATCAAGAACGCTGCCTGAAT    60
                                                          |
CCCCAAGCATTCTTGGGCATGACAGAGCACGATGCTACTGCGATCGCCCCGACCACTCCC   120
Putative terminator
CAGCCGATTTCTGCCTAAGGTGCATCTCTAGCGACACTCTTGTAAGTGATCGAGGGCGTT   180
                          AMB R  C  E  Q  L  H  D  L  A  N

TTGATAAAGCGCCACAATGTGATGATCCTGTAGCTGGTAGTAGACATGCCGCCCTTGCTT   240
 Q  Y  L  A  V  I  H  H  D  Q  L  Q  Y  Y  V  H  R  G  Q  K

GCGATAGCTCACCAGCCGCAGATTACGGAGCGATCGCAATTGGTGAGACACCGCCGATTC   300
 R  Y  S  V  L  R  L  N  R  L  S  R  L  Q  H  S  V  A  S  E

GGAAACACCAATTGCCTGGGCCAAATCCCCAACACAGAGCTCCGATCGCGCTAACAGGGA   360
 S  V  G  I  A  Q  A  L  D  G  V  C  L  E  S  R  A  L  L  S

CAGCAACCGCAGTCGATTTGGATCGGCCAGCACTGCAAAAAATTCGGCTAGCGATTGGGC   420
 L  L  R  L  R  N  P  D  A  L  V  A  F  F  E  A  L  S  Q  A

AACTTCGGGTGCGATCGCTTGAAGCTCCGAGGCGATCGCCGCATGAGTCCCTTGGCAGAC   480
 V  E  P  A  I  A  Q  L  E  S  A  I  A  A  H  T  G  Q  C  V

                              smtB  transcript start  |
TACCGTCTCTCCGTCCTGCAGCACTGGTTTTGTCATGAGCCAATCACGGTTTGTCCACCC   540
 V  T  E  G  D  Q  L  V  P  K  T  M        S.D.

 -10                                                Direct repeat
ACCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGATATT   600
        Inverted repeat      -10        |  smtA transcript start

   S.D.          M  T  S  T  T  L  V  K  C  A  C  E  P  C  L
CAAAGGAGTTGCTGTCATGACCTCAACAACGTTGGTCAAATGCGCTTGTGAGCCCTGTCT   660

  C  N  V  D  P  S  K  A  I  D  R  N  G  L  Y  Y  C  S  E  A
CTGCAACGTCGATCCCAGCAAAGCGATCGATCGCAACGGTCTGTACTACTGCAGCGAAGC   720

  C  A  D  G  H  T  G  G  S  K  G  C  G  H  T  G  C  N  C  H
CTGTGCCGATGGCCACACCGGTGGTAGCAAAGGCTGCGGCCACACCGGCTGTAACTGCCA   780

  G  OCH
CGGCTAATCAACTGTTTCCCTGCTAATCCCCCATCAATCGAAAACCGCTGGCTCCTCAAT   840
                                                    Putative
CATGGGCCAGCGGTTGATTATTTATAGGAGGTGCGATCGCGCAGCTTTACAACCCCTACT   900
   terminator          |
CGCCGGTGATCGAGAGACCTTCGACCCAAACAGCCGGCGAAATCCCACCGGGCGTGATCT   960

GGGCGATCGCATCGACTTGGACGATATGTCGCAACAGTTCCCGAAAATCGCCTGCAACGG  1020

TTGCTGATTCGATGATGACGCGATCGCGTTTGATTGACCAACCAGCCATCAAAGGGCAGC  1080

GAGAACGAGCCTTGCAAGGACTGAACACCCGCATGGAGCGCTTGCAGATCATCGATCAAG  1140

ATCACAGACTCGGCCGTATCGAGGCTCAGTTCCGATCGTGCTTCCGCGGCTTCGACTACC  1200

CCAAGTGGGGGCTAACGGTGACTTTAGCCCCAAGGTTGGCGTGACCGGTCGGGCTGGCAC  1260

CCAGTCGCTTAGCCGTCCCCGCACTATGCAAGAATTTTTAAGTACACCGGCTTCAATCAA  1320

CGGGAG 1326
```

Fig. 1. Nucleotide sequence of the *smt* locus. A 1326 bp portion of the insert in pJHNR49 containing the divergent *smtA* and *smtB* genes with encoded polypeptides. Note that the sequence of SmtB is written from carboxy- to amino-terminus. The transcriptional start sites are shown (vertical lines) for both genes, and features in the operator–promoter region are underlined and annotated. Sequences predicted to form stem-loop structures and act as transcriptional terminators are also underlined. Predicted transcript termination points are marked (vertical lines). These sequence data appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession number X64585.

Results

Cloning the smt *region of* Synechococcus *PCC 7942 and nucleotide sequence analysis*

A PCR-amplified region of the *smtA* gene from *Synechococcus* PCC 6301 (Robinson *et al.*, 1990) was used to screen a *Synechococcus* PCC 7942 genomic library (unlike *Synechococcus* PCC 6301, *Synechococcus* PCC 7942 is readily transformable). A clone (pJHNR49) was isolated that contained a 1.8 kb fragment including the complete *smtA* coding sequence and flanking regions.

The MT locus (*smt*) contains two open reading frames (ORFs) arranged in a divergent orientation (Fig. 1). The smaller ORF has been designated *smtA* and the directed polypeptide sequence (56 amino acids in length, Fig. 1) corresponds to the sequence of the cyanobacterial MT

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

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

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

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

Fig. 2. Multiple alignment with SmtB. Primary amino acid sequences included are SmtB, 267ArsR (*S. xylosus* plasmid pSX267), 773ArsR (*E. coli* plasmid R773), 258ArsR (*S. aureus* plasmid pI258), 258CadC (*S. aureus* plasmid pI258) and OF4CadC (*B. firmus* strain OF4). A consensus sequence was constructed with a plurality of 5. The underlined region of the SmtB sequence delineates a predicted helix-turn-helix motif.

previously reported (Olafson *et al.*, 1988), with two modifications. Two additional amino acids, histidine and glycine, are present at the *C*-terminus and serine substitutes for Cys-32 (Ser-33 in the SmtA sequence), altering a cysteine–cysteine pair to cysteine–serine.

Immediately upstream of *smtA* is a 7 bp inverted repeat with a 2 bp 'hyphen' 5'-CTGAATC-AA-GATTCAG-3' and a 6 bp direct repeat with a 2 bp 'hyphen' 5'-TATTCA-GA-TATTCA-3' (Fig. 1). A sequence similar to an *E. coli* –10 promoter consensus sequence occurs 4 bp downstream of the hyphenated inverted repeat. There is no region corresponding to a consensus *E. coli* –35 sequence. A putative *smtA* transcription terminator structure was identified (Fig. 1).

The divergent ORF, designated *smtB*, has two upstream sequences similar to an *E. coli* –10 promoter sequence, one of which lies upstream (relative to *smtB*) and adjacent to the hyphenated inverted repeat, and a second which lies 1 bp downstream of the hyphenated inverted repeat. A putative transcription terminator is marked in Fig. 1. The deduced polypeptide product of *smtB* is 122 amino acids in length and contains three cysteine residues, none of which is arranged in Cys–Xaa–Cys motifs. The OWL 14.0 Protein Database was searched for sequences similar to SmtB and the *E. coli* ArsR and *Staphylococcus aureus* CadC sequences were the most significant (Fig. 2). A multiple sequence alignment was produced which includes ArsR and CadC proteins from other organisms (Fig. 2). The SmtB primary sequence contains a 20-amino-acid region 69 to 88 inclusive (amino acids 62 to 81 inclusive in SmtB) which scores highly (5.5) on the helix-turn-helix distinction matrix of Dodd and Egan (1990) (Fig. 2).

Determination of smtA *and* smtB *transcriptional start sites by primer extension*

The transcriptional start sites for *smtA* and *smtB* were located (Fig. 3). RNA was isolated from *Synechococcus* PCC 7942 cells exposed to 2.5 μM $CdCl_2$ for 2 h and used as template in primer extension reactions. The predicted *smtA* transcript length is 305 bases and the size estimated from Northern blots was *c.* 300 bases. The predicted *smtB* transcript length is 491 bases. The *smtB* transcript was *c.* 500 bases, estimated from Northern blots, and *smtB* transcript abundance increased (slightly) with elevated Cd (data not shown).

Increase in abundance of smtA *transcripts following exposure of* Synechococcus *PCC 6301 to metal ions*

Figure 4 shows the abundance of *smtA* transcripts in *Synechococcus* PCC 6301 cells in response to metal salts. Abundance increased with a 2 h exposure to ionic

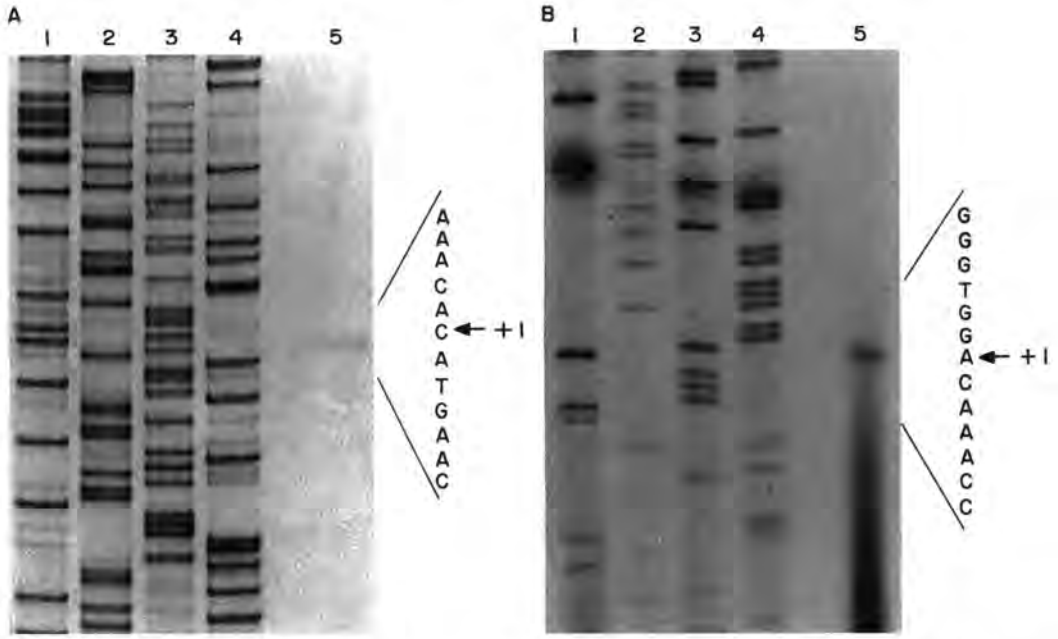


Fig. 3. Transcription start sites. Designation of the transcript start points for *smtA* and *smtB* by primer extension. The sequencing ladders were generated using the same primers.

species of a range of metal ions including Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn. Maximal induction in response to Cd, Co, Cr, Cu, Hg and Zn was observed at 2.5, 5, 1, 5, 2.5, 5 μM respectively. Transcript abundance declined at higher concentrations of some metals (e.g. Hg), which is assumed to correlate with loss of viability. Similar increases in transcript abundance were seen in response to all concentrations of Ni used. Induction by Pb was slight. This may reflect relative insolubility of Pb salts in aqueous solution.

Analysis of smtA *transcript stability*

smtA transcript abundance increased with time over a 1 h period in cultures of *Synechococcus* PCC 7942 strain R2-PIM8 (hereafter referred to as R2-PIM8) exposed to Cd (2.5 μM) in contrast to cultures exposed to rifampicin and Cd (Fig. 5). The rate of *smtA* transcript degradation was similar in cells treated with either Cd and rifampicin, or rifampicin alone (Fig. 5).

Upstream regions of smtA *can confer metal-dependent expression upon a promoterless* lacZ *gene in R2-PIM8*

The *smtA* 5' sequence (600 bp) was amplified by PCR, cloned, and sequenced. The PCR primer introduced a *Bam*HI site, adjacent and upstream of the *smtA* ribosome-binding site. This was ligated to pLACPB2 (Scanlan *et al.*, 1990), carrying a promoterless *lacZ*, to create pLACPB2(*smt*-5') (Fig. 6). The vector contains sequences allowing independent replication in both *Synechococcus* and *E. coli* and carries resistance to chloramphenicol.

R2-PIM8 was transformed to chloramphenicol resistance with pLACPB2 and pLACPB2(*smt*-5'). Growth experiments were performed in triplicate to assess maximal permissive concentrations (MPCs) and minimum inhibitory concentrations (MICs) for Cd (1.5, 2.0 μM), Co (3.0, 4.0 μM), Cr (25.0, 30.0 μM), Cu (9.0, 10.0 μM), Hg (0.025, 0.03 μM), Ni (3.0, 4.0 μM), Pb (this gave very high and variable MPC/MIC values probably because of the poor solubility of Pb salts) and Zn (11.0, 12.0 μM). Beta-galactosidase assays were repeated following 2 h exposures to the concentrations of the metals listed above, with the exception of Pb. At these biologically significant levels, induction of β-galactosidase was observed for Zn > Cu = Cd, with slight induction by Co and Ni ions (Fig. 7). Equivalent increases in β-galactosidase activity on exposure to metal ions were not detected in the control strain containing pLACPB2 without the *smtA* operator–promoter fragment (data not shown). There were no detectable changes in plasmid copy number in cultures exposed to metal ions (data not shown). In addition, metal-dependent β-galactosidase activity was not detected using equivalent constructs containing the *smtB* operator–promoter fused to *lacZ* (data not shown).

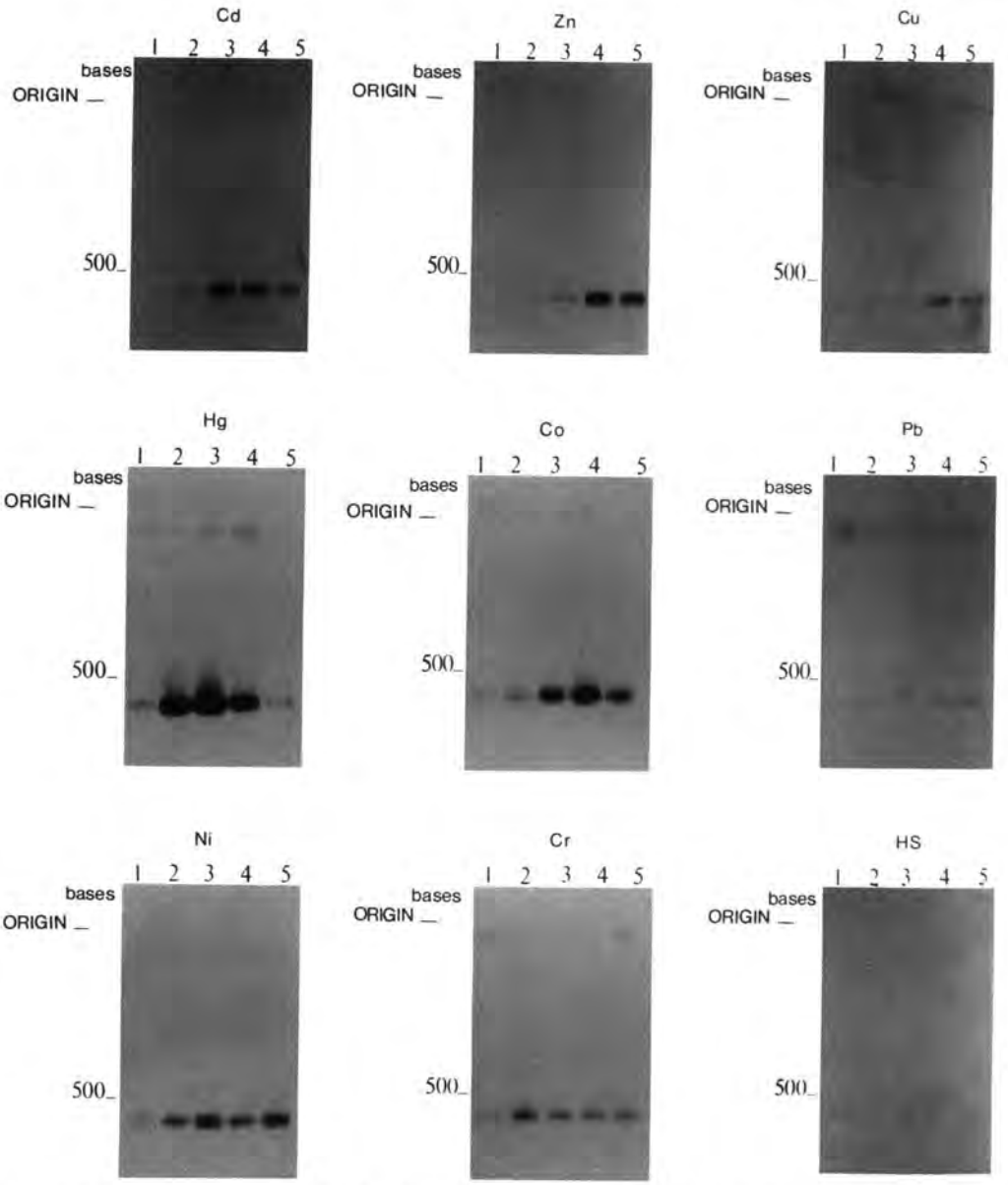


Fig. 4. Metal induction of *smtA* transcripts. Northern blots of RNA from *Synechococcus* PCC 6301 showing the increase in *smtA* transcript abundance in response to 2 h of exposure to exogenous metal ions (Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn) at various concentrations. Lane 1, 0 μM; 2, 1 μM; 3, 2.5 μM;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

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

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

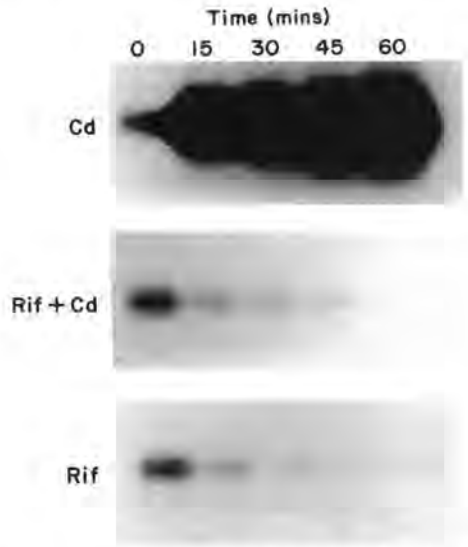


Fig. 5. Stability of the *smtA* transcript. Northern blots of *smtA* transcripts extracted from *Synechococcus* PCC 7942 after exposure to 2.5 μM Cd (Cd), 2.5 μM Cd + 400 μg ml^{-1} rifampicin (Rif + Cd) and to 400 μg ml^{-1} rifampicin alone (Rif). Samples were extracted every 15 min over a 1 h period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

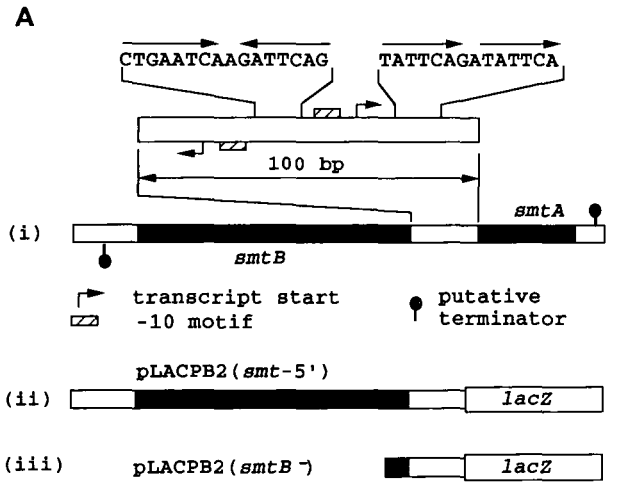
shown). Most significantly, there was highly elevated basal expression of β-galactosidase and loss of metal dependency in the *smt* mutant strain, R2-PIM8(*smt*), containing pLACPB2(*smtB*$^-$). These cells are devoid of a functional plasmid or genomic *smtB*.

Discussion

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

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

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



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

B (i) pLACPB2

```
CTGCAGGTCGACGGATCCCCGGGAATTCATCGAGCAACATATTAATGAGCCAGAGAAATGCTGGCGGCACTG
 PstI  SalI BamHI SmaI EcoRI

AAAGTTTTTGTACAAGCCGATGAAAGCGGCGACGCGCAGTTAATCCCACAGCCGCCAGTTCCGCTGGCGGCA

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

(ii) *smt*

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

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

(iii) *smt-lacZ* fusion

```
                                                                    BamHI
CCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGATATTCAGGATCCCCGGGAA

TTCATCGAGCAACATATTAATGAGCCAGAGAAATGCTGGCGGCACTGAAAGTTTTTGTACAAGCCGATGAAAGC

GGCGACGCGCAGTTAATCCCACAGCCGCCAGTTCCGCTGGCGGCATTTTAACTTTCTTTATCACACAGGAAACA

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

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

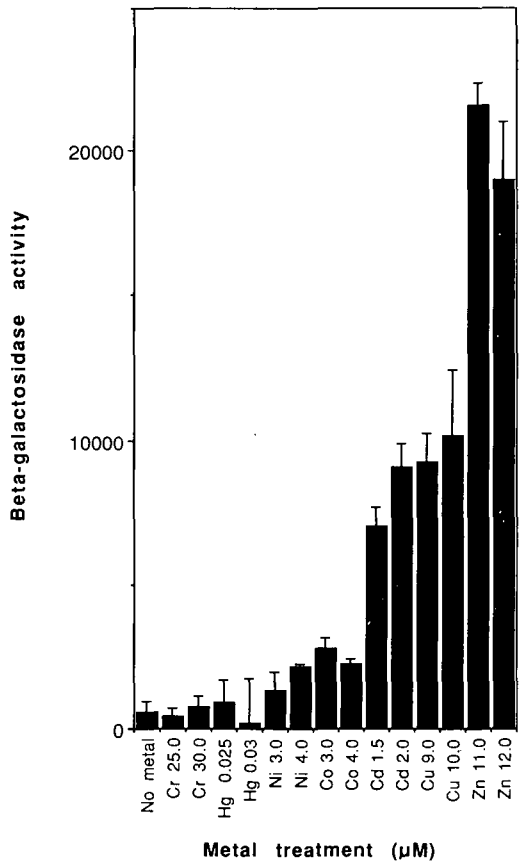


Fig. 7. Metal-induced expression of β-galactosidase.
Beta-galactosidase activity in cells containing pLACPB2(*smt*-5') exposed to maximum permissive concentrations (first bar) and to minimum inhibitory concentrations (second bar) of each metal. Data are the means of three separate determinations with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

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

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

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

Experimental procedures

Cyanobacterial culture and transformation

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

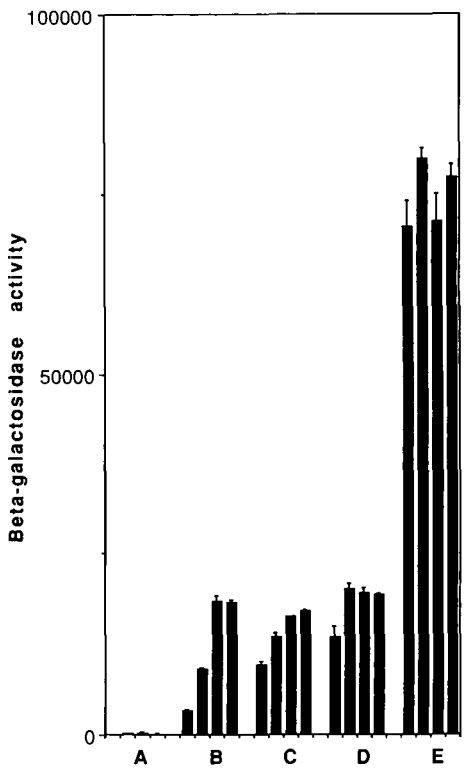


Fig. 8. Beta-galactosidase activity in R2-PIM8 and the *smt*-deleted mutant, R2-PIM8(*smt*). Beta-galactosidase activity of: A, R2-PIM8 containing pLACPB2 only as a control; B, R2-PIM8-containing pLACPB2(*smt*-5'), which includes plasmid and chromosomal *smtB*; C, R2-PIM8 containing pLACPB2(*smtB*$^-$),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

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

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

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

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

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

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

RNA isolation, analysis and primer extension

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

The use of rifampicin to inhibit transcription

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

Construction of smt-lacZ *fusions*

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

Determination of β*-galactosidase activity*

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

Acknowledgements

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

Protein extraction

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

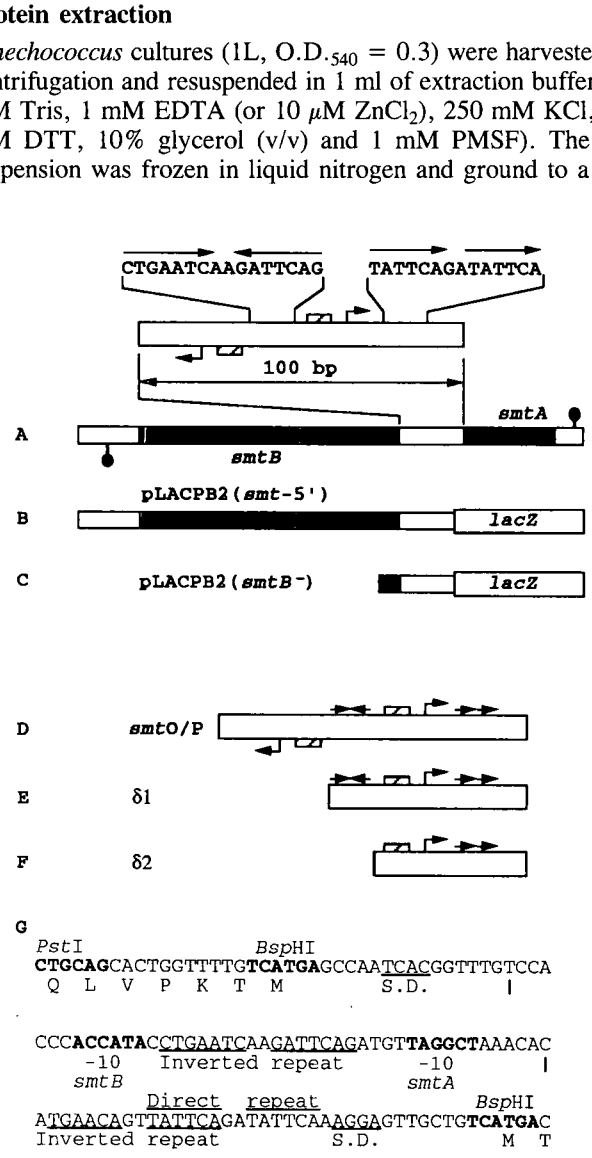

Figure 1. Organisation of the *smt* locus, reporter gene fusions and EMSA probes/competitors. A: *smt* locus. The divergent genes *smtA* and *smtB* are shown as black rectangles. The 100 bp operator-promoter region is expanded to show the sequence of the hyphenated inverted repeat (converging arrows)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

Electrophoretic mobility shift assays

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

β-galactosidase assays

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

Computer analysis

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

RESULTS

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

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

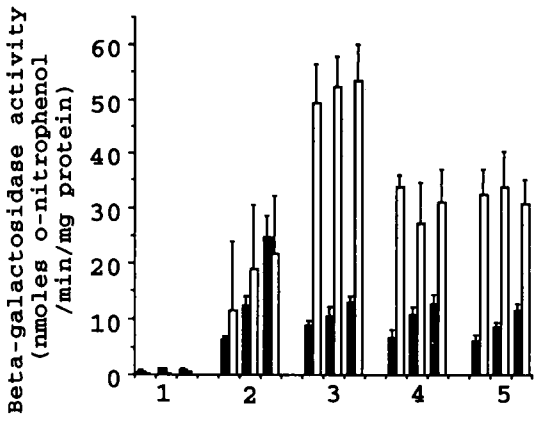

Figure 2. β-galactosidase activity measured in *Synechococcus* strains containing *smtA-lacZ* reporter gene fusions and deletions. Closed columns represent a R2-PIM8 background and open columns represent an R2-PIM8(*smt*) background Each block of three values corresponds to 0, 2.5 and 11 μM Zn exposure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

pLACPB2(*smtB*$^-$), showed elevated basal expression (in media containing no metal supplements) and significantly diminished maximal expression (in 11 μM Zn) (Figure 2). Highly elevated basal β-galactosidase activity and loss of metal-dependency was seen in R2-PIM8(*smt*) containing pLACPB2(*smtB*$^-$), devoid of a functional plasmid or genomic *smtB* (Figure 2)(4). All three sequential deletion derivatives (Figure 1C,E and F) showed similar activity in R2-PIM8, however, R2-PIM8(*smt*) containing pLACPB2(δ1 and δ2) had diminished activity in comparison to the equivalent strain carrying pLACPB2(*smtB*$^-$). The larger errors seen in assays of the R2-PIM8(*smt*) strain are possibly due to the lack of a functional *smtA* gene and consequent aberrant Zn homeostasis.

Identification of complexes which bind to the *smt* operator-promoter region

Three *smt* operator-promoter associated complexes (MAC1, MAC2 and MAC3) were detected using standard protein extracts from R2-PIM8 by electrophoretic mobility shift assays (EMSA) (Figure 3A) with *smt*O-P as probe (shown in Figure 1D). At high concentrations (0.3 μg μl^{-1}) of non-specific competitor DNA, MAC3 is more stable, while MAC1 is less stable, in reactions using extracts from Zn exposed cells (compare tracks 6 and 11, Figure 3A). To localise the DNA binding sites the experiment was repeated using different concentrations (0.01 to 0.5 μg μl^{-1}) of *smt* operator-promoter deletions, δ1 and δ2, in pGEM4Z,

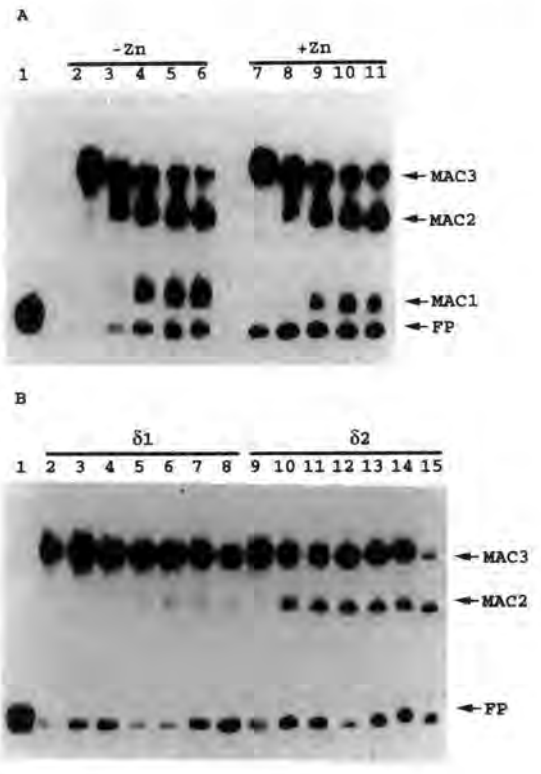

Figure 3. EMSA with *smt*O/P probe. A: Track 1 contains probe alone, tracks 2–6 and 7–11 correspond to reactions containing increasing amounts of poly-dIdC (0, .05, 0.1, 0.2, 0.3 μg/μl). Tracks 2–6 show free probe (FP) and three complexes (MAC1, MAC2 and MAC3) forming with protein extracts from non-Zn exposed cells. Tracks 7–11 show equivalent complexes with protein extracts from cells exposed to Zn *in vivo*. B: Track 1 contains probe alone. Tracks 2–8 and 9–15 correspond to reactions containing increasing concentrations (.01, .05, .1, 0.2, 0.3, 0.4 and 0.5 μg/μl) of pGEM4Z containing δ1 and δ2 respectively.

(Figure 1E and 1F) as specific competitors (Figure 3B). MAC1 was lost in reactions containing either δ1 or δ2. MAC2 was diminished in reactions containing δ1 compared to reactions containing δ2. MAC3 was retained in reactions containing either competitor.

Loss of MAC1 in extracts from an *smt* mutant

MAC2 and MAC3 form with the *smt*O-P probe using standard protein extracts from R2-PIM8(*smt*) (Figure 4A). MAC1 was not detected using extracts from these mutants.

A single complex (C1) was formed with the *smt* operator-promoter deletion fragment δ2 using a protein extract from R2-PIM8 (Figure 4B). This complex was absent when extracts from R2-PIM8(*smt*) were used (Figure 4B, compare tracks 1–3 with 4–6). The complex was again detected using extracts from R2-PIM8(*smt*) containing pLACPB2(*smt*-5′), which reintroduces a plasmid borne *smtB*, but not using extracts from R2-PIM8(*smt*) containing pLACPB2 alone (Figure 4B, compare tracks 7–9 with 10–12).

Effects of Zn on complexes formed with *smt* operator-promoter deletion δ2

To further examine the effects of Zn on complexes forming with the *smt* operator-promoter region extracts were prepared with EDTA free buffers supplemented with $ZnCl_2$ (5 μM), and binding reactions performed in the absence of EDTA. A single major complex (C1) was again formed with promoter deletion δ2 (Figure 5, track 2) using extracts from cells which had not been exposed *in vivo* to elevated Zn. Using extracts from cells exposed (1 h) *in vivo* to $ZnCl_2$ (10 μM) the abundance of C1 declined (Figure 5, compare tracks 2 and 6), and was absent in extracts from R2-PIM8(*smt*) (Figure 5, track 10). Treatment of identical reactions with increasing concentrations (0.1 mM and 1 mM) of a metal chelator, 1,10-phenanthroline resulted in increased abundance of C1 in extracts from R2-PIM8 (Figure 5, compare track 2 with 3 and 4; track 6 with 7 and 8), again no complex was seen using extracts from R2-PIM8(*smt*) (Figure 5, tracks 11 and 12). It is also noted that in the presence of the highest concentration of 1,10-phenanthroline, a second prominent complex (C2) was detected in reactions containing extracts from R2-PIM8 cells which had not been exposed to elevated Zn (Figure 5, track 4). Furthermore, a minor higher M_r complex was also seen in all extracts.

Multiple alignment of SmtB with similar proteins

The OWL 18.0 database was searched for proteins whose primary amino-acid sequence was similar to SmtB. Sequences were aligned and a consensus sequence generated with a plurality of 6 (Figure 6). The accession numbers of the included proteins are OWL: SmtB-S19927; 773ArsR-P15905; 2258ArsR-M86824; CadC-P20047; MerR-S23610; NolR-S19675. GenBank: 267ArsR-M80565; OF4CadC-M90750.

DISCUSSION

Abnormal over expression from the *smt* operator-promoter is (partially) complemented in R2-PIM8(*smt*) by plasmid borne *smtB*, demonstrating that SmtB is a *trans*-acting repressor of *smtA* (figure 2)(4). A direct interaction between SmtB and DNA may be mediated by residues 61 to 81, inclusive, which score highly on a prediction matrix for the helix-turn-helix DNA-binding motif (4). Such inducible negative control would be similar to that

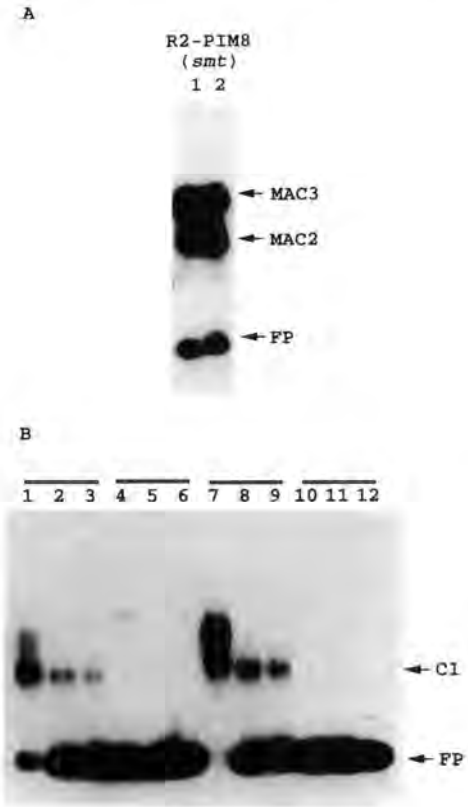

Figure 4. EMSA with protein extracts from R2-PIM8(*smt*) and derivative *smtB* complemented strains. A: Tracks 1 and 2 correspond to reactions containing the *smt*O/P probe with 0.2 and 0.3 $\mu g/\mu l$ of poly-dIdC respectively. The protein extract was from non-Zn exposed R2-PIM8(*smt*). B: The $\delta 2$ probe was used with protein extracts from non-Zn exposed R2-PIM8 (1–3), R2-PIM8(*smt*) (4–6), R2-PIM8(*smt*) containing pLACPB2(*smt*-5′) (7–9) and R2-PIM8(*smt*) containing pLACPB2 (10–12). Each set of three reactions contained increasing concentrations (0, 0.1 and 0.2 $\mu g/\mu l$) of poly-dIdC. Free probe(FP), MAC2, MAC3 and C1(MAC1) are labelled.

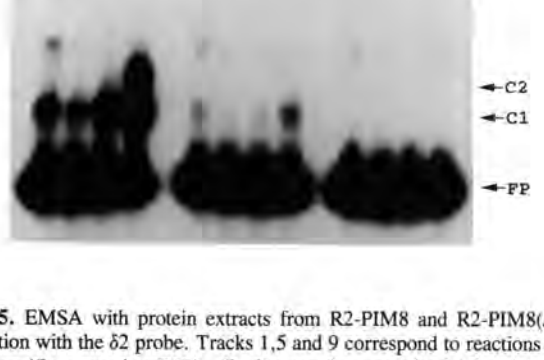

Figure 5. EMSA with protein extracts from R2-PIM8 and R2-PIM8(*smt*) in conjunction with the $\delta 2$ probe. Tracks 1,5 and 9 correspond to reactions devoid of non-specific competitor DNA, all other reactions contained 0.2 $\mu g/\mu l$ of poly-dIdC. Each set of 4 tracks correspond to reactions containing 0,0,0.1 and 1mM 1,10-phenanthroline. Free probe(FP), C1(MAC1) and C2 are labelled.

demonstrated for the arsenic resistance operon (*ars*) from *E.coli* in which ArsR mediated transcriptional repression is alleviated by exposure to the metal-oxyanions arsenate, arsenite, antimonite and bismuthite (9).

```
                1                                                  50
    SmtB  MTKPVLQDGE TVVCQGTHAA IASELQAIAP EVAQSLAEFF AVLADPNRLR
773 ArsR  .......... .......... .......... MLQLTPLQLF KNLSDETRLG
267 ArsR  .......... .......... .......... MSYKELSTIL KVLSDPSRLE
258 ArsR  .......... .......... .......... MSYKELSTIL KILSDSSRLE
OF4 CadC  ....VNKKDT CEIFCYDEEK VNRIQGDLKT IDIVSVAQML KAIADENRAK
258 CadC  ....MKKKDT CEIFCYDEEK VNRIQGDLQT VDISGVSQIL KAIADENRAK
 MerR  .......... ...MKSPALA GSLATAEVPC THPDTTARFF RALADPTRLK
 NolR  .......... ...MNFRMEH TMQPLPPEKH EDAEIAAGFL SAMANPKRLL
Consensus ---------- ---------- ---------- ---------L --L-D--RL-

               51                                                 100
    SmtB  LLSLL.ARSE LCVGDLAQAI GVSESAVSHQ LRSLRNLRLV SYRKQGRHVY
773 ArsR  IVLLLREMGE LCVCDLCMAL DQSQPKISRH LAMLRESGIL LDRKQGKWVH
267 ArsR  ILDLL.SCGE LCACDLLEHF QFSQPTLSHH MKSLVDNELV TTRKNGNKHM
258 ArsR  ILDLL.SCGE LCACDLLEHF QFSQPTLSHH MKSLVDNELV TTRKDGNKHW
OF4 CadC  ITYALCQDEE SCVCDIANII GITAANASHH LRTLHKQGIV RYRKEGKLAF
258 CadC  ITYALCQDEE LCVCDIANIL GVTIANASHH LRTLYKQGVV NFRKEGKLAL
 MerR  LLQFI.LRGE RTSAECVEHA GISQPRVSVH LSCLVDCGYV SARRDGKKLR
 NolR  ILDSL.VKEE MAVGALAHKV GLSQSALSQH LSKLRAQNLV STRRDAQTIY
Consensus I---L----E LC--D----- --S----S-H L--L-----V --RK-G----

               101                                                147
    SmtB  YQLQDH..HI VALYQNALDH LQECR..... .......... .......
773 ArsR  YRLSPHIPSW AAQIIEQAWL SQQDDVQVIA RKLASVNCSG SSKAVCI
267 ArsR  YQL.NH..EF LDYINQNLDI INTSDQGCAC KNMKSGEC.. ........
258 ArsR  YQL.NH..AI LDDIIQNLNI INTSNQRCVC KNVKSGDC.. ........
OF4 CadC  YSLDDEHIRQ IMMIVLEHKK EVNVNV.... .......... ........
258 CadC  YSLGDEHIRQ IMMIALAHKK EVKVNV.... .......... ........
 MerR  YSVGDP..RV ADLVMLARCL AADNAAALDC CTRIPGEGEQ R.......
 NolR  YSSSSD..AV LKILGALSDI YGDDTDAVEE KPLVRKSA.. ........
Consensus Y-L-------- ---I------ ---------- ---------- --------
```

Figure 6. Multiple sequence alignment of SmtB from *Synechococcus* PCC 7942, ArsR from *E.coli* (plasmid R773), ArsR from *Staphylococcus xylosus* (plasmid pSX267), ArsR from *Staphylococcus aureus* (plasmid pI258), CadC from *Bacillus firmus* OF4, CadC from *Staphylococcus aureus* (plasmid pI258), MerR from *Streptomyces lividans* and NolR from *Rhizobium meliloti*. A concensus sequence is shown with a plurality of 6. The putative DNA-binding region of SmtB is underlined.

There is a significant loss of inducible reporter gene activity in R2-PIM8 containing pLACPB2(*smtB*$^-$), lacking ca. 500 bp of *smtA* 5′-sequence, (figure 1C). This either reflects the loss of a 'remote' *cis*-acting element essential for maximal expression and/or the loss of *trans*-acting plasmid borne *smtB* and hence inequity between copies of SmtB and its target site. The latter might result in increased basal *smtA* expression and hence reduced available endogenous Zn which may explain the observed patterns of expression in short time course assays such as these. Further deletion of the *smt* 5′-region, in pLACPB2($\delta 1$), resulted in loss of induction which was only apparent in the *smt* mutant background. A corresponding loss of induction in R2-PIM8, where the major element of transcriptional control (SmtB) was still functional, was not seen. The region lost corresponds to the proposed MAC3 binding site and represents a *cis*-acting activatory region. Computer analysis has identified a 5 bp overlapping direct repeat (CCACC) immediately upstream of the 7-2-7 hyphenated inverted repeat, which is a candidate binding site for MAC3. Further deletion, pLACPB2($\delta 2$), (loss of the 7-2-7 hyphenated inverted repeat) did not significantly alter the induction in either R2-PIM8 or R2-PIM8(*smt*). The 7-2-7 hyphenated inverted repeat corresponds to the proposed MAC2 binding site (see below). This feature may play a role in the regulation of *smtB* alone, and/or only slightly modify *smtA* expression.

Three complexes, MAC1, MAC2 and MAC3, form with the *smt* operator-promoter region using protein extracts from R2-PIM8. MAC1 was absent from reactions containing competitor $\delta 2$ (and $\delta 1$) demonstrating MAC1 formation with the region lying between the 7-2-7 hyphenated inverted repeat and Shine-Dalgarno sequence of *smtA* (Figure 3B). MAC2 is diminished in reactions containing competitor d1, but not $\delta 2$. These data suggest that MAC2 associates with the 7-2-7 hyphenated inverted repeat which is only present in $\delta 1$. MAC1

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

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

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

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

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

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

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