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Prokaryotic metallothionein locus and cadmium tolerance in *Synechococcus* PCC 6301

by

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A thesis submitted for the degree of Doctor of Philosophy

in the University of Durham

Department of Biological Sciences

November 1992

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- 5 JAN 1993

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

ABSTRACT

The aim of this study was to investigate the molecular mechanism of Cd-tolerance in the cyanobacterium *Synechococcus* PCC 6301 and to establish whether the prokaryotic metallothionein (MT) locus, *smt*, is involved.

Cd-tolerant cell lines of *Synechococcus* PCC 6301 were developed by step-wise selection, of a culture that had undergone prolonged maintenance in liquid medium. The Cd-tolerant cell lines A0.8, A1.3 and A1.7 (tolerant to 0.8, 1.3, 1.7 μM Cd, respectively) were phenotypically different to the non-selected line A0. Genomic DNA from A0 and the Cd-tolerant lines A0.8, A1.3 and A1.7 was analysed by Southern hybridisation. A ca. 4-fold increase in hybridisation to radiolabelled *smtA* (prokaryotic metallothionein gene), relative to A0, was observed in genomic DNA from A1.7. Equivalent amounts of DNA were loaded onto each track, and no difference in hybridisation to a control gene, *psaE* (photosystem I gene), was observed. Indeed, the hybridisation of DNA from A1.7 to *psaE* was slightly less than that observed in A0. Genomic DNA isolated from A0, A0.8, A1.3 and A1.7 was also analysed after 2, 4, 7 and 12 subcultures in the presence of the respective Cd concentrations. An increase in hybridisation to *smtA*, relative to A0, was observed in DNA from all Cd-tolerant cell lines. Additionally, unique additional restriction fragments, both larger and smaller than that in A0, were observed in DNA from A1.3 and A1.7. A similar restriction pattern was observed in 3 independent restrictions of DNA from A1.3 after 2 subcultures.

Cd-tolerant cell lines were also developed from a 'clonal' culture of *Synechococcus* PCC 6301. An increase in tolerance was marked by an increase in growth lag, which reduced upon subsequent maintenance of the Cd-tolerant line in the presence of Cd. Genomic DNA from the non-selected line C0 and Cd-tolerant lines C1.4, C1.8, C2.6 and C3.2 (tolerant to 1.4, 1.8, 2.6, 3.2 μM Cd, respectively) were analysed after 1, 2, 3, 4 and 5 subcultures. In all the Cd-tolerant lines, an increase in hybridisation to *smtA*, and additional larger (ca. 11 kb) and smaller (ca. 5.45 kb) restriction fragments, relative to C0 (ca. 5.8 kb), were observed. However, amplification and rearrangement in DNA from C1.4 were evident only after 2 subcultures. Additionally, restriction fragment equivalent in size to that observed in C0 was lost in C1.8, C2.6 and C3.2, and the presence of Cd did not affect DNA restriction with *Sal*I under *in vitro* and short term *in vivo* conditions..

The rearrangement in Cd-tolerant line C3.2 was observed on a minimal *Hin*dIII-*Sal*I fragment (ca. 350 bp smaller than that in C0) and isolated from size-fractionated genomic libraries. The alteration was mapped by PCR to a 600 bp region in the 5' flank of *smtA*. Nucleotide sequence analysis of the clones identified a deletion of 352 bp within a region of 360 bp encoding the C-terminal end of *smtB* (repressor of *smtA* transcription), rendering it non-functional.

Increased basal level of *smtA* expression (derepressed expression) and indications for complete loss of the excised fragment were observed in Cd-tolerant line C3.2. Rearrangement was detected in DNA from C3.2 even after maintenance in the absence of Cd for 3 subcultures. The clone bank pPLAN Ba1-Ba7 and pPLAN B2 (carrying *Bam*HI restriction fragments of *Synechococcus* PCC 7942 plasmids) were used to study the plasmid/chromosomal localisation of *smtA*. Weak hybridisation of pPLAN Ba2 to *smtA* was observed, but further Southern analysis of plasmid and genomic DNA suggested chromosomal localisation of *smtA*. PCR and Southern hybridisation were used to detect homologues of *smtA* in other cyanobacterial strains. Putative homologues were identified in *Synechococcus* PCC 7942, *Synechococcus* D562, *Oscillatoria* D814 and *Synechocystis* D840 (= PCC 6803) by heterologous probing. However, no hybridisation to *smtA* was observed in DNA isolated from *Calothrix* D184 and *Microchaete* D578.

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ABBREVIATIONS

°C	degrees Celsius
%	percent
pH	hydrogen ion potential
g	gram
mg	milligram
µg	microgram
ng	nanogram
kb	kilo base
bp	base pair
l	litre
ml	millilitre
µl	microlitre
M	molar
mM	millimolar
µM	micromolar
v/v	volume for volume
w/v	weight for volume
h	hour
min	minute
s	second
m	meter
cm	centimeter
$g\ l^{-1}$	grams per litre
$mg\ l^{-1}$	milligrams per litre
As	arsenic
Ca	calcium
Cd	cadmium
Co	cobalt
Cr	chromium
Cu	copper
Fe	iron
Hg	mercury
K	potassium
Mn	manganese
N	nitrogen
Ni	nickel
Pb	lead
Pd	palladium
PO_4	phosphate
S	sulphur
Se	selenium
Zn	zinc
MT	metallothionein
PAR	photosynthetically active radiation
PCC	Pasteur Culture Collection
PCR	polymerase chain reaction
psi	pounds per square inch
rpm	revolutions per minute
Chl a	chlorophyll a
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
CsCl	caesium chloride
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
SDS	sodium dodecyl sulphate
Tris	tris(hydroxymethyl)methylamine

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oṁ ajñāna-timirāndhasya
jñānānjana-salākayā
cakṣur unmilitaṁ yena
tasmai śrī-gurave namaḥ
(Bhagavad-Gita)

(I was born in the darkest ignorance, and my "master" opened my eyes with the torch of knowledge. I offer my respectful obeisance's unto him)

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Chapter 1

GENERAL INTRODUCTION

Anthropogenic mobilisation of toxic trace metals into the biosphere, and the consequent adaptation of certain organisms to supra-optimal concentrations of these metals, has been extensively documented (original references cited in Antonovics *et al.*, 1971; Bradshaw, 1984). Cyanobacteria have been isolated from metal-polluted sites, and some of these isolates tolerate higher concentrations of metal in subsequent culture than do cyanobacterial strains isolated from environments not enriched with metal (Shehata & Whitton, 1981). Cyanobacteria have also been selected in the laboratory for increased tolerance to a number of different metals. However, the mechanisms of metal tolerance in metal-adapted cyanobacteria have not been fully described, and no genes which confer metal tolerance have been identified in cyanobacteria. Additionally, amplification and rearrangement of metallothionein genes has been observed in a wide range of eukaryotic cell lines selected for Cd tolerance (Robinson & Jackson, 1986; Section 1.631).

1.1 Occurrence and distribution of metals

Elevated levels of metal and metalloid compounds such as As, Cd, Co, Cr, Cu, Ni, Pb, Se and Zn often occur naturally in soils overlying ore bodies and mineralisations. They also occur in the wastes from metal extraction industries and in surface soils and lake sediments around base-metal smelters (Schultz & Hutchinson, 1991). Metal-contaminated wastes are often mixed with municipal wastes prior to treatment. This could result in high levels of metals in the output from some waste water treatment plants, and potentially dangerous levels of toxic metals in agricultural land surrounding industrial areas (Adriano, 1986).

1.2 Essential and toxic metals

Metals can be organised into two groups: essential (bulk and trace) and toxic (non-essential) metals. Essential bulk metals (s-block elements, Na and K, Mg and Ca, groups

IA and IIA, respectively) are present in relatively high concentrations in biological systems, for example making up 99% of all metal ions found in man (Hughes, 1981; Hughes & Poole, 1989). They are distributed selectively, with K and Mg concentrated inside the cell and Na and Ca outside the cell. Among various functions, these ions form cross-membrane chemiosmotic gradients, and in the generation of trigger and activation mechanisms such as the transmission of nerve impulses, muscle contraction and hormone secretion. These metals are found as cofactors to many enzymes, and are often important structurally in hard tissues and in the stabilisation of large polymerised complexes.

The essential trace (or ultra-trace) metals are vital for most living systems and are present often at extremely low concentrations. This group includes the 3d group of transition metals (Co, Cr, Cu, Fe, Mn, Ni, V, Zn and the second row transition metal Mo). The d-block elements form complexes much more strongly than do the IA and IIA cations. Two of the more abundant of trace metals, Fe and Cu, have several well-characterised functions, such as the transfer of respiratory gases (Fe in haemoglobin; Cu in haemocyanin), and roles in redox reactions (Co is also involved and also to a lesser extent Cr). Cu and Zn are found as cofactors to a wide variety of enzymes. Zn is found as a cofactor to representatives of all six classes of enzyme (IUPAC classification), and is known to play a role in: gene expression, structural stabilisation of proteins and nucleic acids, maintenance of the integrity of sub cellular organelles, participation in transport processes (Lee *et al.*, 1991; Vallee & Auld, 1990).

The essential elements may also exert toxic effects if their concentrations are raised to supra-optimal levels. The toxic metals (Cd, Hg, Pb, Sn, Tl, As) do not have defined biological functions, and are toxic at very low concentrations. However, Cd and Pb, amongst the toxic metals, have been suggested to play some role in cell metabolism (Schwartz, 1977; Price & Morel, 1990). Therefore, the categories of essential and toxic metals may change.

1.3 Basis of toxicity of metal ions

In addition to the chemical properties of an element the toxic effects of a metal would depend upon (Stokes, 1983; Collins & Stotzky, 1989):

a) Activity of the metal determined by the form of the metal (its chemical speciation), which affects its mobility and ability to bind to cell surfaces;

b) pH of the environment: different hydroxylated species of toxic metals are formed at different pH values (concentration of H^+ changes) and thus there is an effect on their competition for ionogenic sites on the cell surface (charge of ionogenic groups is also affected by pH);

c) Influence of other ions (cations and anions) and other trace metals and nutrients on metal uptake: trace metals form co-ordination complexes with inorganic anions (e.g. OH^-, Cl^-) and thus undergo a change in their chemical speciation. The presence of other inorganic cations (e.g. Ca^{2+}, H^+) results in a state of competition with the cationic forms of the toxic metals for anionic sites on the cell surface. The presence of chelators (natural - EDTA; or synthetic - humic and fulvic acids) also affects the toxicity of metals.

The toxicity of metals can be due to (Ochiai, 1987):

a) Liganding to, and thereby affecting the function of, biologically important molecules.

b) Participation in oxidation and reduction reactions, causing interference with cellular metabolism.

1.4 Metal-binding sites (groups) of biological molecules

For the purpose of liganding, different metal ions prefer different groupings (Hughes, 1981):

O	O/N/S	N/S
Na, Al	V, Pb	Co, Hg
K	Cr, Ga	Ni
Mg	Mn, Tl	Cu
Ca	Fe, Cd	Zn
	Mo	

These preferences for certain ligand environments provide an immediate distinction between the behaviour of individual metals that must be incorporated into the design of selective sites. Metal binding sites must have the following characteristics: (a) a region with a high concentration of metal-liganding atoms (O, N or S), (b) a sufficient number of such atoms to stabilise the metal (2 to 8 depending on the metal ion), and (c) these must be arranged in the correct three-dimensional configuration to allow space for the metal ion (reviewed by Hughes & Poole, 1989). Furthermore, in biological systems metal-binding molecules must have the correct affinity for the ion such that it is not stripped from the binding site by other molecules, but conversely in some instances may be released for donation to other ligands. The ability of metallothionein (metal-binding protein, Section 1.6) to donate Zn, or of thionein to remove Zn from other Zn proteins is constrained by thermodynamic and kinetic considerations (Zeng *et al.*, 1991b). The rates at which the clusters (thiolate clusters) transfer metals upon exposure to chelating agents and exchange metals among metallothionein isoforms are much higher than expected on the basis of Zn mercaptide chemistry. This kinetic lability of Zn thiolate clusters in conjunction with their thermodynamic stability has been proposed to allow the thionein/metallothionein couple to have a responsive dynamic metabolic role (Zeng *et al.*, 1991b). Binding of metal ions with varying affinities is therefore achieved with ligands that have evolved specific configurations.

Bacteria carry out transformations (including oxidation, reduction, methylation and demethylation) of metal ions, and these transformations are sometimes by-products of normal metabolism conferring no known advantage upon the participating organism (Silver & Misra, 1983). The tolerance mechanisms in prokaryotes can be broadly categorised into four groups:

1) Exclusion or reduced influx (e.g. Ag resistance by extracellular complexation),

2) Enhanced metal ion efflux (e.g. Cd resistance, Section 1.51, Arsenate resistance),

3) Intracellular detoxification (e.g. Hg resistance: enzymatic detoxification of mercurials into volatile species), and

4) Internal sequestration (e.g. internal sequestration by metallothioneins, Section 1.6)

Moreover, internal metal ion sequestration by metallothioneins is not well documented in prokaryotes, although a number of studies have suggested that MTs 'may' occur in diverse microbes.

1.5 Resistance towards Zn, Cu and Cd

1.51 Resistance mechanisms in non-cyanobacterial prokaryotes

Zn is an essential trace metal, but at supra-optimal concentrations is toxic to micro-organisms. Zn-transport other than by a broad-specificity Mg transport system has not been properly demonstrated in bacteria (Silver & Walderhaug, 1992). However, resistance to Zn has been demonstrated in various studies. Resistance to Co, Zn and Cd in *Alcaligenes eutrophus* CH34 is conferred by the plasmid pMOL30 (Mergeay *et al.*, 1985). This resistance results from inducible, energy-dependent cation efflux (Nies & Silver, 1989) encoded by the *czc* determinant which has been cloned and sequenced (Nies *et al.*, 1987; 1989). The products of *czc* genes may function as a main pump protein (CzcA), a cation funnel (CzcB), a modulator of substrate specificity (CzcC), and a protein involved in regulation of *czc* (CzcD) (Dressler *et al.*, 1991). Subsequently, it has been shown that an additional component, *czcR*, is required for the full expression of the *czc* determinant (Nies, 1992). The *czcR* is oriented in the opposite direction compared to the genes *czcCBAD*. The CzcR protein contains three potential metal-binding sites and is thought to be a DNA-associated protein. The *czcR* gene product is essential for full expression of *czc* and is postulated to act as an activator of *czc* transcription. Furthermore, CzcR and CzcD have been suggested to form a two-component regulatory system (Nies, 1992).

Cu is toxic in excess due to its capacity to catalyse adverse redox reactions, such as hydroxyl radical generation. Cu can also prevent adequate functioning of proteins by direct binding of the metal to amino acid side chains (especially histidine and cysteine) (cited in Brown *et al.*, 1991). Cu-resistant bacteria have been isolated from many sources, but only Cu-resistant *Escherichia coli, Pseudomonas syringae* and *Xanthomonas campestris* have been characterised further. In the Cu-resistance determinant from

Pseudomonas syringae the first two (*copA* and *copB*) genes are needed for partial Cu-resistance, but the second two (*copC* and *copD*) are also needed for full resistance (Mellano & Cooksey, 1988a). Cooksey (1990) and Cha & Cooksey (1991) proposed a mechanism of resistance involving periplasmic binding and extracellular sequestration of the Cu cations, decreasing free concentrations, and protecting the cellular cytoplasm from exposure to toxic levels. The Cop system is specifically induced by Cu and not by other divalent cations (Ca, Mn, Fe, Zn, Cd, Hg, Pb) (Mellano & Cooksey, 1988b). The plasmid determinant of Cu-resistance from *Xanthomonas campestris* has genes similar in number and characteristics to those in *Pseudomonas syringae* system (Bender *et al.*, 1990; cited in Silver & Walderhaug, 1992).

Cu-resistant mutants of *E. coli* were characterised and mapped to seven complementation groups, *cutA-cutF* (*c*opper *u*ptake and *t*ransport) and *cutR* (*r*egulation) (cited in Brown *et al.*, 1991). Under normal physiological conditions, Cu transport and equilibrium are mediated by the CutA and CutB (influx proteins), followed by intracellular Cu-binding proteins (CutE and CutF) and by the CutC and CutD (efflux) proteins. CutA-CutF are products of structural genes localised on the chromosome and responsible for Cu metabolism in the cell, and CutR is the protein regulating their expression. A plasmid-borne Cu-resistance determinant, different from that of *Pseudomonas* and *Xanthomonas*, was identified in isolates of *E. coli*. Both plasmid and chromosomal gene products interact in Cu transport, intracellular binding and efflux (Rouch *et al.*, 1989a; Brown *et al.*, 1992). The plasmid-determined Cu resistance has been ascribed to the *pco* (*p*lasmid-borne *co*pper resistance) determinant. This resistance determinant, *pco*, contains at least four genes, *pcoARBC*, that are required for Cu-resistance (Rouch *et al.*, 1985; 1989a). The PcoA and PcoB plasmid-encoded proteins function in Cu efflux, PcoC is involved in intracellular Cu-binding, and *pcoR* determines a *trans*-acting regulatory factor. Furthermore, products CutA (uptake) and CutD (efflux) required for normal Cu metabolism are also required for resistance to Cu (Rouch *et al.*, 1989a; 1989b; 1992).

Cd accumulation occurs via the chromosomally determined Mn transport system in sensitive cells of *Staphylococcus aureus* (Weiss *et al.*, 1978). Rather than a direct

blocking of Cd-uptake, an energy-dependent efflux system functions in resistant cells (Tynecka *et al.*, 1981). Two systems which confer Cd-resistance by efflux have been characterised: *czc* (for resistance to Cd, Zn and Co, described above) and the plasmid-encoded *cad* operon of *S. aureus* plasmid pI258, which contains two open reading frames (Novick & Roth, 1968; Novick *et al.*, 1979; Nucifora *et al.*, 1989). The first, *cadA*, encodes a membrane protein 727 amino acids in length, and the second, *cadC*, encodes a soluble protein 122 amino acids in length. The CadA polypeptide sequence has strong homology to the E1-E2 class of ATPases, found in membranes of animal cells, plants, lower eukaryotes and bacteria (Nucifora *et al.*, 1989; Silver *et al.*, 1989). The current model for CadA membrane ATPase is based on the better studied members of this class, the Ca^{2+} ATPase of animal sarcoplasmic reticulum and the Na^{+}/K^{+} ATPase of animal cell plasma membranes (Silver *et al.*, 1989). The polypeptide sequence contains a series of recognisable motifs and key conserved residues, such as recognition sites for Cd, hairpin structures considered to be involved in Cd cation translocation, and ATP binding sites etc. (Silver & Walderhaug, 1992).

The CadC polypeptide is unrelated to other sequences, but has weak homology to ArsR sequences (regulator of the *ars* system: arsenate-arsenite-antimony resistance determinant) and to CadX, an undefined open reading frame in the sequence of an unrelated Cd^{2+} resistance system, CadB.

Strains of *Staphylococcus aureus* harbouring the CadA resistance system also have at-least two alternative Cd-resistance systems. The *cadB* determinant present on plasmid pII147, but not on plasmid pI258 (Novick & Roth, 1968; Smith & Novick, 1972; Novick *et al.*, 1979) confers resistance to Cd and Zn and does not involve cation efflux. However, *cadB* is associated with enhanced Cd-binding to the cell (Perry & Silver, 1982). The *cadB* determinant contains two open reading frames, *cadB* and *cadX* (cited in Silver & Walderhaug, 1992). The amino acid translation sequence of *cadX* has similarity to CadC of the *cadA* ATPase system and to ArsR regulatory protein of the As-resistance determinant. Witte *et al.* (1986) reported a chromosomal system of Cd resistance not related to the *cadA* system, in a special group of methicillin-resistant *S. aureus* strains,

which also exhibited chromosomally determined resistance to methicillin and mercury salts. Furthermore, Cd resistance in these strains is not associated with Zn resistance, but is associated with a cation efflux mechanism.

Other mechanisms of Cd-resistance have been reported in bacterial species. Surowitz *et al.* (1984) suggested reduced Cd accumulation as a Cd resistance mechanism in *Bacillus subtilis*. Whilst, Aiking *et al.* (1982; 1984) for *Klebsiella aerogenes*, and Macaskie & Dean (1984) and Macaskie *et al.* (1987) for *Citrobacter* sp. have demonstrated that resistance towards Cd involves precipitation of Cd as sulphide or phosphate. The reduction of lag period induced in *E. coli* by Cd, with repeated sub-culturing, has been suggested to involve the repair of Cd-induced DNA single strand breaks (Mitra *et al.*, 1975). In addition, *E. coli* is also suggested to possess Cd-binding proteins (Mitra, 1984; Khazaeli & Mitra, 1981).

1.52 Resistance in cyanobacteria

Contamination of rivers and estuaries with Pb, Hg, Cd, Zn and Cu has been documented in numerous surveys (Say & Whitton, 1981; Forstner, 1983). Algae have been used as biological indicators to monitor toxic trace metal pollution in aquatic environments (Phillips, 1977; Whitton, 1984) and in a limited number of cases they have been applied to the purification of water contaminated with metals (Kessler, 1986). Eukaryotic algae and cyanobacteria are often abundant, with cyanobacteria being the dominant form, in Zn-enriched waters of high pH (Whitton, 1980). Strains isolated from such sites are highly resistant to Zn (Shehata & Whitton, 1982). Various effects of metal toxicity have been documented in metal-tolerant strains isolated from metal polluted sites and developed in the laboratory. A brief introduction to the literature on effects of metals on metabolic processes in cyanobacteria is presented.

Zn-uptake in *Anacystis nidulans* (= *Synechococcus* PCC 6301) is concentration dependent and increases with time during growth (Shehata & Whitton, 1982). Upon exposure of wild-type and Cd-tolerant *A. nidulans* to Cd, a four fold higher uptake rate and concentration of Cd is shown in the wild-type than the Cd-tolerant culture. Similarly,

metal ions of Cd and Cu have a growth inhibitory effect on *Anabaena* PCC 7120 (Laube *et al.*, 1980), and some concentrations of Cu lysed *Anabaena* PCC 7120 cells at early, but not late, stages of growth. The exposure of *Anacystis nidulans* IU 625 to Cd resulted in inhibition of both NH_4 and PO_4 uptake, where NH_4 uptake was more sensitive to Cd than PO_4 uptake (Singh & Yadava, 1984).

Significant inhibition of various metabolic processes (for e.g. growth, nutrient uptake, photosynthesis, ATP content, nitrate reductase, glutamine synthetase and urease activities) following metal supplementation have been extensively documented (reviewed by Whitton, 1980; Rai *et al.*, 1981; Vymazal *et al.*, 1985; Reed & Gadd, 1990). Cd exposure induces an increase in filament length, heterocyst frequency, and loss of cellular contents from filament apical cells, suggesting that metal toxicity resulted from transport of Cd into the cell rather than being bound at the cell surface. In exponentially growing wild-type cultures of *A. nidulans* (*Synechococcus* PCC 6301) separation of cells occurred soon after division, but in old cultures chains of two or four cells were frequent (Shehata & Whitton, 1982). Tolerant strains exhibited an increased average length of the rods, and at sub-inhibitory levels of Zn, filaments were produced. However, under partially inhibitory levels of Cu, the wild-type, and Cu and Zn-tolerant strains formed sub-spherical units, in contrast to normal rod shapes, and formed the bulk of the population (Whitton & Shehata, 1982). Similarly, Chintamani & Mohanty (1989) observed elongation and adherence of cells after division in *Synechococcus* PCC 6301 grown under elevated levels of Zn. In addition to the various morphological changes, an increase in the carotenoid/chlorophyll ratio was observed in cultures treated with Zn. Chl a, phycocyanin and total protein contents (per ml of culture) of *Synechococcus* PCC 6301 were less in metal-treated cultures, but on a per cell basis Chl a and total protein contents were higher in treated samples (Chintamani & Mohanty, 1989).

Polyphosphate bodies, implicated in sequestration of Cd, Co, Cu, Hg, Ni, Pb and Zn (Jensen *et al.*, 1982a), were enlarged after Cd exposure, whilst exposure to Co reduced the number of polyhedral bodies. However, in cultures of *Chlorella saccharophila,* a lower eukaryotic alga, exposed to low concentrations, Zn was present only in

polyphosphate body cell sectors, and not in any cell sectors away from the polyphosphate bodies (Jensen *et al.*, 1982b). Furthermore, it has been demonstrated that the Cd adsorbed on cell surface and transported into the cells, decreases with decrease in pH (Skowronski, 1986). The amount of Cd available to cells is limited at alkaline pH due to Cd sorption by hydrolysis of the micro nutrients (constituents of the medium), and formation of precipitates, mainly metal hydroxides (Skowronski *et al.*, 1988; 1991). Zn-toxicity decreased with a fall in pH for Zn- sensitive and tolerant populations (Say & Whitton, 1977). Additionally, increase in levels of Mg, Ca and PO_4 were effective in reducing Zn-toxicity with Zn-tolerant populations than with Zn-sensitive ones. Ca had a proportionately greater effect in reducing Cd-toxicity than Zn-toxicity (Say & Whitton, 1977; Shehata & Whitton, 1982). Co, Ni, Cu and Pb individually had an additive rather than synergistic effect leading to increased Zn toxicity. PO_4 starved cells of *A. nidulans* were slightly more sensitive than PO_4 rich cells at low environmental levels of inorganic PO_4, but less sensitive at higher environmental levels. Reduction in toxicity was less affected when PO_4 was added after exposure of cells to Zn (Shehata & Whitton, 1982). Furthermore, organic phosphates supported growth, but were less effective at reducing Zn-toxicity.

No detectable Zn uptake was observed in cells of *Anabaena variabilis* grown under phosphorus-starved conditions and exposed to Zn, but was detected when Zn was part of the complete culture medium or in cells grown in complete medium for 4 h post P-starvation and then Zn was added. Under different P-starvation conditions and exposed to Cu, cells contained S, suggesting release of S from other cellular constituents, such as protein, and is sequestered in the polyphosphate bodies, coupled with some loss of K (Jensen *et al.*, 1982a; 1986). Additionally, it has been demonstrated that more Cu binds under anaerobic conditions, and this extra uptake was associated with, but not stoichiometrically related to, an increased loss of K by the cells, suggesting that the release of K is due to a graded response of a barrier, normally of low permeability, to increasing amounts of bound Cu. However, exposure of *A. flos-aquae* to Cd caused a loss of Mg

and Ca from the polyphosphate bodies (Rachlin *et al.*, 1984) resulting in ionic changes in the elemental composition of these cellular inclusions.

Cd has an acute sea surface depletion and exhibits best correlation with a major algal nutrient (P) (Boyle *et al.*, 1976; Bruland *et al.*, 1978). In sea water with low Zn concentrations, Cd substitutes for Zn in certain macromolecules of the marine diatom *Thalassiosira weissflogii* (Price & Morel, 1990). The growth-promoting properties of Cd are only evident in the absence of Zn from the medium, the distribution of Cd and Zn among the soluble cellular constituents is remarkably similar (suggesting a metal substitution in the same protein rather than an enzyme substitution), and the quantity of Cd per cell decreases in the presence of Zn and increases in the absence of Zn.

Tolerant strains of *Anacystis nidulans* exhibited an increase in growth lag when cultured in medium supplemented with Zn after maintenance for 20 subcultures in the absence of Zn, but original resistance was regained by the second subculture in the presence of Zn (Whitton & Shehata, 1982). Repeated culturing of cyanobacterial isolates in the absence of Cu results in loss of tolerance for most species within a few subcultures; however, original tolerance recovered following one subculture in medium containing an intermediate level of Cu (Takamura *et al.*, 1989; 1990). Strains of *A. nidulans* tolerant to Co, Ni, Cu, Zn and Cd also partial tolerance to another metal (Shehata & Whitton, 1982). However, in freshwater algal isolates from metal-polluted sites, Cu-tolerant strains also exhibited co-tolerance to Cd and Zn, and *vice versa*, though strains showing specific resistance to Zn or to Cd were also obtained (Takamura *et al.*, 1989). A Cd-resistant strain of *Nostoc calcicola* developed by step-wise adaptation exhibited cross-resistance to the antibiotics, neomycin and chloramphenicol, but not to streptomycin, and also tolerated elevated levels of other metals like Zn and Hg (Singh & Pandey, 1982).

Different studies, as introduced above, have shown various effects of metal exposure on metabolic processes in cyanobacteria. Additionally, different mechanisms for metal tolerance in cyanobacteria have been postulated.

Extracellular polysaccharides have been suggested to act as natural metal chelators and reduce metal toxicity (Crist *et al.*, 1981). Purified sheath fractions of *Calothrix*

parietina and *C. scopulorum* were similar in chemical composition, and bound metals (upto atleast 0.7% of sheath dry weight) with the effectiveness Fe>Zn>Cu>Ni>Mn>Mo>Co, and Ni, Cu, Zn and Fe were highly enriched relative to their concentration in the medium (Weckesser *et al.*, 1988). Additionally, Manzini *et al.* (1984) observed that alginates extracted from seaweeds and bacteria exhibit Cu binding ability, as does pectate, a polymer of ß-D-galacturonic acid found in plant tissue.

Oxygenic photosynthesis in wild-type *Nostoc calcicola* is extremely sensitive to Cu (Verma & Singh, 1991). However, a Cu-resistant mutant grew at high concentrations of Cu due to mutational acquisition of an energy-dependent efficient Cu-efflux system, which rendered Cu-inhibited oxygenic photosynthesis fully reversible. The efficiency of the Cu-efflux system in the wild-type was negligible, but in the resistant strain the rate of Cu-efflux was rapid during the first 5 min and gradually declined with time, resulting in reduction of the intracellular Cu pool.

Cu complexation by organic ligands influences the toxicity of Cu in natural waters (Sunda & Lewis, 1978). Fe limitation increases the extracellular concentration of Cu-complexing agents in cultures of *Anabaena flos-aquae* and *A. cylindrica*, and the Fe-algal exudate complex is more stable than the Cu-complex, suggesting that the strong Cu-complexing agents released by filamentous cyanobacteria are siderophores (McKnight & Morel, 1979; 1980). However, siderophore excretion is not suggested to be a mechanism by which cyanobacteria overcome the toxic effects of Cu. Murphy *et al.* (1976) suggested that eukaryotic algae cannot assimilate Fe from the Fe-hydroxamate siderophore complex and cyanobacteria monopolize low Fe concentrations by excretion of hydroxamate siderophores. Furthermore, the biological uptake of Fe by Fe-limited *Anabaena* populations was blocked by addition of Cu (Murphy & Lean, 1975).

Although, different mechanisms of metal tolerance have been suggested in different organisms, no molecular mechanism for metal tolerance has been identified in cyanobacteria.

1.6 Metallothionein

The passage of metals through cells and organisms is linked to their association with specific metal-binding macromolecules (cited in Kägi & Schaffer, 1988). The mode of metal-binding in metalloproteins varies widely yielding structures of divergent chemical and biological specificity (Kägi & Schaffer, 1988). One such super family of metal-binding macromolecules are the metallothioneins (MTs).

MT was discovered by Margoshes and Vallee (1957) in an attempt to identify a tissue component responsible for the natural accumulation of Cd in mammalian kidney. Since this initial description, MTs have been isolated from a wide range of sources, both eukaryotic and a prokaryote. MTs are thus considered to be ubiquitous and are characterised by low molecular weight, high cysteine content (thus a high content of sulphur), unique amino acid sequence (characteristic distribution of cysteinyl residues such as Cys-X-Cys), selective capacity to bind and be induced by heavy metal ions (such as Zn, Cd and Cu), and low in aromatic amino acids (Kägi & Vallee, 1960; Kägi & Vallee, 1961; Karin, 1985). Extensive biochemical and genetic analyses has since been conducted and several reviews provide an extensive documentation of the various studies involving the MT system (Karin, 1985; Hamer, 1986; Robinson & Jackson, 1986; Palmiter, 1987; Kägi & Schaffer, 1988; Kägi, 1991; Vallee, 1991). A summary of the various characteristics and information available on the MT system is presented.

1.61 Classification and occurrence of MTs

Historically, the term 'metallothionein' was used to designate Cd-, Zn-, and Cu-containing sulphur rich protein from equine renal cortex. The conspicuous features of MTs have prompted a variety of similar metal-thiolate polypeptides to be included under the generic term 'metallothionein'. Therefore, "Polypeptides resembling equine renal metallothionein in several of their features can be designated as *metallothionein*" (Kojima, 1991).

MTs have been divided into three classes (Kägi & Kojima, 1987) on the basis of their structural characteristics: Class I MTs are defined as polypeptides with locations of cysteine closely related to those in equine renal MT (such as *Neurospora* and *Agaricus* MTs), Class II MTs being the polypeptides with locations of cysteine only distantly related to those in equine renal MT (such as *Saccharomyces* and *Synechococcus* MT). The class III MTs are the atypical, nontranslationally synthesised metal-thiolate polypeptides, such as phytochelatins. Additionally, all the class I MTs isolated from vertebrates can be sub-divided into isoforms MT-I and MT-II (based on their ionic charge differences), each isoform potentially comprising several isoproteins.

Most studies in animal MT systems have focused on liver and kidney, the primary sites of heavy metal accumulation, but MT is also detectable in other organs and in various cultured cell types (cited in Hamer, 1986). MT is usually detected by virtue of its high metal content (usually Cd, Zn, Cu). However, the composition of the MT would vary from one organism/tissue to the other, and would depend upon the history of metal exposure. For example, exclusively Zn containing MT has been isolated from human liver, whilst Cd and Cu are normally components of those isolated from the kidneys. Mehra *et al.* (1988) isolated an exclusive Cu-thionein from the lower eukaryote *Candida glabrata*. Similarly, MTs have also been isolated from lower eukaryotes, for e.g. *Saccharomyces cerevisiae* (Karin *et al.*, 1984), and *Neurospora crassa* (Lerch, 1980). MT-like proteins have also been reported in a few eukaryotic algae (cited in Robinson, 1989; Section 1.7), and various prokaryotes have also been suggested to produce MT-like proteins (cited in Silver & Misra, 1988). One such protein from a cyanobacterium was purified to homogeneity and characterised (Olafson *et al.*, 1988). The corresponding gene has subsequently been isolated and characterised from the cyanobacterium *Synechococcus* PCC 7942 (Section 1.7). Furthermore, in higher plants, genes capable of encoding proteins with homology to class I MTs have been described, but their putative products remain to be identified *in planta* (deMiranda *et al.*, 1990; Evans *et al.*, 1990).

1.62 Structure and metal binding characteristics of MTs

Most research concerning the structure of MT relates to the mammalian MT, and serves as model for MTs in other systems. The mammalian MT is a 61- or 62-amino-acid peptide of which 20 are cysteine residues. From the 14 mammalian MT molecules for which protein or DNA data is available, the consensus sequence derived is as follows:

MDPNCSCATGGSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCVCKGASDKCSCCA

Beta domain Alpha domain

(Invariant cysteine residues are shown in bold type, reproduced from Hamer (1986) which includes original references).

The metals in MT are contained in two distinct, polynuclear clusters. The A cluster contains 11 cysteines and is contained within the carboxy-terminal α domain, whilst the B cluster contains nine cysteines, and is contained in the amino-terminal ß domain. 2D NMR spectroscopic measurements have proved beneficial in providing data for the spatial structure of mammalian MT and the organisation of metal-thiolate clusters (Braun *et al.*, 1986; Arseniev *et al.*, 1988; Schultze *et al.*, 1988). Comparison of low energy bands in the far UV absorption spectra of MT with those of tetrahedral halide complexes, suggests a tetrahedral co-ordination of metal ions in MT (Vasak *et al.*, 1981). These results were further confirmed by studies involving various spectral techniques, such as, EXAFS (Hasnain *et al.*, 1987) and PAC (Vasak & Bauer, 1982). Metals are associated with MT exclusively through thiolate bonds to all 20 cysteine residues. The metals can be removed by exposure to low pH and the resulting apothionein can be reconstituted with 7 atoms of Cd or Zn, the α domain binding 4 and the ß domain binding 3 divalent metal ions. In the rat Cd-, Zn-MT-II, the A cluster contains four Cd atoms of which two are bonded by three bridging and one terminal sulphur, and the other two by two bridging and two terminal sulphurs. The B cluster contains one Cd and two Zn atoms, all of which are bonded by two bridging and two terminal sulphurs (cited in Hamer, 1986).

In addition to binding Zn and Cd, mammalian MTs are also known to bind Cu *in vivo*. Cu binds in the +1 rather than the +2 valence state, and EXAFS measurements for the

binding stoichiometry suggests that 12 Cu ions may each be co-ordinated to three cysteines in a triangular structure, rather than tetrahedral, and alters the folding and tertiary structure of the protein (Winge, 1987; Hamer, 1986). The α and ß domains of MTs can be separated by digestion of the linking amino-acids using subtilisin, and each domain binds metal ions in the same way as the complete molecule, using *in vitro* substituted MT, were able to demonstrate the relative binding affinities for metal-MT complexes were shown to be in the order: Hg(II)>Cu(I)>Cd(II)>Zn(II)>Ni(II),Co(II) (Winge & Miklossy, 1982; Nielson & Winge, 1983; Nielson *et al.*, 1985).

Cu-MTs isolated from *Neurospora crassa* (Beltrami & Lerch, 1983) and from *Agaricus bisporus* (Munger & Lerch, 1985) have been designated as class I MTs, and their cysteine residues align perfectly with those of the N-terminal region of mammalian MTs. However, MT-like proteins isolated from other lower eukaryotes (i.e. the Cu-MTs of *Saccharomyces cerevisiae* and of *Candida glabrata*), and the Cd/Zn MT isolated from the cyanobacterium *Synechococcus* PCC 7942, do not align with vertebrate MTs (thus classified as class II MTs), and generally show little sequence similarity to one another. Nevertheless, these proteins do contain the Cys-Cys, Cys-X-Cys and Cys-X-X-Cys motifs which are characteristic of MTs.

1.63 Proposed functions of MTs

Much of the interest in MTs has focused on the regulation of transcription of the genes that encode them, and the biological roles of MTs. Kägi & Vallee (1960), in one of their first papers on MT, speculated that MTs might play an important role in "....catalysis, storage, immune phenomena, or detoxification....". The conservation of the structure of MTs, its ubiquitous nature, and its ability to both bind and be induced by metal ions, strongly suggests that MTs must play a major role in some fundamental metal-related biological process (Hamer, 1986). Defining this role has proven difficult. However, it is important to emphasise that the structurally distinct MTs occurring in different organisms may have different roles.

1.631 Role in metal detoxification

The protective effects of MT have been studied extensively both in animal and cell culture systems, but detoxification of metal ions is not universally accepted as the primary function of MT (Karin, 1985). Several studies suggest its involvement as a major component in detoxification of metal ions. Mammalian MT synthesis is induced by a range of metal ions (Ag, Au, Bi, Cd, Cu, Hg, Pb, Pt, and Zn), and the protein is found to be in association with the metal that induces its synthesis (Webb, 1987). The association/involvement of MT molecules in the induction by Cd and subsequent high-affinity sequestration of the metal ion by the induced protein is well documented (Kägi & Kojima, 1987; Webb, 1987).

Mammalian cell lines in culture, that fail to produce the MT protein exhibit decreased tolerance to Cd (Compere & Palmiter, 1981; Crawford *et al.*, 1985). These cell lines, apart from their loss of capacity to produce MT due to gene hypermethylation, were normal in all other respects. Similarly, in the yeast *Saccharomyces cerevisiae* cells, *CUP1* sequences (gene coding for the Cu-MT) were replaced by a heterologous marker gene (Hamer *et al.*, 1985). These recombinant strains were hypersensitive to Cu, but were normal in all other aspects of cellular metabolism, such as doubling time, mating, diplophase growth, sporulation and germination. Additionally, transfection of *CUP1*-deleted cells with plasmids containing the *CUP1* gene or a heterologous monkey MT-I gene under control of *CUP1* regulatory sequences, provides protection to the cells from Cu toxicity (Thiele *et al.*, 1985).

A wide variety of cultured cells have been selected for Cd resistance by continuous exposure of the cells to step-wise increase in Cd in the medium. During selection, the levels of MT mRNA greatly exceeds the maximum that can be induced in the unselected cells (Durnam & Palmiter, 1987). Although a number of mechanisms could attribute for increased MT mRNA production, it has almost invariably been associated with MT gene amplification (Beach & Palmiter, 1981; Gick & McCarty, 1982; Hayashi *et al.*, 1983; Durnam & Palmiter, 1987; review by Palmiter, 1987). Similarly, increased resistance to

Cu ions, resulted from amplification of the *CUP1* gene in *Saccharomyces cerevisiae* (Karin *et al.*, 1984). In *Candida glabrata* cells selected for Cu resistance, there is stable chromosomal amplification of MT-II gene to around 30 copies (Mehra *et al.*, 1990). The increased resistance is due to much greater MT synthetic capacity and metal resistance declines with the loss of the extra genes.

Depending on the cell type, Cd resistance can be completely stable in the absence of selection or decay with a half-life of ca. a week (Beach & Palmiter, 1981; Mayo & Palmiter, 1982). These extra copies if they have an origin of replication, may persist as extra-chromosomal elements; alternatively, they may integrate into the chromosome (Schimke, 1984). The amplification units in mammalian cells have not been precisely identified, but are very large, and thus the closely linked MT genes are invariably linked together. The mechanism of MT amplification is unknown but probably results from aberrant replication of the MT locus during the cell cycle (cited in Palmiter, 1987). The observation of MT gene amplification to be associated with Cd resistance argues strongly that MT plays an important role in metal detoxification. Additionally, cell lines that over-produce MT accumulate larger quantities of Zn and Cu then do normal cells. This hypothesis is further supported by the fact that Cd resistance can be conferred in cells by transfer of MT genes on a self-replicating plasmid. Cell lines containing high copy number of BPV-MT (Bovine papilloma virus-MT) recombinants have been shown to be highly resistant to Cd due to MT over-production (Karin *et al.*, 1983). Furthermore, in cell cultures of *Datura innoxia* selected for resistance to various concentrations of $CdCl_2$, addition of Cd and Cu results in the rapid synthesis and accumulation of sulphur-rich, metal-binding polypeptides (Jackson *et al.*, 1987). Additionally, in Cd-resistant cultures a direct correlation between the amount of Cd incorporated and the degree of Cd tolerance, and a correlation between the maximum amount of metal-binding polypeptide synthesised and the level of resistance to different concentrations of metal ions has been demonstrated. Similarly, non-protein cysteine-rich polypeptides have been shown to accumulate in high amounts when Cd-resistant tomato cells, selected for tolerance to elevated concentrations of Cd, are grown in the presence of Cd (Steffens *et al.*, 1986). The importance of MT

gene amplification and other studies relating to MT gene amplification are further illustrated in detail in Chapter 8.

1.632 Alternative functions of MTs

The role of MTs in metal detoxification is well documented, but MTs seem likely to be involved in processes other than protection against heavy metals. MTs isolated from liver and kidney contain a high concentration of Zn and Cu, and may serve as a major storage form for these metals. Additionally, MTs are expressed in an inducible manner in essentially every tissue (Searle *et al.*, 1984). These observations are suggestive of both extracellular (homeostatic) and intracellular control of Zn and Cu metabolism. Furthermore, the intracellular level of Zn regulates the turn-over rate of MTs; when Zn is in short supply, MTs are rapidly degraded (Karin *et al.*, 1981). However, Hamer (1986) has summarised that the sole function of Cu-MT of *Saccharomyces cerevisiae* is to maintain a low level of free intracellular Cu concentration.

Many biological processes involve the use of Zn-requiring enzymes, and as a major Zn-binding protein MTs could potentially modulate these processes either directly, by interaction with inactive apoenzymes, or indirectly by regulating the intracellular Zn. Zn-requiring apoenzymes were reactivated by the transfer of Zn from MTs to the apoenzymes (Udom & Brady, 1980). Zeng *et al.* (1991a; 1991b) were able to inhibit DNA binding of the Zn-dependent transcription factors Sp1 and TFIIIA *in vitro* by the addition of apo-MT, thus abolishing transcription activation in an *in vitro* assay. Therefore, it was postulated that under *in vivo* conditions, similar activity could provide a mechanism of control for a large subset of genes requiring Zn-dependent transcription factors. Different variants of MT could therefore carry Zn to different intracellular compartments, thus modulating the metabolic and proliferative status by alterations in the intracellular distribution pattern of Zn (Karin, 1985). Recent studies have examined the temporal and spatial expression of MT genes. Animal MT expression varies widely in different tissue types and is dependent also on the stage of development of the organism, raising additional possibilities of the involvement of MT in the control of cellular growth. Kern *et al.* (1981) have

demonstrated a programmed mode of MT gene regulation in rat foetal development. Changes in the sub-cellular localisation of MT in cultured hepatocytes have recently been demonstrated (Tsujikawa *et al.*, 1991). The localisation of MT shifts from the cytoplasm to the nucleus in the early S-phase. The functional human MT gene cluster is split in leukemic cells of patients (Lebeau *et al.*, 1985). One half of the gene cluster, normally present on the long arm of chromosome 16 is translocated to the short arm of the same chromosome. Additionally, the breakpoint always occurs in the middle of the MT gene cluster. This led to the suggestion that a resident cellular oncogene on the short arm of the chromosome is activated by enhancer elements associated with the MT (Karin *et al.*, 1984).

Inducers of MT biosynthesis are known to include factors other than heavy metals (e.g. UV, X-ray irradiation, infection, administration of substances as diverse as chloroform and glucocorticoids), suggesting possible role of MTs in cellular adaptation mechanisms. Interferon and Interleukin 1, activate macrophages and neutrophils, which release active oxygen species. These are extremely cytotoxic and can cause severe tissue damage in the host in the absence of protective measure (such as superoxide dismutase). Zn and Cd-MTs scavenge free hydroxyl ions (but not superoxide radicals unlike superoxide dismutase), suggesting a direct participation in the detoxification of this reactive species (Thornalley & Vasak, 1985). Additionally, MT over-expressing mutants of cultured mammalian cells are resistant to X-ray damage, suggesting role of MT in protection of cells against ionizing radiation (Bakka & Webb, 1981; Karin, 1985), possibly by scavenging free radicals or as sources of Zn for DNA repair enzymes that are activated after irradiation.

1.7 Metal-binding proteins in algae and cyanobacteria

Two main Cu binding molecules contributing to metal detoxification and regulation, were reported in *Euglena gracilis* (Piccinni *et al.*, 1985). These two molecules were different in chain weight (6932 and 3590), and amino acid composition, but had a relatively high percentage of cysteine (13 and 20%, respectively). Peptide no. 1 also had a

high content of aspartic acid. A family of arginine- and asparagine-rich polypeptides are synthesised in Zn deprived *Euglena* cells (Vallee & Falchuk, 1981), and also, in *Euglena* a reduction in Zn content is induced upon Cu treatment (Albergoni *et al.*, 1980). It is noted that cyanophycin is a polymer of aspartic acid and arginine, and in cyanobacteria is produced in quantity in response to Ni or Cu exposure (Wood, 1983). Similarly, two Cd-binding proteins Cd-BP-I and Cd-BP-II are produced in Cd-exposed cells of *E. gracilis* (Gingrich *et al.*, 1984; 1986), and these cells also have a large pool of very low molecular weight Zn species. The following properties of the Cd-BP-I and Cd-BP-II have been demonstrated: lower Mr values and more negatively charged than MTs, lack of cross-reactivity to mammalian MT antibodies, low Zn content (despite high levels in growth medium) and a high content of sulphide ions (Weber *et al.*, 1988). The presence of sulphide ions in high concentrations suggested its relation to the sulphide containing peptides of *Schizosaccharomyces pombe* and similar plant peptides, known commonly as phytochelatins, cadystins or metallochelatins (Robinson *et al.*, 1987; Shaw III *et al.*, 1988).

Cu-tolerance in *Scenedesmus acutiformis*, involves binding of Cu to proteins which were similar in size to those identified in mammalian systems and yeast (Stokes *et al.*, 1977). Similarly, in *S. quadricauda*, Reddy & Prasad (1989) identified a protein of approximately 8 kDa only in cells exposed to Cd, and suggested it to be similar to that in *Dunaliela* (10 kDa) (Heuillet *et al.*, 1988). A Cd binding protein from *Chlorella ellipsoidea* was characterised and from its amino acid composition suggested its similarity to phytochelatins (Nagano *et al.*, 1984). Similarly, Gekeler *et al.* (1988) examined organisms from six classes of Phycophyta and concluded that Cd-binding proteins similar to phytochelatins (class III MTs) are ubiquitous in the division of algae, and that algae sequester heavy metals by an identical mechanism as higher plants, namely via complexation to phytochelatins.

A Cd- and Zn-binding material was detected in *A. nidulans* (Maclean *et al.*, 1972). Subsequently, Olafson *et al.* (1979a) reported the isolation of a Cd-inducible metal-binding protein from Cd-exposed cells of a marine cyanobacterium *Synechococcus* sp. strain

RRIMP NI. Subsequently, this protein was purified to homogenity from *Synechococcus* TX-20 and characterised. Amino acid analysis suggested a high cysteine content (lower than that reported for eukaryotic metallothioneins), but was consistent with the metal content analysis. Furthermore, a single methionine, and elevated levels of lysine and serine were observed. Various chemical and structural characteristics of the prokaryotic MT from *Synechococcus* TX-20 revealed superficial features similar to eukaryotic MTs (Olafson, 1986; Olafson *et al.*, 1988).

TSTTLVKCACEPCLCNVDPSKAIDRNGLYYCCEACADGHTGGSKGCGHTGCNC

Amino acid sequence of the prokaryotic MT from *Synechococcus* sp. (Reproduced from Olafson *et al.*, 1988)

The prokaryotic MT molecule complexes Cd, Zn and Cu, but its synthesis is induced only by Cd and Zn, with induction controlled at the level of transcription (Olafson *et al.*, 1980), a phenomenon previously described in the crustacean *Scylla serrata* (Olafson *et al.*, 1979b). Although the prokaryotic MT has a high content of cysteine residues and characteristic clusters of Cys-X-Cys, Cys-X-X-Cys and Cys-Cys sequences, primary structural analysis did not show any relationship to MTs from higher organisms. The conservation of certain characteristic features, despite a lack of sequence similarity suggested that cyanobacterial MT may have a convergent evolutionary relationship with eukaryotic MTs (Olafson *et al.*, 1988). Moreover, the *Synechococcus* MT showed similarity to *Saccharomyces* MT in having a high hydrophobicity, where *Saccharomyces* MT has 5 hydrophobic residues, whilst the *Synechococcus* MT has 8 residues, making it the most hydrophobic MT to be described. However, unlike the yeast MT, the hydrophobic residues in *Synechococcus* MT are distributed throughout the first half of the molecule with a pair of adjacent tyrosine residues situated in the middle of the protein. Additionally, the prokaryotic MT lacks the adjacency of hydroxylated and basic residues with cysteine residues. Secondary-structure analysis shows similarity to eukaryotic MTs in the metal-thiolate complex region. The cyanobacterial MT did not exhibit the presence of any helical structures, but indicated that ca. 61% of the molecule was in ß-pleated sheet, with 29% in the form of ß-turns, accounting for the reduced ellipticity in the 200 nm

region as compared to the mammalian protein. The metal-thiolate cluster of the prokaryotic MT might be similar to the eukaryotic proteins, but in a single domain (Olafson *et al.*, 1988).

Since the isolation and characterisation of the first prokaryotic MT protein from *Synechococcus* TX-20 (Olafson *et al.*, 1988), and immediately prior to and during the course of this study, the corresponding gene encoding the MT has been isolated and characterised from *Synechococcus* PCC 6301 and PCC 7942 (Robinson *et al.*, 1990; Huckle *et al.*, in press). The structural gene, designated *smtA*, encodes a protein of 56 amino acids, similar to that purified from *Synechococcus* TX-20 by Olafson *et al.* (1988) with two modifications. Two additional amino acids, histidine and glycine, are present at the C-terminus and serine substitutes for cysteine$_{32}$ (serine$_{33}$ in SmtA sequence), altering a cysteine-cysteine pair to cysteine-serine. The structural features within the *smt* operator/promoter region include a 7-2-7 hyphenated inverted repeat and a 6-2-6 hyphenated direct repeat. Furthermore, a second divergent open reading frame upstream of the *smt* region was identified and designated *smtB*. The deduced SmtB polypeptide contains 122 amino acids. Sequence similarity exists between SmtB and ArsR (protein regulating the transcription of *E. coli* and *Staphylococcus aureus ars* operons, which code for the arsenic, arsenate and antimonite efflux systems), and SmtB and CadC (encoded by *cadC* and encodes an ATP-dependent Cd(II) efflux system in *S. aureus*). The gene structure, similarity to a known transcriptional regulator and the presence of a DNA-binding motif are suggestive of a putative role for SmtB as a regulator of *smtA* transcription (also Section 8.3).

Transcript abundance of *smtA* has been shown to increase following exposure to Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn, in contrast to the induction of yeast MT gene *CUP1*, which is induced only by Cu and Ag (Huckle *et al.*, in press). Additionally, exposure to Cd was shown to have no effect on transcript stability of *smtA*. Shi *et al.* (1992) have shown a higher affinity of the cyanobacterial MT for Zn (lower pH of half displacement), than that estimated for equine renal MT. These observations have led to the suggestion of a role for SmtA in Zn homoeostasis (Huckle *et al.*, in press), consistent with the

observations that the cyanobacterial MT *in vivo* in *Synechococcus* TX-20 (= PCC 6301) is induced by, and associated with, Zn (Olafson *et al.*, 1988).

Olafson *et al.* (1980) observed that *Synechococcus* sp. (Section 1.8) cells previously cultured in Cd, exhibited tolerance when recultured in the same medium, with no marked growth lag and increased MT synthesis. Furthermore, the Cd-exposed cells exhibited a marked growth lag as compared to cells grown in the absence of Cd, and onset of growth was coincident with increase in MT. However, MT levels reduced to near basal values following repeated subculture in Cd-free media, and suggested the phenomenon to be due to repression of MT synthesis in the absence of metal (Olafson, 1986). These cells on re-transfer to Cd-supplemented medium grew with no observable lag phase, whilst the wild type cells grew with the usual growth lag in Cd-supplemented medium. Furthermore, acquisition of Cd resistance in *Synechococcus* was thought unlikely to be related to a chromosomal mutation event, since the frequency for a chromosomal mutation would be very high. The phenomenon of metal resistance has thus been speculated to involve the amplification of an extrachromosomal gene (Olafson, 1986). Although cyanobacteria are reported to have plasmids (Lau & Doolittle, 1979; Laudenbach *et al.*, 1983), no plasmid-encoded functions have so far been demonstrated (Ciferri *et al.*, 1989). *Synechococcus* PCC 6301 is known to contain two plasmids of ca. 8.0 kb and 48.5 kb, which could potentially harbour the *smtA* gene. At an intermediate stage of this study, another researcher in the laboratory analysed total DNA isolated from R2-PIM8, a small plasmid cured (R2-SPc) derivative of *Synechococcus* PCC 7942, and showed that this strain contains *smtA* (Turner *et al.*, 1992), indicating *smtA* to be either chromosomal or present on the 48.5 kb plasmid. Furthermore, it was also found that the sizes of *Sal*I, *Hin*dIII and *Bam*HI restriction fragments containing the *smtA* gene in DNA isolated from *Synechococcus* PCC 6301 (Robinson *et al.*, 1990) do not correspond to the known sizes of *Sal*I, *Hin*dIII and *Bam*HI restriction fragments of the 48.5 kb plasmid (Laudenbach *et al.*, 1983).

1.8 A note about taxonomic designations

In view of the uncertain state of cyanobacterial taxonomy, this brief note outlines the taxonomic designations used in this thesis. In the literature, different taxonomic names have been appended to the same strain by different authors: *Synechococcus* PCC 6301 (*Synechococcus leopoliensis, Anacystis nidulans, Anacystis nidulans* TX-20, *Anacystis nidulans* UTEX 625, *Anacystis nidulans* UTEX 1550); *Synechococcus* PCC 7942 (*Anacystis nidulans* R2). Strains designate alternative names to *Synechococcus* PCC 6301, all originated from the single isolate of Kratz and Myers (1955b) and are therefore identical. The name *Agmenellum quadruplicatum* implies membership of a different genus for a strain which has been described as similar in most respects to *Synechococcus* PCC 6301 (Rippka *et al.*, 1979). Similarly, strains PCC 6301 and PCC 7942 have been designated the binomial *Anacystis nidulans* and are suggested to belong to one and the same species (Golden *et al.*, 1989; Wilmotte & Stam, 1984). However, PCC 7942 has a higher transformation efficiency than PCC 6301, and is thus the organism of choice for genetic manipulations.

Aims

The main aim of this research was to identify a molecular mechanism of metal tolerance in cyanobacteria. Laboratory strain of *Synechococcus* PCC 6301 was initially to be used as a model system. It was planned to develop Cd-tolerant cell lines of *Synechococcus* PCC 6301 by step-wise adaptation to increasing concentrations of Cd, and to investigate the role of the prokaryotic metallothionein gene, *smtA*, in acquisition of Cd tolerance. It was also planned to look for evidence of *smtA* homologues in axenic cyanobacterial strains isolated from metal-polluted sites, and to investigate whether similar mechanisms of metal tolerance operate in isolates from metal-polluted sites.

This study was made possible by the isolation of the first prokaryotic metallothionein gene, *smtA*, by another researcher in the laboratory. Further characterisation of the *smt* locus, whilst this study was in progress, has affected the plan of this research and has

helped in designing future experiments, and interpreting results at different stages. The information obtained from this research may identify a molecular mechanism of Cd-tolerance which could operate in cyanobacteria selected for growth in metal polluted environments.

Chapter 2

GENERAL MATERIALS AND METHODS

2.1 Materials

2.11 Cyanobacterial cultures and growth media

Cyanobacterial strains were obtained from the Durham University Culture Collection. The cultures of *Synechococcus* D33, *Synechococcus* D839 and *Synechocystis* D840 had originally been obtained from sources other than the Pasteur Culture Collection. However, these strains are believed to have originated from similar sources as the cultures held in the Pasteur Culture Collection. For simplicity, in this thesis, *Synechococcus* D33 (= PCC 6301), *Synechococcus* D839 (= PCC 7942) and *Synechocystis* D840 (= PCC 6803) are referred to by their Pasteur Culture Collection designation (also Section 1.8).

Cyanobacteria strains were cultivated in AC medium and MJH medium. The AC medium (ACM) was that of Kratz and Myers (1955a), modified according to Shehata and Whitton (1982). The mineral composition was as given in Table 2.1. MJH medium was as given in Table 2.2. Trace elements for both AC and MJH medium were the BG-11 formula of Rippka *et al.* (1979) (Table 2.3). Buffering capacity was provided by 2.5 mM (0.6 g l^{-1}) HEPES with the pH adjusted to 7.6 using 1.0 M NaOH. Cyanobacterial strains were cultivated under constant light (100 μmol photon m^{-2} s^{-1} PAR) at 32°C, the light source being cool white fluorescent tubes. Cyanobacterial strains were cultured in 100 ml Erlenmeyer conical flasks fitted with silicone-rubber bungs to facilitate gaseous exchange. Larger quantities of cultures were grown in 2 L Erlenmeyer conical flasks, and constantly aerated by bubbling sterile aeration.

Strain	Medium used	Country of origin	Details of site
Synechococcus sp. D33 (= PCC 6301)	ACM		
Synechococcus sp. D839 (= PCC 7942)	ACM		
Synechocystis sp. D840 (= PCC 6803)	ACM		
Synechococcus sp. D562	ACM + 2 mg l^{-1} Cd	U.S.A	Elvins Tailings Dam, Missouri Environment: Zn, 8 mg l^{-1}, Growth temperature: 25°C Initially grown in AC medium + Zn, 5 mg l^{-1} + PO_4 EDTA Isolated by: F.H.A. Shehata in 1979 (Whitton *et al.*, 1981)
Microchaete sp. D578	MJH + 0.25 mg l^{-1} Cd	U.S.A	Elvins Tailings Dam, Missouri Tolerates Zn, 20+ mg l^{-1} Growth temperature: 25°C Initially grown in Chu10E medium + Zn, 5 mg l^{-1} (Whitton *et al.*, 1981) Isolated by: J.W. Simon in 1979
Calothrix parietina D184	MJH + 0.25 mg l^{-1} Cd	England	Laboratory Zn tank Tolerates Zn, 9 mg l^{-1} Growth temperature: 25°C Initially grown in AD medium + P, 1 mg l^{-1} + Fe, 0.4 mg l^{-1} Isolated by: V.P. Singh in 1972
Oscillatoria sp. D813	MJH + 0.25 mg l^{-1} Cd	England	Gillgill Burn, Tyne Basin *Pseudanabaena*-like morphology Growth temperature: 25°C Growth medium: MJH + Cd, 0.25 mg l^{-1}, Isolated by: M.J. Hutchinson in 1988
Oscillatoria sp. D814	MJH + 0.25 mg l^{-1} Cd	England	Gillgill Burn, Tyne Basin Growth temperature: 25°C Growth medium: MJH + Cd, 0.25 mg l^{-1}, Isolated by: M.J. Hutchinson in 1988

2.12 *E. coli* strains and growth medium

The *E. coli* (K12) strains used were JM101: [*sup*E, (*lac-pro*AB), {F'*tra*D36, *pro*AB, *lac*I^qZ M15}, (r_{k+}, m_{k+}), *mcr*A(+)], and 'Sure': *mcrA*, (*mcrBC-hsdRMS-mrr*)*171*,

supE44, thi-1, lambda-*gyrA96, relA1, lac, recB, recJ, sbcC, umuC*::Tn5 (*kan*^{r}), *uvrC,* [F', *proAB, lac*^{q}*Z M15,* Tn10, (tet^r)]. JM101 was obtained from Northumbria Biologicals Ltd, Cramlington, Co. Durham, and 'Sure' was obtained from Stratagene Ltd, Cambridge, UK. *E. coli* strains were grown in Luria-Beltrami (LB) medium (Sambrook *et al.,* 1989).

2.13 Plasmids

The following commercially used plasmids were used: pUC19 and pGEM4z. pUC19 was obtained from Boehringer Mannheim, Lewes, UK, and pGEM4z was obtained from Promega Ltd, Enterprise Rd., Southampton, UK. Other plasmids used during the course of this research were pJHNR11 (Robinson *et al.*, 1990; 144 bp PCR fragment corresponding to the *smtA* coding region, in pUC19), pJHNR49 (Huckle *et al.*, in press; 1.8 kb *Hin*dIII-*Sal*I fragment containing the *smt* locus, in pGEM4Z), pJHNR61 (Gupta *et al.*, 1992; 215 bp PCR fragment corresponding to the coding region of *psaE,* photosystem I gene from *Synechocystis* PCC 6803: reported by Chitnis *et al.*, 1989). A clone bank representing the pANL (large 48.5 kb plasmid of *Anacystis nidulans* R2) genome and the small plasmid (ca. 8.0 kb), constructed in pDPL13 (Gendel *et al.*, 1983) was obtained by the courtesy of Dr. D.E. Laudenbach. The plasmid bank pPLAN Ba1-Ba7 contain the 11.7, 10.6, 9.0, 6.2, 4.7, 3.7 and 2.25 kb *Bam*HI fragments of the pANL plasmid, respectively. Plasmid pPLAN B2 contains the entire ca. 8.0 kb plasmid (Laudenbach *et al.*, 1983; 1985). The plasmids pPLAN Ba1-Ba7 and pPLAN B2 were transformed into transformation competent 'SURE' cells, and transformants checked by endonuclease restriction.

2.14 Chemicals, reagents and other consumables

General laboratory chemicals were obtained from Sigma Chemical Co., Poole, Dorset, and BDH (NELS), Newton Aycliffe, Co. Durham. Other chemicals and reagents are as below:

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

Deoxynucleotide triphosphates; Boehringer Mannheim UK, Lewes, Sussex, UK.

Radiochemicals, hybridisation membranes ('Hybond N' and 'Hybond N+'); Amersham International Ltd, Bucks., UK.

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

3MM chromatography paper; Whatman Ltd, Maidstone, Kent, UK.

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

Bacto-Agar; Difco, Detroit, Michigan.

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

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

Phenol (re-distilled); International Biotechnologies Inc., Newhaven, Connecticut.

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

Restriction enzymes. DNA modification enzymes, IPTG, Xgal; Northumbria Biologicals Ltd, Cramlington, Co. Durham, UK, and New England Biolabs, Inc., Bishop's Stortford, Herts., UK.

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

The water used in growth media and for work with DNA and RNA was double-deionised (MilliQ - 17-18 MΩ cm^{-1} resistivity; Millipore, Watford, UK.). Water used for RNA manipulations was further treated for denaturation of RNAses by addition of diethylpyrocarbonate (0.1% v/v), incubation at 25°C for 16 h, followed by autoclaving (15 psi, 20 min).

Glassware used for cyanobacterial culture, preparation and storage of metal stock solutions, and experiments involving use of metals was soaked in 4% Nitric acid (v/v in distilled water), 2 h, rinsed thoroughly with distilled water and dried before use.

2.2 Buffers and metal solutions

2.21 Buffers

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

2.22 Metal solutions.

Metals were used as the following salts:

$CdCl_2$; $ZnCl_2$; $CuCl_2.2H_2O$. Stock solutions were prepared in MilliQ water, and autoclaved (15 psi, 20 min) prior to use.

2.3 Methods

2.31 Growth and cell counts

Growth of cyanobacteria was monitored by measuring absorbance at 540 nm, using a Titretek micro-titre plate reader (Flow Laboratories). Cell counts for a measure of cell density ml^{-1} was calculated using a Improved Neubauer haemacytometer (depth 0.1 mm).

2.32 General molecular biology methods

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

2.321 Preparation of nucleic acids from cyanobacteria

2.321a Preparation of total nucleic acids (non-CsCl method)

This method was used for preparation of genomic DNA (extracted as total nucleic acid) from cell lines A0, A0.8, A1.3 and A1.7 (described in Section 4.3). This method has

previously been described for the isolation of nucleic acids from plant cell cultures (Robinson *et al.*, 1988), but excluding CsCl gradients.

a) Cyanobacterial cells (1000 ml culture) were harvested by centrifugation (8000 x g, 10 min).

b) Cells were resuspended in 1.0 ml DNA extraction buffer (1.4 M NaCl, 100 mM Tris.Cl pH 8.0, 20 mM Na_2EDTA), pipetted dropwise into liquid nitrogen and ground to a fine powder, using a pestle and mortar pre-cooled with liquid nitrogen.

c) The powder was transferred to sterile Corex tube with a sterile spatula. To the fine powder was added 100 µl ß-mercaptoethanol and boiling DNA extraction buffer (same volume as the packed cell volume).

d) Before the sample thaws, an equal volume of PCA (1:4:5 DNA extraction buffer: Equilibrated phenol: chloroform/Isoamyl alcohol- 24:1 v/v) was added, mixed well by inversion and allowed to thaw.

e) The sample was subjected to centrifugation at 5,000 x g, 5 min (Beckman JA-20 rotor), room temperature, and the upper aqueous phase collected.

f) PCA extraction followed by chloroform/isoamyl alcohol extraction was repeated till a clear interface was obtained.

g) The total nucleic acids were precipitated after the last extraction by adding 1/5 volume Ammonium acetate (5 M) and 2.5 volumes of -20°C 100% ethanol, then stored overnight at -20°C.

h) The nucleic acids were pelleted by centrifugation, 10000 x g, 20 min, washed twice with 70% ethanol (in water), partially dried in a vacuum, and resuspended in sterile MilliQ water.

2.321b Preparation of genomic DNA from cyanobacteria (CsCl method).

This method was used for isolation of genomic DNA from cell lines C0, C1.4, C1.8, C2.6 and C3.2 (described in Section 5.3). Steps a-e (described above) were essentially the same as described in Section 2.321a, followed by:

f) To every 4.0 ml of the supernatant was added 4.3 g of CsCl, and gently mixed till all the CsCl goes into solution.

g) To the above solution was added 0.25 ml ethidium-bromide stock (10 mg ml^{-1}).

h) The solution was placed in 1/2 x 2 inch Beckman quick seal centrifuge tubes and the tubes sealed.

i) The tubes were placed in VTi65 rotor and centrifuged (Sorvall OTD65B) at 50000 x g, 15°C for at least 12 h.

j) After termination of spin the tubes were visualised under ultra-violet lamp, and the top band corresponding to genomic DNA was eluted by the method described in Sambrook *et al.* (1989).

k) Ethidium-bromide from the eluted solution was removed by several extraction's with equal volumes of isoamyl alcohol (saturated with water and CsCl), the upper layer being that of isoamyl alcohol.

l) Salts were removed from the solution containing genomic DNA and CsCl by dialysis against large volumes of TE buffer (10 mM Tris.Cl, 1 mM EDTA, pH 8.0) at 4°C for atleast 16 h, with continuous stirring.

m) Genomic DNA was precipitated by adding 1/5 volume of ammonium acetate (5 M) and 2.5 volumes of -20°C 100% ethanol, mixed well by inversion, and left overnight at -20°C.

n) The nucleic acid was pelleted by centrifugation (10000 x g, 20 min, 4°C, Beckman JA-20 rotor), washed with 70% ethanol, partially dried under vacuum and resuspended in sterile MilliQ water.

2.321c Preparation of RNA from cyanobacteria

The RNA from cyanobacteria was isolated as total nucleic acids using essentially the same method as described by Dzelzkalns *et al.* (1988).

a) Cyanobacterial cells were harvested (50-100 ml culture), 8000 x g, 10 min, 4°C.

b) Cells were resuspended in small volume of TE buffer (pH 8.0), pipetted dropwise into liquid nitrogen and ground to a fine powder using a sterile precooled (with liquid nitrogen) pestle and mortar.

c) Ground cells were transferred to sterile Corex tubes, equal volume of prewarmed (37°C) lysis buffer (50 mM Tris.Cl, 100 mM NaCl, 20 mM EDTA, 2% w/v SDS, 60 mM ß-mercaptoethanol, 10 μg ml^{-1} Proteinase K, pH 8.0) added, mixed well and incubated at 37°C, 30 min.

d) The solution was passed several times through a 19 gauge needle if found to be very viscous.

e) The solution was extracted with phenol/chloroform (5:1, lysis buffer saturated) until a clear interface was obtained, aqueous layer collected and adjusted to 0.5 M ammonium acetate.

f) RNA was precipitated by adding 2.5 volumes -20°C 100% ethanol, and incubated at -20°C 2 h (or -70°C 30 min).

g) Nucleic acids were pelleted by centrifugation at 12000 x g, 30 min, pellet washed with 70% ethanol, partially dried under vacuum and resuspended in small volume of sterile MilliQ water (10-20 μl). Store at -20°C.

The integrity of the RNA was checked, and approximate quantity estimated by electrophoresis on a 1% agarose mini-gel. Prior to separation of RNA by a denaturing formaldehyde-agarose gel electrophoresis (Section 2.328), the RNA sample was mixed in a solution containing 50% formamide, 1X MOPS (final concentrations), incubated at 70°C for 10 min, then chilled on ice.

2.322 Plasmid mini-preparations from *E. coli* by alkaline lysis

(modified from Birboim and Doly, 1979)

a) *E. coli* cultures (5 ml) were grown in LB broth for 16 h at 37°C, with appropriate selection, dependent on the plasmid concerned.

b) An aliquot (1.5 ml) was removed to an Eppendorf microfuge tube, cells harvested by centrifugation (12000 x g, 1 min), and the supernatant removed.

c) The cells were resuspended in 100 μl of ice cold buffer (50 mM glucose, 25 mM Tris.Cl (pH 8.0), 10 mM EDTA and incubated at room temperature for 5 min.

d) 200 μl of alkaline SDS solution (0.2 N NaOH, 1% SDS) was added, the contents mixed by inversion and incubated on ice for 10 min.

e) The solution was mixed and 150 μl of high salt solution (3M sodium acetate, pH 4.8) added, the contents mixed thoroughly by inversion and placed on ice for a further 10 min.

f) After microcentrifugation (12000 x g, 5 min), the supernatant was removed to a fresh tube, RNAse A was added to a final concentration of 20 μg ml^{-1}, and incubated at 37°C for 30 min.

g) An equal volume of phenol chloroform (1:1 v/v) was added, the contents mixed, centrifuged at 12000 x g, 5 min., and the aqueous phase transferred to a fresh tube.

h) 2.5 volumes of -20°C 100% ethanol was added and incubated at -70°C for 30 min.

i) The plasmid DNA was recovered by microcentrifugation (12000 x g, 30 min), the pellet washed with 70% ethanol (twice) and partially dried under vacuum.

j) Plasmid DNA was resuspended in 16 μl of water, 4 μl of 4 M NaCl and 20 μl of 13% polyethylene glycol added, and incubated on ice for 30 min.

k) The purified plasmid DNA was pelleted by centrifugation, washed with 70% ethanol, partially dried under vacuum and finally resuspended in 20 μl of sterile MilliQ water.

2.323 Plasmid maxi-preparations from *E. coli*

Large-scale preparations of plasmid DNA was essentially a scale-up of steps (a-i) described in Section 2.322. 500 ml of *E. coli* culture grown under appropriate selection was harvested (by centrifugation at 4000 x g; Beckman centrifuge). Centrifugation in the steps to follow was performed using 30 ml glass Corex tubes (10000 x g; Beckman JA-20 rotor). The following steps were incorporated (after step i of Section 2.322) when DNA of greater purity was required.

a) The dried pellet was resuspended in 8 ml of TE buffer (pH 8.0), and 8.6 g of CsCl added, followed by 0.45 ml of ethidium bromide solution (10 mg ml^{-1}).

b) The solution was placed in two 1/2 x 2 inch quick-seal centrifuge tubes (ca. 5 ml capacity), and the tubes heat sealed.

c) Centrifugation was performed at 50000 x g, 15°C for 16 h using a Sorvall OTD65B ultracentrifuge.

d) Plasmid bands were visualised under UV lamp, and the plasmid band (lower) eluted by the procedure described in Sambrook *et al.* (1989).

e) Ethidium bromide was removed by several extraction's with isoamyl alcohol (saturated with CsCl and water), and the salts removed by dialysis against TE buffer (pH 8.0) at 4°C.

f) Plasmid DNA was precipitated using 2.5 volumes of 100% ethanol, washed with 70% ethanol, partially dried under vacuum and resuspended in sterile MilliQ water.

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

The method used for the preparation of frozen transformation competent *E. coli* cells was as described by Alexander *et al.* (1984). Transformation of *E. coli* competent cells was performed as follows:

a) An aliquot of competent cells (200 µl) was thawed on ice. Plasmid DNA (1-10 ng)/ligation reaction was diluted to 100 µl in TE buffer (pH 8.0) and mixed with competent cells.

b) The tube was incubated on ice for 30 min., followed by heat shock for 5 min at 37°C.

c) The transformation reaction was diluted to 2 ml with 2 XL broth, prewarmed to 37°C, and incubated for 2 h at 37°C (under shaking conditions).

d) Aliquots of transformed cells were then plated onto LB agar containing the desired selective agent.

2.325 Agarose gel electrophoresis of DNA and isolation of DNA restriction fragments from agarose gels

Agarose gel electrophoresis of DNA was performed as described by Sambrook *et al.* (1989). Generally 0.7% agarose gels were used, but varying concentrations upto a maximum of 2% were used depending on the size of fragment to be separated. Maxi and mini gels were cast using TBE buffer (0.089 M Tris-borate, 0.002 M EDTA). DNA was loaded into wells of the gel after addition of loading dye (0.25% w/v each of bromophenol

blue and xylene cyanol, 15% w/v Ficoll 400). Generally the size markers used were lambda phage DNA restricted with *Pst*I, or DNA kilobase marker (GIBCO-BRL, Life Technologies Ltd., Renfrewshire, UK.).

Restriction fragments were isolated from agarose gels by either of the methods described below:

a) Electroelution.

Gel slice containing the restriction fragment of interest was cut from the gel using a clean scalpel blade, and DNA eluted from the agarose block by electroelution (Sambrook *et al.*, 1989).

b) Silica fines method.

Gel slice containing the DNA was placed in an Eppendorf tube and 1 ml of sodium iodide solution (6.05 M sodium iodide, 0.11 M sodium sulphite; filter sterilised and saturated with sodium sulphite) added. The tube was placed at 65°C until the gel block melts. 10 µl of silica fines (quantity depending on amount of DNA present) were added (silica fines were a courtesy of Dr. R.G. Alexander. Now available as 'Finebind': Amersham International Ltd, Bucks., UK.). The solution was mixed and incubated at room temp for 20 min., fines pelleted by centrifugation at 12000 x g for 15 s, washed with 70% ethanol and resuspended in 50 µl TE buffer (pH 8.0). The DNA was eluted at 37°C for 30 min. with intermittent mixing by inversion. The fines were pelleted at 12000 x g for 15 s, and the supernatant collected.

The DNA eluted from either of the two procedures was purified by phenol extraction and precipitated using 2.5 volumes of 100% ethanol in a final concentration of 1 mM ammonium acetate. For DNA less than 1 kb in size, 1 mM glycogen was added to assist precipitation. The precipitated DNA was washed with 70% ethanol, dried under vacuum and resuspended in sterile MilliQ water.

2.326 Manipulations of DNA fragments

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

2.327 Radioactive labelling of DNA fragments

Double-stranded DNA fragments were labelled by random priming using (α-^{32}P)dCTP with Klenow polymerase. The protocol used was the same as described by Feinberg and Vogelstein (1983). Following the labelling reaction (16 h, room temp), unincorporated radioactivity was separated from the labelled DNA fragments by Sephadex G-50 gel permeation chromatography columns (10 ml volume).

2.328 Formaldehyde-agarose gel electrophoresis of RNA

Electrophoresis of RNA was done by preparation of Formaldehyde-agarose gels and electrophoresis as described by Sambrook *et al.* (1989). Ribosomal RNA bands were used as size markers for these gels. The rRNA bands produced by the electrophoresis of RNA extracted from *Synechococcus* and their sizes are indicated where gels are presented.

2.329 Southern and northern blotting

DNA and RNA was transferred to nylon hybridisation membranes (Hybond-N) essentially as described by Sambrook *et al.* (1989). DNA was denatured prior to transfer by soaking the gel in an excess of denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 1 h, followed by neutralisation of the gel by soaking in an excess of neutralisation solution (1.5 M NaCl, 0.5 M Tris; pH 7.5) for 1 h. RNA gels were soaked in distilled water to remove formaldehyde prior to transfer. Gels were blotted for 16 h using 10X SSC (0.15 M NaCl, 0.015 M sodium citrate; pH 7.0), after which complete transfer of nucleic acids had occurred. DNA was fixed to the membrane by baking, whereas RNA was fixed with UV

illumination, followed by baking, under vacuum at 80°C for 1 h prior to hybridisation to radioactive probes.

Alternatively, DNA was transferred to nylon hybridisation membranes (Hybond-N+) by blotting for 16 h using 0.4 N NaOH as the transfer buffer. The protocol does not require denaturation and neutralisation of gel prior to transfer, and no fixation step is required prior to hybridisation.

2.3210 Hybridisation of radioactive DNA probes to filter-immobilised nucleic acids

All hybridisation reactions were carried out in heat sealed polyethylene bags contained in plastic boxes, and placed in temperature controlled water baths. Northern blots were hybridised to radioactive probes at 42°C, whilst Southern blots were hybridised at 65°C, and hybridisation's continued for 16 h. In both cases, filters were prehybridised for 1 h prior to addition of probe. The solutions used for prehybridisation and hybridisation of Southern and northern blots were as described by Sambrook *et al.* (1989). After hybridisation, the filters were washed using 2X SSC, 0.1% SDS (10 min, room temp.), followed by washing with 1X SSC, 0.1% SDS (10 (RNA)/20 (DNA) min, 42/65°C). The filters were then sealed in polyethylene bags, placed on 3MM paper, oriented using several spots of radioactive ink, and exposed to X-ray film. After film development, the filter could be washed to a higher stringency, or completely stripped of radioactivity by pouring boiling 0.1% SDS onto the filter and allowing it to cool to room temperature (2X). The filter could then be re-probed as desired.

2.3211 Quantification of genomic and plasmid DNA

DNA concentrations for routine manipulations were estimated by measurement of absorbance at 260 nm. However, DNA concentration in samples of genomic DNA and plasmid (pJHNR11) to be used for gene copy number reconstructions was estimated accurately by the Fluorometric Diaminobenzoic acid (DABA) method of Thomas and Farquhar (1978). Aliquots (1-5 µl) of standard DNA (Lambda) concentrations and the unknown samples were taken in Eppendorf tubes and dried under vacuum. DABA was

then dissolved at 400 mg ml^{-1} in H_20 immediately prior to use. 20 µl of DABA was added to each tube containing DNA samples, mixed well and incubated at 60°C, 30 min. Samples were quickly cooled on ice and diluted with 1 ml, 1 M HCl. Fluorescence was read at an excitation wavelength of 405 nm and emission wavelength of 505 nm. Concentration of DNA in unknown samples was calculated from the standard calibration curve.

2.3212 Estimation of gene copy equivalents

Gene copy equivalents were calculated from first principles as follows:

1 mole of single copy gene is contained in the gram equivalent weight of the haploid genome. (Genome size of *Synechococcus* PCC 6301 = 3.212×10^6 bp, Herdman *et al.*, 1979.)

1 mole = $3.212 \times 10^6 \times 625$ g, where Mol. wt. of 1 bp = 625

6×10^{23} sequences = $3.212 \times 10^6 \times 625$ g, where 6×10^{23} is the Avogadro No.

$$1 \text{ sequence} = \frac{3.212 \times 10^6 \times 625}{6 \times 10^{23}}$$

Therefore, in 10 µg of genomic DNA there are

$$\frac{6 \times 10^{23} \times 10 \times 10^{-6}}{3.212 \times 10^6 \times 625} \text{ sequences}$$

Now, vector (pUC19) + insert (*smtA*) = 2686 + 144 = 2830 bp

Therefore,

$$1 \text{ sequence} = \frac{2830 \times 625}{6 \times 10^{23}} \text{ grams}$$

$$\frac{6\text{x}10^{23}\text{x}10\text{x}10^{-6}}{3.212\text{x}10^6\text{x}625} \text{ sequences} = \frac{2830\text{x}625}{6\text{x}10^{23}} \text{ x } \frac{6\text{x}10^{23}\text{x}10\text{x}10^6}{3.212\text{x}10^6\text{x}625} \text{ grams}$$

$$= \frac{2830 \times 10 \times 10^{-6}}{3.212 \times 10^{6}} \text{ grams}$$

$$= 8.8107 \times 10^{-9} \text{ grams}$$

$$= 8.8107 \text{ ng}$$

Therefore, 10 μg of genomic DNA would contain 2.988×10^9 copies of the genome, and an equivalent number of copies of *smtA* would be contained in 8.81 ng of pJHNR11 DNA.

2.3213 Use of polymerase chain reaction (PCR) for *in vitro* amplification of DNA

PCR reactions for *in vitro* amplification of *smtA* and *psaE* (to be primarily used for preparation of radioactive probes) were carried out essentially as described by Saiki *et al.* (1988) with minor modifications as described by Fordham-Skelton *et al.* (1990). Reaction conditions were as follows: 200 μM each of dATP, dTTP, dCTP and dGTP, 50 mM KCl, 10 mM Tris.Cl (pH 8.3), 1.5 mM $MgCl_2$), 0.01% gelatin, in a final reaction volume of 50 μl. Primers used in amplification reactions were as described by Robinson *et al.* (1990) and Gupta *et al.* (1992). Taq polymerase was added to the reaction last, contents mixed and overlaid with mineral oil. Reactions were carried out using a Hybaid intelligent heating block. Reactions were subjected to 28 cycles of the following series of temperatures and times: denaturation 92°C for 1.5 min, annealing 55°C for 1.5 min, extension 72°C for 1.5 min. Amplified DNA samples were stored at -20°C prior to further analysis. Any deviations from these reaction conditions are stated for individual reactions.

2.3214 Automated DNA sequence analysis

Direct sequencing of plasmid clones was performed by the dideoxy-sequencing method of Sanger *et al.* (1977), using fluorescent dye-linked universal M13 primers. Sequences were analysed using an applied Biosystems 370A DNA sequencer. Plasmids were sequenced in both directions using forward and reverse primers. Reactions were prepared according to protocols described by the manufacturer (Model 370A DNA sequencing system, User's manual version 1.3A, October 1988).

2.3215 Synthesis of oligonucleotides

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

TABLE 2.1: Mineral salt composition of modified AC medium.

Compound/ element	Molecular/ atomic wt.	stock conc. ($g\ l^{-1}$)	medium conc. ($mg\ l^{-1}$)	(mM)	total element conc. ($mg\ l^{-1}$)	(mM)
$CaCl_2.2H_2O$	147.020	19.86	19.86	0.135		
Ca	40.080		5.414		5.414	0.135
Cl	35.453		9.578		24.072	0.679
NaCl	58.440	46.000	23.000	0.394		
Na	22.989		9.048			
Cl	35.453		13.953			
KNO_3	101.110	100.000	500.000	4.945		
K	39.098		193.344			
N	14.007		69.266		69.266	4.945
$MgSO_4.7H_2O$	246.47	50.000	250.000	1.014		
Mg	24.305		24.653		24.653	1.014
S	32.060		32.519		32.559	1.015
$K_2HPO_4.3H_2O$	228.230	13.103	13.103	0.057		
K	39.098		4.489		197.834	5.060
P	30.974		1.778		1.778	0.057
Na_2EDTA	372.240	1.667	1.667	0.005		
Na	22.989		0.206		28.800	1.253
EDTA	326.262		1.461		1.461	0.005
$FeCl_3.6H_2O$	270.300	1.210	1.210	0.005		
Fe	55.847		0.250		0.250	0.005
Cl	35.453		0.476			
Buffering NaOH	40.000	40.000	34.000	0.850		
Na	22.989		19.541			

TABLE 2.2: Mineral salt composition of MJH medium.

Compound/ element	Molecular/ atomic wt.	stock conc. ($g\ l^{-1}$)	medium conc. ($mg\ l^{-1}$)	(mM)	total element conc. ($mg\ l^{-1}$)	(mM)
$Ca(NO_3)_2$	236.150	117.840	117.840	0.499		
Ca	40.080		20.000		20.000	0.499
N	14.007		13.978		26.987	1.926
NaCl	58.440	15.960	15.960	0.273		
Na	22.989		6.278			
Cl	35.453		9.682			
$MgSO_4.7H_2O$	246.47	101.410	101.410	0.411		
Mg	24.305		10.000		10.000	0.411
S	32.060		13.192		13.192	0.411
KNO_3	101.110	93.910	93.910	0.928		
K	39.098		36.310			
N	14.007		13.009			
KH_2PO_4	136.090	7.820	7.820	0.057		
K	39.098		2.246		38.556	0.986
P	30.974		1.778		1.778	0.057
Na_2EDTA	372.240	13.350	3.337	0.008		
Na	22.989		0.412		26.231	1.141
EDTA	326.262		2.924		2.924	0.008
$FeCl_3.6H_2O$	270.300	9.700	2.425	0.008		
Fe	55.847		0.501		0.501	0.008
Cl	35.453		0.954		10.636	0.300
Buffering NaOH	40.000	40.000	34.000	0.850		
Na	22.989		19.541			

TABLE 2.3: Mineral salt composition of microelements for modified AC and MJH medium.

Compound/ element	Molecular/ atomic wt.	stock conc. ($g\ l^{-1}$)	medium conc. ($mg\ l^{-1}$)	(mM)	total element conc. ($mg\ l^{-1}$)	(mM)
$CoSO_4.7H_2O$	281.100	0.042	0.0420	0.0002		
Co	58.933		0.0088		0.0088	0.0002
S	32.060		0.0048			
$CuSO_4.5H_2O$	249.680	0.079	0.0790	0.0003		
Cu	63.546		0.0201		0.0201	0.0003
S	32.060		0.0101			
H_3BO_3	61.830	2.86	2.8600	0.463		
B	10.810		0.5000		0.5000	0.0463
$MnCl_2.4H_2O$	197.920	1.810	1.8100	0.0091		
Mn	54.938		0.5024		0.5024	0.0091
Cl	35.453		0.6484		0.6484	0.0182
Na_2MoO_4	241.950	0.390	0.3900	0.0016		
Na	22.989		0.0741		0.0741	0.0032
Mo	95.940		0.1546		0.1546	0.0016
$ZnSO_4.7H_2O$	287.550	0.222	0.2220	0.0008		
Zn	65.380		0.0505		0.0505	0.0008
S	32.060		0.0248		0.0397	0.0012

Chapter 3

LOCALISATION OF *smtA*

3.1 Introduction

It has previously been proposed that the rapid development of Cd-tolerance in *Synechococcus* TX-20 may involve the amplification of an MT gene located extrachromosomally (Section 1.7), since *Synechococcus* sp. is known to have plasmids (Section 1.7). However, amplification of MT genes (initially chromosomal), associated with metal tolerance, has also been described in metal-tolerant eukaryotic cell lines (Section 1.631). An understanding of chromosomal or extrachromosomal localisation of *smtA* is clearly important. The work presented below was performed to establish *smtA* localisation.

3.2 Results

3.21 Analysis of genomic DNA, pPLAN Ba1-Ba7 and pPLAN B2

Restriction of plasmids pPLAN Ba1-Ba7 and pPLAN B2 with *Bam*HI released the various *Bam*HI fragments of the 48.5 kb plasmid and the small (ca. 8.0 kb) plasmid, from the vector pDPL13 (Fig. 3.1A). Genomic DNA isolated from *Synechococcus* PCC 6301 was restricted with *Bam*HI and used as a control.

The restricted DNA was analysed by Southern hybridisation to radiolabelled *Hin*dIII-*Sal*I fragment isolated from pJHNR49. Hybridisation to the *smt* locus was observed in *Bam*HI restricted genomic DNA from *Synechococcus* PCC 6301, but no hybridisation was detected to any of the *Bam*HI restriction fragments of the 48.5 kb or the ca. 8.0 kb plasmid (Fig. 3.1B).

The filter was stripped of radioactivity and re-hybridised to a radiolabelled PCR amplification product (144 bp) corresponding to the coding region of *smtA*. This identified hybridisation of *smtA* to the genomic DNA. Additionally, weak hybridisation was observed only to the 10.6 kb *Bam*HI fragment released from pPLAN Ba2 (Fig. 3.1C).

However, the size of pPLAN Ba2 does not correspond to the size of the hybridising fragment in genomic DNA.

3.22 Analysis of pPLAN Ba2 and genomic clone

Restriction endonuclease *Pst*I restricts within the coding region and upstream of *smtA*, releasing a fragment of 213 bp. *smt* clone pJHNR49 and pPLAN Ba2 were restricted with *Pst*I and fragments separated on an agarose gel (Fig. 3.2A). Low molecular weight (corresponding to 213 bp and smaller) products and a single high molecular weight product (corresponding to the vector plus the rest of the *smt* locus) was observed in pJHNR49, whilst, large numbers of low and high molecular weight products were observed with pPLAN Ba2 (Fig. 3.2A). However, the low molecular weight products of pPLAN Ba2 did not exactly correspond in size to the 213 bp fragment of pJHNR49.

The *Pst*I restricted DNA from pJHNR49 and pPLAN Ba2 was hybridised to radiolabelled 144 bp *smtA* fragment (Fig. 3.2B). Strong hybridisation was observed to the 213 bp restriction fragment of pJHNR49 and weak hybridisation to the high molecular weight fragment (vector plus the remaining *smt* locus). In pPLAN Ba2 only weak hybridisation was observed to two restriction fragments of ca. 2.0 and 10.0 kb. Moreover, no hybridisation was observed to any of the low molecular weight (ca. 200 bp) restriction products (Fig. 3.2B).

3.23 Genomic DNA analysis

Genomic DNA isolated from *Synechococcus* PCC 6301 was restricted with *Hin*dIII-*Sal*I, separated on agarose gels (Fig. 3.2C) and hybridised to radiolabelled 2.0 kb *Pst*I fragment of pPLAN Ba2 (identified by hybridisation to *smtA*; Section 3.22). The 2.0 kb *Pst*I fragment identified its homologue in the form of two hybridising fragments of ca. 1.8 and 6.0 kb (Fig. 3.2D). Re-hybridisation of the filter with radiolabelled 144 bp *smtA* fragment revealed only a single hybridising fragment of 1.8 kb (Fig. 3.2E). The second fragment of ca. 6.0 kb observed upon hybridisation to the 2.0 kb *Pst*I fragment of pPLAN Ba2 was not detected when hybridised to *smtA*.

3.3 Summary

The observations can be summarised as follows:

1) No hybridisation of *Bam*HI fragments from pPLAN Ba1-Ba7 and pPLAN B2 to radiolabelled *Hin*dIII-*Sal*I fragment released from pJHNR49 was observed.

2) Weak hybridisation of pPLAN Ba2 was observed to radiolabelled 144 bp *smtA* amplification product.

3) The restriction fragment in pPLAN Ba2 hybridising to *smtA* does not correspond in size to that observed in the genomic DNA from *Synechococcus* PCC 6301.

4) Restriction of pPLAN Ba2 with *Pst*I did not release any fragment corresponding to that in pJHNR49 (213 bp).

5) *Pst*I restricted pJHNR49 showed strong hybridisation of the 213 bp fragment to *smtA*, whilst weak hybridisation to two different larger restriction fragments was observed in pPLAN Ba2.

6) The sizes of the hybridising fragments in *Pst*I restricted pPLAN Ba2 and pJHNR49 did not correspond.

7) Hybridisation of *Hin*dIII-*Sal*I restricted genomic DNA from *Synechococcus* PCC 6301 to radiolabelled 2.0 kb *Pst*I fragment from pPLAN Ba2 identified two restriction fragments of ca. 1.8 and 6.0 kb.

8) Hybridisation of *Hin*dIII-*Sal*I restricted genomic DNA from *Synechococcus* PCC 6301 to *smtA* always identifies only a single restriction fragment of 1.8 kb.

It is concluded that certain sequences with weak homology to *smtA* are present in pPLAN Ba2. However, a lack of correlation in restriction fragment lengths and difference in hybridisation intensities between *smtA* and pPLAN Ba2 suggests that the prokaryotic metallothionein locus, *smt*, in *Synechococcus* PCC 6301 is localised on the chromosome.

Figure 3.1. Localisation of *smtA*. Genomic DNA isolated from: lane 1, *Synechococcus* PCC 6301; and plasmid DNA isolated from: lane 2, pPLAN Ba1; lane 3, pPLAN Ba2; lane 4, pPLAN Ba3; lane 5, pPLAN Ba4; lane 6, pPLAN Ba5; lane 7, pPLAN Ba6; lane 8, pPLAN Ba7 and lane 9, pPLAN B2; was restricted with *Bam*HI. Panel *A*: visualisation of ethidium bromide stained DNA, panel *B*: hybridisation to *Hin*dIII-*Sal*I fragment carrying the *smt* locus, and panel *C*: hybridisation to *smtA*.

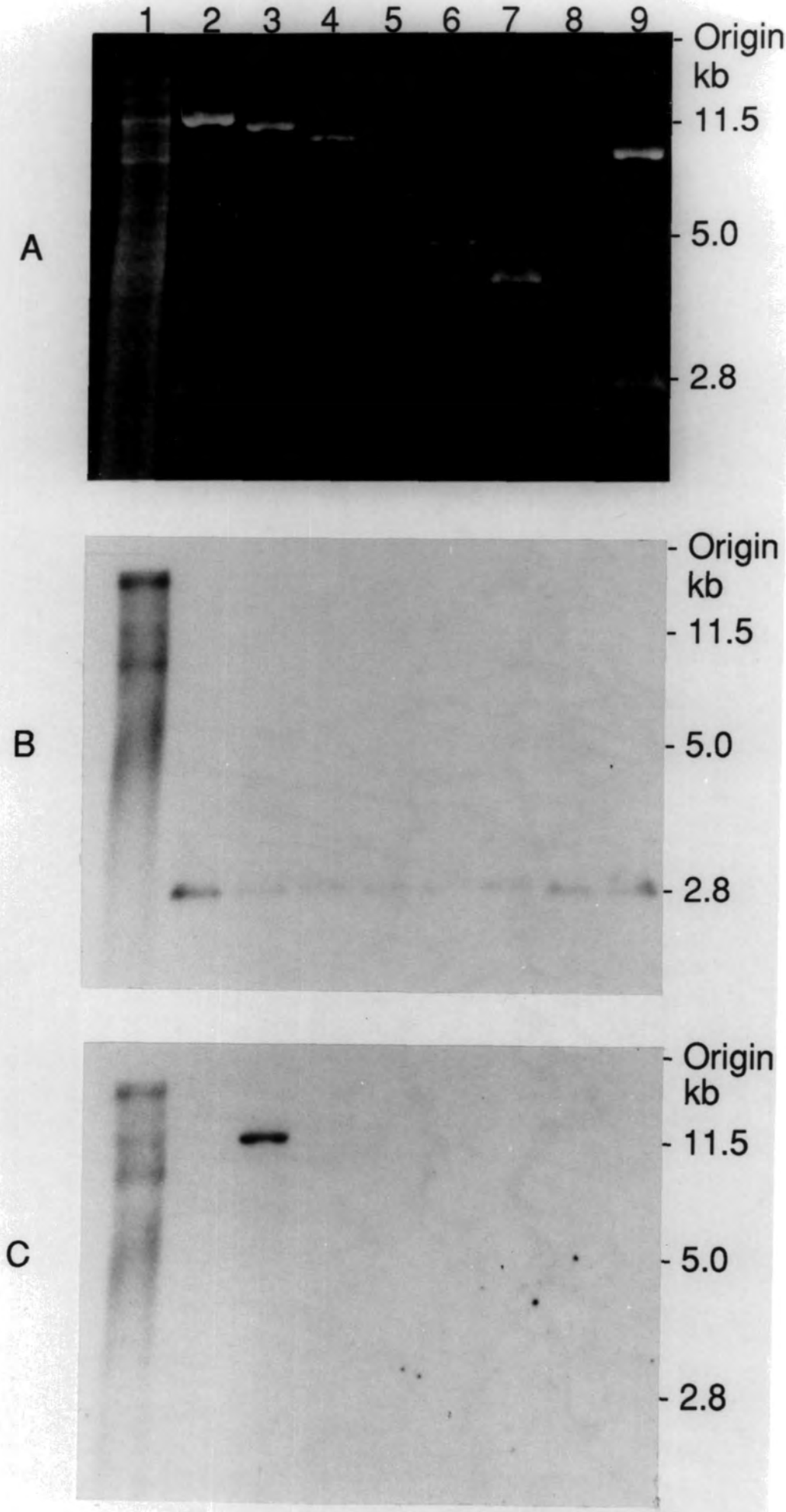
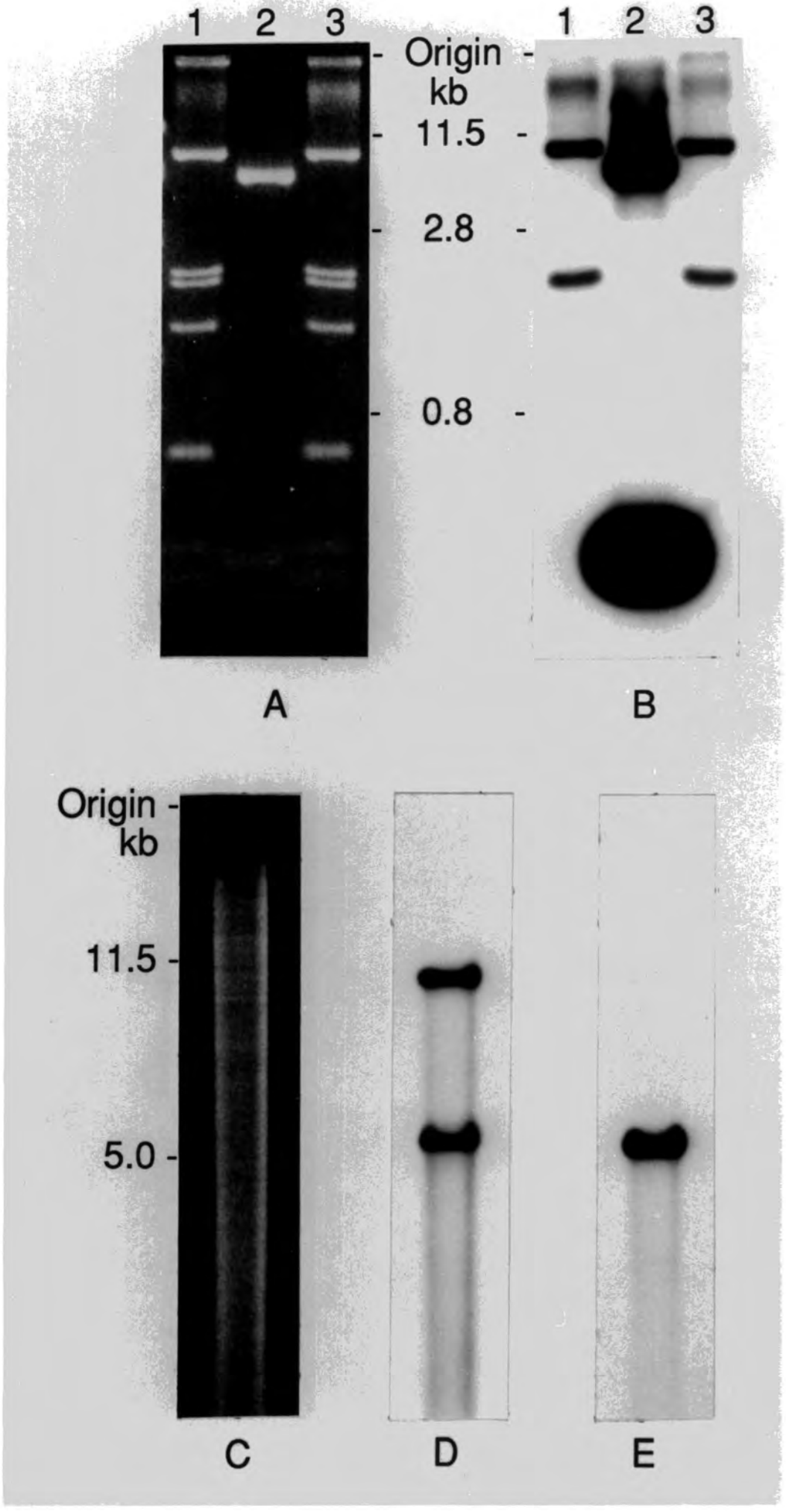

1 2 3 4 5 6 7 8 9
Origin
kb
11.5
5.0
2.8
A
Origin
kb
11.5
5.0
2.8
B
Origin
kb
11.5
5.0
2.8
C

Figure 3.2. Localisation of *smtA*. Plasmid DNA isolated from: lane 1, pPLAN Ba2; lane 2, pJHNR49 and lane 3, pPLAN Ba2; was restricted with *Pst*I and separated on agarose gel. Panel *A*: visualisation of ethidium bromide stained DNA, and panel *B*: hybridisation to *smtA*.

Genomic DNA isolated from *Synechococcus* PCC 6301 (panel *C*) was hybridised to panel *D*: 2.0 kb *Pst*I fragment from pPLAN Ba2, and panel *E*: hybridisation to *smtA*.


1
2
3
Origin
kb
11.5
2.8
0.8
A
1
2
3
B
Origin
kb
11.5
5.0
C
D
E

Chapter 4

STEP-WISE SELECTION OF *Synechococcus* PCC 6301 CELLS TO INCREASED Cd-TOLERANCE

4.1 Introduction

Metal-tolerant cell lines of cyanobacteria and different eukaryotic organisms have previously been developed in the laboratory by step-wise selection (Sections 1.52, 1.631), but genes involved in the acquisition of tolerance have previously not been identified in cyanobacteria.

4.2 Materials and Methods

4.21 Step-wise selection of Cd-tolerant cell lines

A culture of *Synechococcus* PCC 6301 that had undergone prolonged maintenance in liquid medium was used for step-wise selection. *Synechococcus* PCC 6301 cell lines were developed for tolerance to supra-optimal concentrations of Cd by repeated sub-culturing in liquid medium containing a range of Cd concentrations. Cells that grew in the highest concentration of Cd were used as inocula for further subculture. 100 µl of a culture (cell density of culture: ca. $1x10^6$ - $5x10^6$ cells ml^{-1}) was inoculated into 10 ml of fresh medium. The most tolerant cell line obtained after each step of selection was maintained in liquid media supplemented with the respective Cd concentration.

4.3 Results

Step-wise selection of a *Synechococcus* PCC 6301 culture (A0) resulted in cell lines tolerant to 0.8 µM Cd (A0.8), 1.3 µM Cd (A1.3) and 1.7 µM Cd (A1.7), whilst, initial tolerance in non-selected cell line A0 was only at 0.4 µM Cd. Cd-tolerant cell lines were maintained in the respective Cd concentrations and analysed as follows.

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

A linear relationship between increase in absorbance (540 nm) and cell density of a culture of *Synechococcus* PCC 6301 was obtained. Growth (increase in cell density) of A0 and the three Cd-tolerant lines (A0.8, A1.3 and A1.7) was monitored (absorbance at 540 nm) in media supplemented with different concentrations of Cd (0 μM, 0.8 μM, 1.3 μM, 1.7 μM) (Fig. 4.1). The inoculum density was 1 x 10^6 cells ml^{-1}.

The growth of A0 was partially inhibited in media containing 1.3 μM Cd (Fig. 4.1C), but was totally inhibited in the presence of 1.7 μM Cd (Fig. 4.1D). The growth of A0.8 was also partly inhibited at 1.7 μM Cd. However, no significant difference in growth, relative to A0.8 and A0 was observed for A1.3 and A1.7, in media supplemented with 1.7 μM Cd (Fig. 4.1D). The four lines grew equally well in non-supplemented media.

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

Ten micrograms of genomic DNA isolated from A0, A0.8, A1.3 and A1.7 was restricted with *Sal*I. Standard amounts of plasmid DNA (pJHNR11), corresponding to 1-6 and 8 gene copy number equivalents, was restricted with *Eco*RI. The restricted DNA was separated on agarose gel, visualised and transferred to nylon membrane for hybridisation.

An increase in hybridisation to *smtA* was observed in genomic DNA isolated from A1.7. The increase in hybridisation was ca. four-fold in A1.7, relative to that for A0, as judged from gene copy number reconstruction's (Fig. 4.2A). However, ethidium-bromide stained gel shows that equivalent amounts of genomic DNA from the four lines was loaded on the gel (Fig. 4.2C).

The filter was stripped of radioactivity and re-hybridised to a radiolabelled fragment of the *psaE* gene (released from clone pJHNR61) (Fig. 4.2B). No increase in hybridisation of *psaE* to A1.7, relative to A0, was observed; indeed, a slight decrease in hybridisation to DNA isolated from A1.7 was evident.

4.33 Analysis of genomic DNA from A0, A0.8, A1.3 and A1.7 after subsequent maintenance

Genomic DNA isolated from the non-selected A0 and Cd-tolerant lines A0.8, A1.3 and A1.7 was further analysed by Southern hybridisation after 2, 4, 7 and 12 subcultures in liquid media supplemented with the respective Cd concentrations (Fig. 4.3). Genomic DNA was first hybridised to radiolabelled *smtA*, followed by hybridisation to *psaE*. All Southern blots were done with gene copy number reconstruction's but data has only been shown in Fig. 4.1.

smtA hybridisation of genomic DNA isolated after two subcultures shows increased hybridisation of *smtA*, relative to A0, to the DNA from Cd-tolerant cell lines A1.3 and A1.7 (Fig. 4.3A, panel 2). In addition, unique restriction fragments both larger and smaller than that detected in A0 were observed in DNA from A1.3. Moreover, only larger unique restriction fragments in addition to that observed in A0, were observed in DNA from Cd-tolerant line A1.7 (Fig. 4.3A, panel 2).

After 4 and 12 subcultures, an increased hybridisation of *smtA*, relative to that observed in A0, was observed in the DNA isolated from all the Cd-tolerant cell lines (Fig. 4.3A, panels 4, 12). Additionally, unique larger and smaller restriction fragments to that observed in A0, were repeatedly detected in DNA from the Cd-tolerant lines. The pattern of additional unique restriction fragments was similar to that observed for DNA isolated after two subcultures. However, in DNA isolated after seven subcultures a similar restriction pattern to A0 also occurs in all the Cd-tolerant lines (Fig. 4.3A, panel 7). Nevertheless, prolonged exposure to x-ray sensitive film reveals both larger and smaller additional *smtA* restriction fragments in the tolerant lines, although these fragments were relatively less abundant.

No evidence of any rearrangement was observed for DNA isolated from A0 at any stage of DNA isolation, when hybridised to *smtA*. Southern blots of genomic DNA isolated from the four lines after two, four, seven and twelve subcultures, were re-hybridised to the control gene, *psaE* (Fig. 4.3B). Furthermore, no indication of any

rearrangement was observed for DNA isolated from any of the lines (A0, A0.8, A1.3, A1.7), at any stage of sub-culturing, when probed with *psaE*. In some Southern blots slight variation in *psaE* hybridisation (between DNA isolated from A0 and that from Cd-tolerant lines) was observed (e.g. Fig. 4.3B, panel 4). This slight difference coincides with slight differences in the amount of genomic DNA loaded onto gels. Additionally, the magnitude of difference in *psaE* hybridisation was much less than that observed for *smtA* hybridisation.

4.34 Analysis of genomic DNA isolated from A1.3

The appearance of unique larger and smaller *smtA* restriction fragments could potentially be an effect generated from anomalous, and possibly incomplete, restriction. To substantiate whether or not the appearance of additional restriction fragments in A1.3 were reproducible, genomic DNA isolated from A1.3 after two subcultures was independently restricted three times with *Sal*I restriction endonuclease (Fig. 4.4A, B & C). A similar banding pattern of larger and smaller *smtA* restriction fragments to that observed previously (Fig. 4.3A, panel 2), was obtained in the three restrictions.

4.4 Summary

The results can be summarised as follows:

1) Cd-tolerant cell lines (A0.8, A1.3, A1.7) of *Synechococcus* PCC 6301 were developed by step-wise selection to increasing concentrations of Cd.

2) The Cd-tolerant cell lines were phenotypically distinct from the non-selected cell line A0.

3) An increase in hybridisation to *smtA* was observed in *Sal*I restricted genomic DNA isolated from Cd-tolerant cell line A1.7. Gene copy number reconstruction's suggested an increase of ca. four-fold.

4) Hybridisation to a control gene, *psaE*, suggested a slightly less abundance, relative to that in A0, in genomic DNA isolated from A1.7.

5) Genomic DNA isolated from the Cd-tolerant cell lines and the non-selected cell line was analysed after 2, 4, 7 and 12 subcultures.

6) An increase in hybridisation, relative to that in A0, was observed in all the Cd-tolerant cell lines.

7) Additional unique larger and smaller restriction fragments, relative to that in A0, were repeatedly observed in A1.3 and A1.7.

8) After 7 subcultures, a similar restriction pattern to A0 was observed in DNA from all the Cd-tolerant cell lines, but prolonged exposure reveal additional restriction fragments in both A1.3 and A1.7.

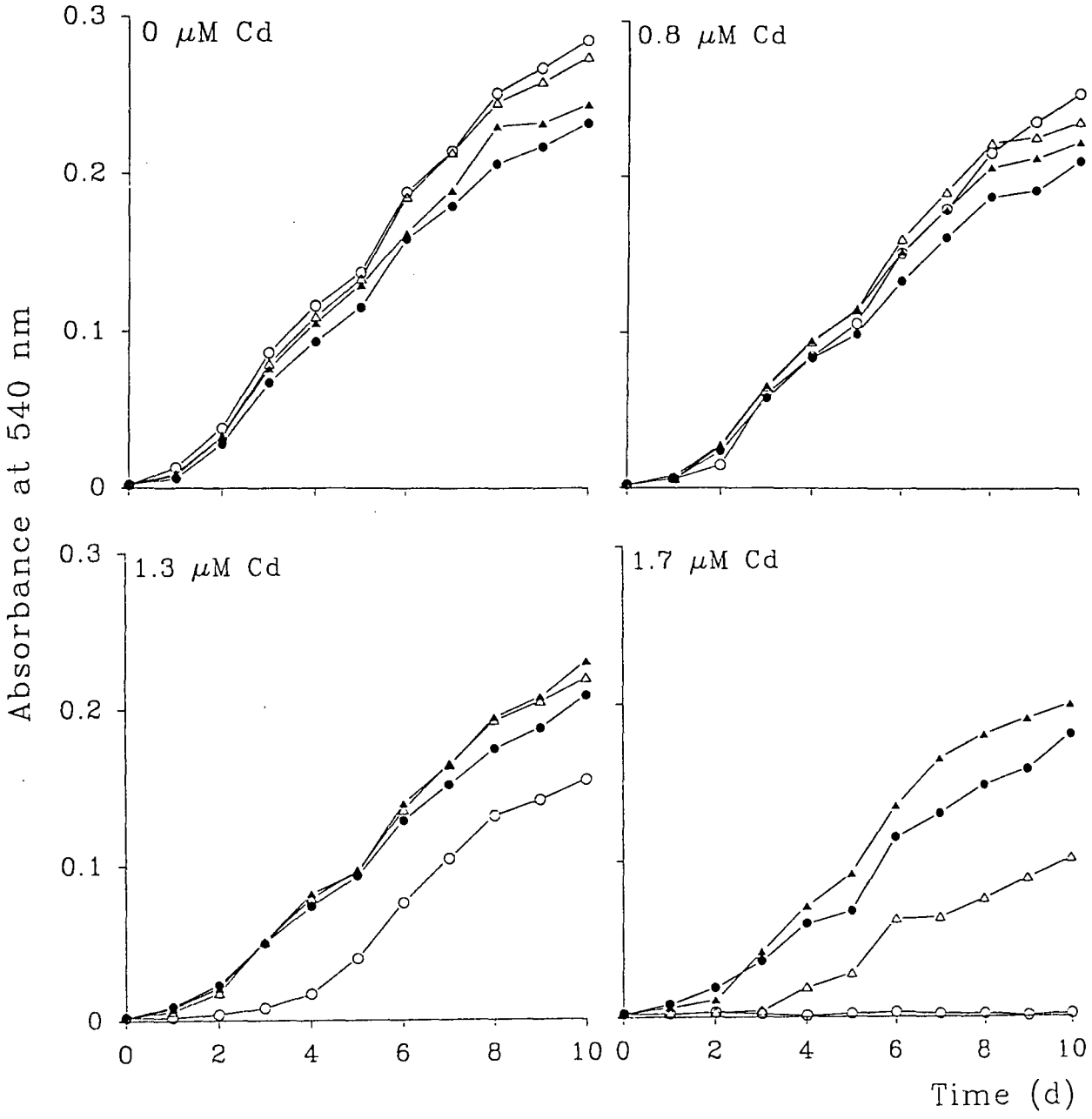
9) Hybridisation to a control gene, *psaE*, did not suggest any increase in hybridisation or appearance of unique restriction fragments. No unique restriction fragments were observed in A0 during selection or subsequent maintenance.

10) A similar pattern of restriction fragments was observed in three independent *SalI* restrictions of DNA from A1.3 after 2 subcultures.

From the results described above, it was concluded that step-wise selection Cd-tolerant cell lines of *Synechococcus* PCC 6301 are phenotypically different from the non-selected line. Furthermore, an increase in hybridisation intensity to *smtA* and unique additional restriction fragments was observed in Cd-tolerant lines. An equivalent response of increase in hybridisation and additional unique restriction fragments was not observed when hybridised to another gene, *psaE*. This suggests that in step-wise selected Cd-tolerant cell lines of *Synechococcus* PCC 6301 there is amplification and rearrangement of the *smt* locus.

Figure 4.1. Growth of non-selected A0 (o-o) and Cd-tolerant lines (Δ - Δ A0.8; ▲ - ▲ A1.3 and ●-● A1.7) in different concentrations of Cd. Panel *A*: growth in 0 μM Cd, panel *B*: growth in 0.8 μM Cd, panel *C*: growth in 1.3 μM Cd, and panel *D*: growth in 1.7 μM Cd.

Growth in different Cd concentrations



Cd-tolerant lines: ○ A0, △ A0.8, ▲ A1.3, ● A1.7

Figure 4.2. Analysis of genomic DNA isolated from A0 and Cd-tolerant lines. Panel *A*: hybridisation to *smtA*, panel *B*: hybridisation to *psaE*, and panel *C*: visualisation of ethidium bromide stained DNA. *Sal*I restricted genomic DNA was isolated from: lane 1, A0; lane 2, A0.8; lane 3, A1.3 and lane 4, A1.7. Lanes 5-11 contain standard amounts of plasmid (pJHNR11) DNA, equivalent to 1, 2, 3, 4, 5, 6 and 8 gene copies, respectively. Panel *B*: shows two bands: upper band corresponding to *smtA*, and the lower band corresponding to *psaE*.

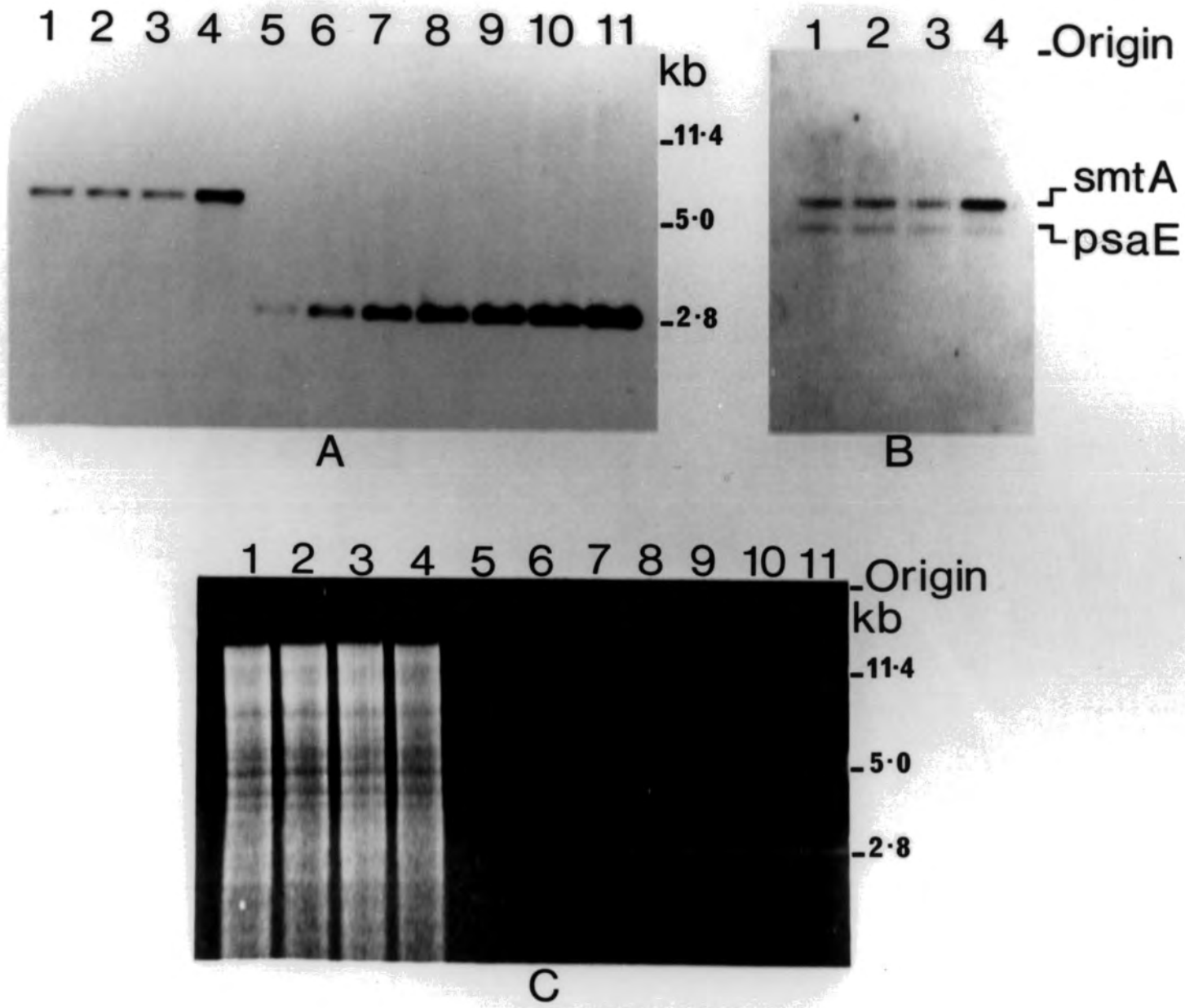

1 2 3 4 5 6 7 8 9 10 11
kb
11·4
5·0
2·8
A
1 2 3 4
Origin
smtA
psaE
B
1 2 3 4 5 6 7 8 9 10 11
Origin
kb
11·4
5·0
2·8
C

Figure 4.3. Analysis of genomic DNA isolated from A0 and Cd-tolerant lines (A0.8, A1.3 and A1.7) after 2, 4, 7 and 12 subcultures. Equivalent amounts of DNA isolated from: lane 1, A0; and Cd-tolerant lines - lane 2, A0.8; lane 3, A1.3 and lane 4, A1.7; was restricted with *Sal*I. Panel *A*: hybridisation to *smtA*, panel *B*: hybridisation to *psaE*.

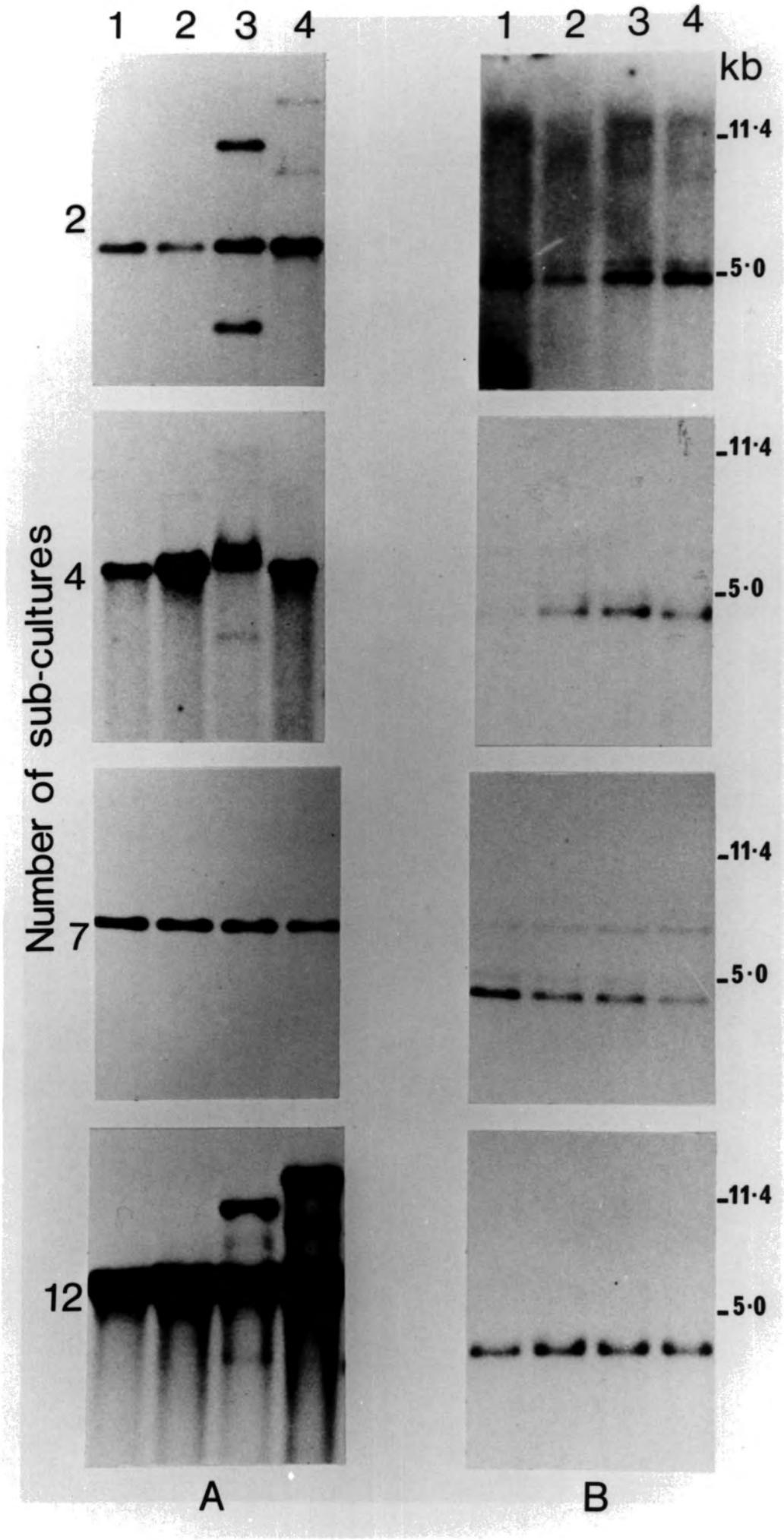
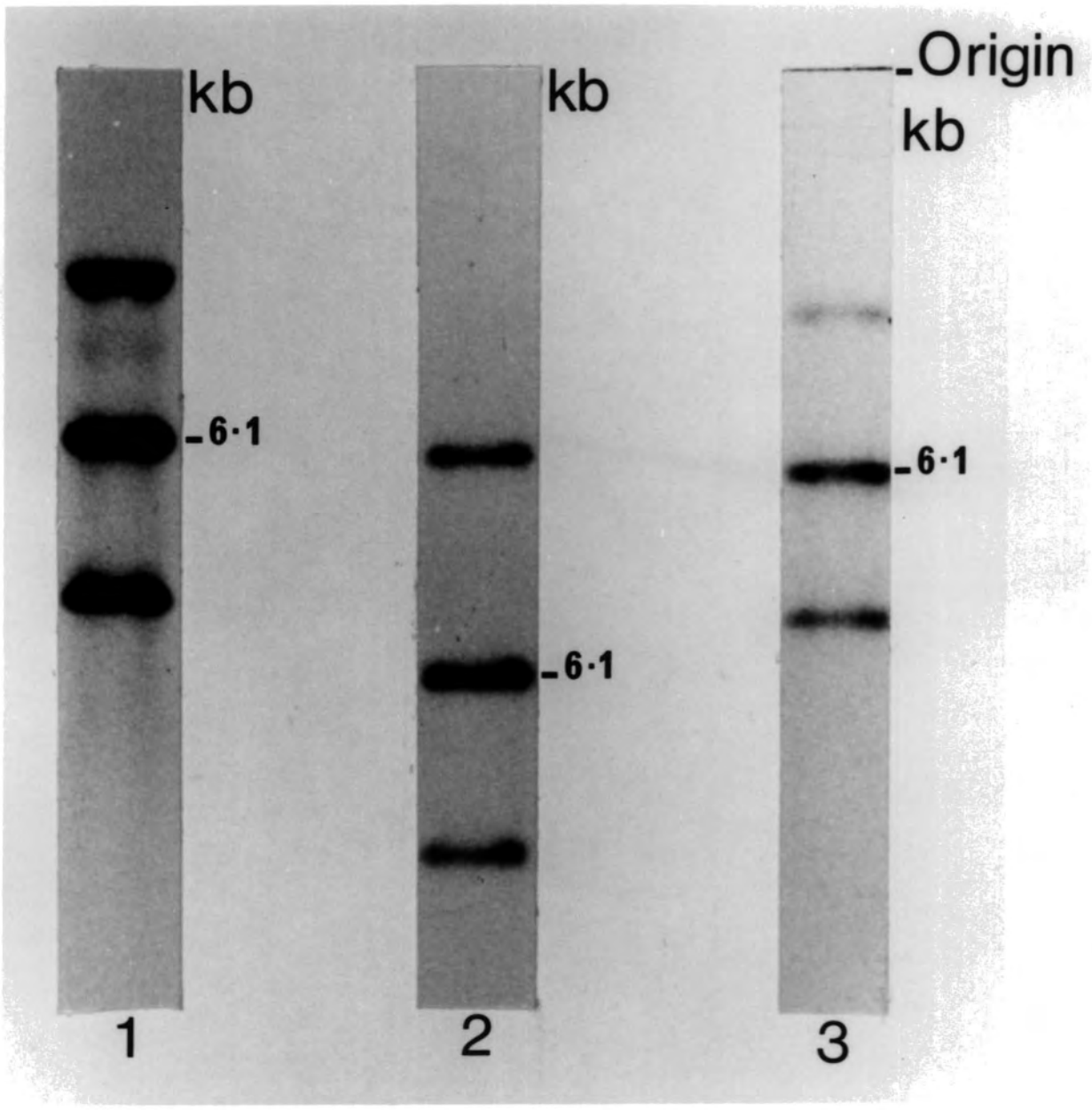

1 2 3 4
1 2 3 4
kb
11·4
5·0
2
4
7
12
Number of sub-cultures
A
B

Figure 4.4. Three independent restrictions of genomic DNA isolated from Cd-tolerant line A1.3 after 2 subcultures and hybridisation to *smtA*.


kb
6·1
1
kb
6·1
2
Origin
kb
6·1
3

Chapter 5

STEP-WISE SELECTION OF Cd-TOLERANT CELL LINES FROM A CLONAL *Synechococcus* PCC 6301 CULTURE

5.1 Introduction

The results described in Chapter 4 provide evidence for amplification and rearrangement of the *smt* locus in Cd-tolerant cell lines of *Synechococcus* PCC 6301. This may possibly be due to positive selection of mutants from a genetically diverse culture. It was thus thought necessary to repeat selection of Cd-tolerant cell lines from a culture of *Synechococcus* PCC 6301 to minimise initial genetic variability.

5.2 Materials and Methods

5.21 Step-wise selection of Cd-tolerant cell lines

A culture of *Synechococcus* PCC 6301 generated from a single plated colony (to minimise initial genetic variability) was step-wise adapted for selection of Cd-tolerant cell lines. The re-selection protocol was essentially the same as described in Section 4.21. The inoculum and harvesting cell densities for cultures were 2×10^5 and 2×10^8 cells ml^{-1}, respectively, throughout selection and subsequent maintenance of non-selected and Cd-tolerant cell lines.

5.3 Results

Upon step-wise selection of a clonal culture of *Synechococcus* PCC 6301 (C0), four lines tolerant to 1.4 (C1.4), 1.8 (C1.8), 2.6 (C2.6) and 3.2 (C3.2) μM Cd were obtained. Coincident with the development of tolerance to increasing Cd concentrations, an increase in growth lag was observed. This increased growth lag decreased upon subsequent maintenance of Cd-tolerant lines in medium supplemented with the respective Cd concentration. Figure 5.1 shows the step-wise selection of Cd-tolerant lines and subsequent maintenance of C3.2, the line tolerant to the highest concentration of Cd. The

non-selected line (C0) and the four Cd-tolerant lines (C1.4, C1.8, C2.6 and C3.2) were maintained in the respective Cd concentrations and analysed as follows.

5.31 Analysis of genomic DNA

Ten micrograms of genomic DNA, from each of the non-selected (C0) and Cd-tolerant cell lines (C1.4, C1.8, C2.6 and C3.2) after one, two, three and four subcultures, was restricted with *Sal*I and analysed by hybridisation to radiolabelled *smtA*. All Southern blots were performed with gene copy number reconstruction's, although data have not been shown in figures.

After the first subculture, an increase in *smtA* hybridisation, relative to C0, was observed in DNA isolated from C1.8, C2.6 and C3.2 (Fig. 5.2A, panel 1). Additionally, unique larger and smaller *smtA* restriction fragments (ca. 11.0 and 5.45 kb), to that observed in C0 (5.8 kb), were observed in DNA isolated from C1.8, C2.6 and C3.2. No *Sal*I *smtA* restriction fragments equivalent to that observed in DNA isolated from C0 (5.8 kb) were observed in DNA isolated from C1.8, C2.6 and C3.2. After one subculture, DNA isolated from C1.4 exhibited a restriction pattern similar to C0. No additional *smtA* restriction fragments and no increase in hybridisation to *smtA* was observed for this line (Fig. 5.2A, panel 1).

After two, three and four subcultures, unique larger and smaller restriction fragments (ca. 11.0 and 5.45 kb) were observed in DNA isolated from all the Cd-tolerant lines (Fig. 5.2A, panels 2, 3, 4). However, in DNA isolated from the Cd-tolerant lines, no *Sal*I *smtA* restriction fragments, equivalent to that observed in C0 (5.8 kb) were observed. Furthermore, an increase in *smtA* hybridisation to DNA from all the Cd-tolerant lines, relative to that in DNA from C0, was also observed (Fig. 5.2A, panels 2, 3, 4).

The Southern blots corresponding to DNA isolated after one, two, three and four subcultures were stripped of radioactivity and re-hybridised to radiolabelled fragment of the control gene, *psaE* (Fig. 5.2B). No evidence of rearrangement in the non-selected line or any of the Cd-tolerant lines was observed during any stage of DNA isolation or subsequent maintenance of these lines. Slight differences in hybridisation of *psaE* to C0

and DNA from Cd-tolerant cell lines was observed (e.g. Fig. 5.2B, panels 1, 3). These slight differences correlated with slight differences in the amount of DNA loaded. Also, the difference in magnitude of *psaE* hybridisation between C0 and DNA from Cd-tolerant lines was much less to that observed for *smtA*.

No evidence of rearrangement was ever observed at any stage in DNA isolated from the non-selected line C0.

5.32 Effect of Cd on restriction of genomic DNA with *Sal*I

Genomic DNA preparations from Cd-tolerant lines of *Synechococcus* PCC 6301 maintained in the presence of the respective Cd concentration, may contain minute quantities of Cd which might affect restriction with *Sal*I. Alternatively, Cd might interact with DNA in cultures exposed to Cd, for e.g. by modification of endonuclease recognition site, and thus affect the end result of restriction with *Sal*I. An effect on restriction with *Sal*I could generate artefacts which might be interpreted as gene rearrangements. Therefore, *in vitro* and short term (2 h) *in vivo* effects of Cd on restriction, were investigated.

5.321 *In vitro* effects of Cd

Genomic DNA isolated from a Cd-free culture of *Synechococcus* PCC 6301 (C0) was restricted with *Sal*I restriction endonuclease. Prior to incubation, different Cd-concentrations ranging from 10^{-1} μM to 10^{-10} μM were added to the restriction reactions. A control reaction with no added Cd was also performed. Subsequently, the *Sal*I restricted genomic DNA was analysed by Southern hybridisation to radiolabelled *smtA* and *psaE* (Fig. 5.3A). The presence of Cd had no influence on restriction with *Sal*I, since similar hybridisation of *smtA* or *psaE* to DNA, restricted in either the presence or absence of added Cd, was observed.

5.322 Short term *in vivo* effects of Cd

To study the short term (2 h) *in vivo* effects of Cd on restriction with *Sal*I, a culture of C0 was grown to mid-log phase, and divided into two portions. To one portion, 3.2 μM Cd was added and incubated for 2 h, whereas, the second portion was incubated for 2 h without the addition of Cd. Genomic DNA was isolated from the Cd exposed and unexposed culture after 2 h of incubation. Ten micrograms of genomic DNA was restricted with *Sal*I and analysed by Southern hybridisation to radiolabelled *smtA* (Fig. 5.3B). There was no evidence for any effect of short term (2 h) Cd exposure, on restriction with *Sal*I, since there was no difference in hybridisation of *smtA* to DNA from cultures with or without exposure to Cd.

5.33 Analysis of genomic DNA after subsequent subculture

After five subcultures, genomic DNA from C0 and the four Cd-tolerant cell lines, was analysed by hybridisation to *smtA*. As observed previously (Fig. 5.2A), again unique larger and smaller *smtA* restriction fragments (ca. 11.0 and 5.45 kb) were evident in DNA isolated from Cd-tolerant cell lines (Fig. 5.4A). Additionally, an increase in *smtA* hybridisation, relative to C0, was also observed to the different *smtA* restriction fragments in DNA isolated from Cd-tolerant cell lines. The smaller *smtA* restriction fragments in DNA from all the Cd-tolerant were always ca. 350 bp smaller, compared to that observed in DNA from C0 (5.8 kb). Fragment equivalent to the *smtA* restriction fragment observed in C0 was not apparent in DNA from any of the Cd-tolerant lines.

5.34 Analysis of genomic DNA isolated from C0 and C3.2

Genomic DNA isolated after five subcultures was used to further characterise the observed rearrangement in Cd-tolerant cell line C3.2. Ten micrograms of DNA from C0 and C3.2 was restricted with *Hin*dIII and hybridised to radiolabelled *smtA* (Fig. 5.4B). An increase in hybridisation of *smtA* to DNA from C3.2 was observed. Only a single *Hin*dIII *smtA* restriction fragment was observed in C3.2, in contrast to two *smtA* restriction

fragments observed upon restriction with *Sal*I. However, the hybridising *smtA* restriction fragments in C3.2 was again ca. 350 bp smaller (ca. 2.45 kb), as compared to that in DNA isolated from C0 (2.8 kb).

Since *Sal*I has a recognition site within the *Hin*dIII sites flanking the *smt* locus, it was used in conjunction with *Hin*dIII to obtain a smaller restriction fragment carrying the observed rearrangement. Ten micrograms of genomic DNA from C0 and C3.2 was restricted with *Sal*I followed by *Hin*dIII and hybridised to radiolabelled *smtA* (Fig. 5.4C). A single *smtA* restriction fragment was again observed in DNA isolated from C3.2. The *smtA* restriction fragment in C3.2 was consistently ca. 350 bp smaller (ca. 1.45 kb), in comparison to that observed in DNA isolated from C0 (1.8 kb) (Fig. 5.4C).

5.4 Summary

The results can be summarised as follows:

1) Cd-tolerant cell lines (C1.4, C1.8, C2.6, C3.2) of *Synechococcus* PCC 6301 were developed by step-wise selection of a culture developed from a single plated colony.

2) Coincident with increase in hybridisation, an increase in growth lag was observed. The growth lag decreased upon subsequent maintenance of Cd-tolerant cell lines in the respective Cd concentration.

3) An increase in hybridisation to *smtA*, relative to that in C0, was observed in *Sal*I restricted C1.8, C2.6 and C3.2 DNA after 1 subculture in the presence of Cd. In C1.4, increase in hybridisation was observed only after 2 subcultures.

4) Unique *smtA* restriction fragments, both larger and smaller to that in C0, were observed in all Cd-tolerant lines after 1 subculture, except C1.4 where additional restriction fragments were apparent only after 2 subcultures.

5) In C1.8, C2.6 and C3.2, restriction fragments corresponding to that observed in C0 (ca. 5.8 kb) were absent. Indeed, the smaller restriction fragment in Cd-tolerant lines was ca. 350 bp smaller than that in C0.

6) No increase in hybridisation intensity or the appearance of unique restriction fragments was apparent on hybridisation of genomic DNA from C0, C1.4, C1.8, C2.6 and C3.2 to the control gene, *psaE*.

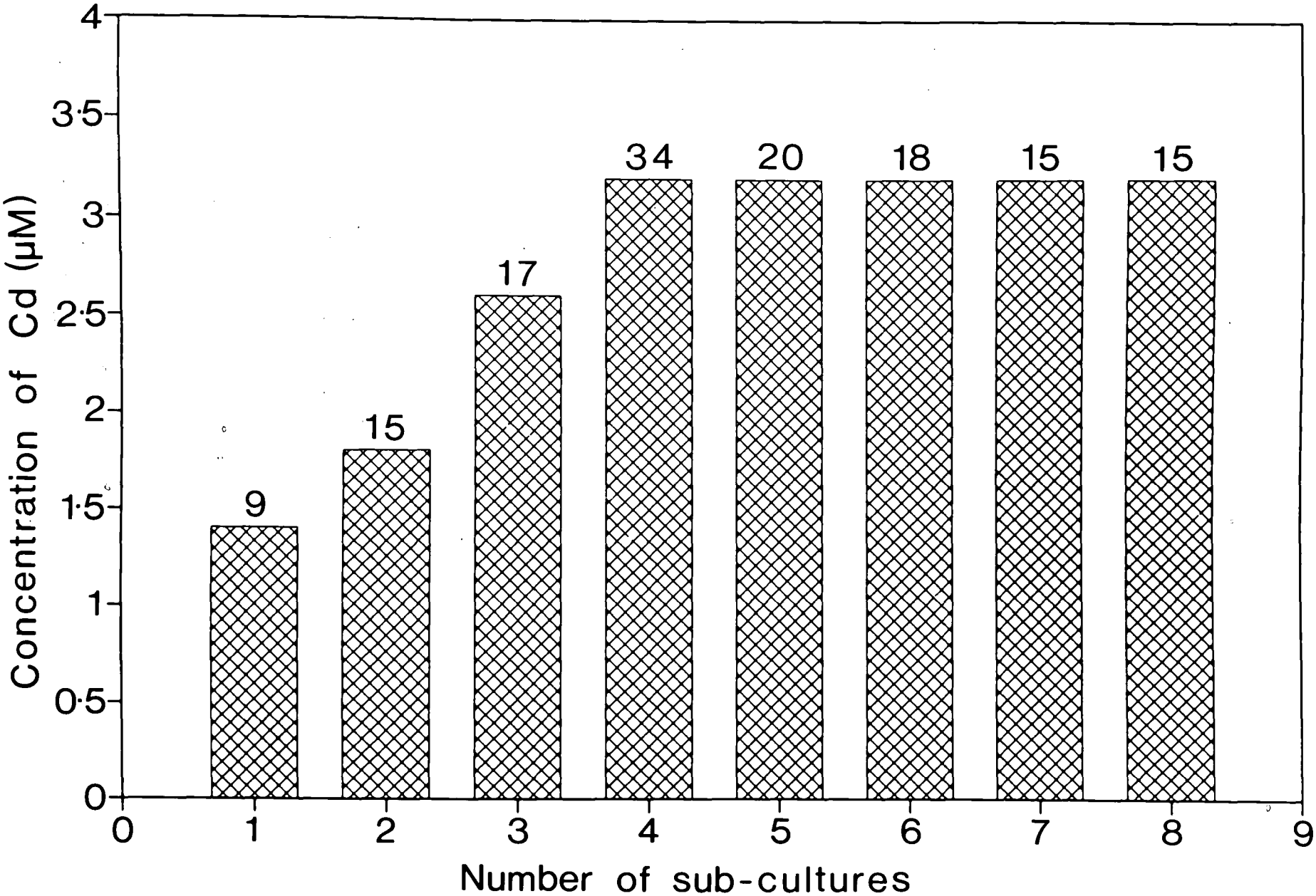
7) Increase in hybridisation or additional restriction fragments was never detected in C0 during selection or subsequent maintenance.

8) The presence of Cd did not affect restriction of genomic DNA with *Sal*I, under *in vitro* or short term (2 h) *in vivo* conditions.

9) The apparent rearrangement was obtained as a single hybridising fragment on *Hin*dIII-*Sal*I restriction of genomic DNA from C3.2. The hybridising fragment was ca. 350 bp smaller than that observed in C0.

These observations suggest that the initial growth lag in Cd-tolerant cell lines selected by step-wise selection decreases following subsequent maintenance in media supplemented with Cd. In Cd-tolerant cell lines an increase in hybridisation, suggestive of amplification, and unique restriction fragments were observed. However, only a single restriction fragment, ca. 350 bp smaller than that in C0, was observed in the Cd-tolerant line C3.2. After 5 subcultures in the presence of Cd no restriction fragment, equivalent in size to that in C0, was observed in any of the Cd-tolerant lines. There was no evidence of amplification or rearrangement of another gene, *psaE*, in Cd-tolerant lines. Furthermore, there was no effect of Cd on restriction of genomic DNA under *in vitro* or short-term *in vivo* conditions. Therefore, it can be concluded that in step-wise selected Cd-tolerant cell lines of *Synechococcus* PCC 6301 there is specific amplification of *smtA* and rearrangement of the *smt* locus.

Figure 5.1. Step-wise selection of a "clonal" culture of *Synechococcus* PCC 6301, C0, and subsequent maintenance of the Cd-tolerant line C3.2. Each bar represents the maximum concentration of Cd at which growth was observed after subculture from previous Cd-concentration. After 4 subcultures, the maintenance of Cd-tolerant line C3.2 is presented. Numbers over each bar represent the number of days taken for the culture growing in the respective Cd concentration to reach a final cell density of 2×10^8 cells ml^{-1}, after subculture.


Concentration of Cd (µM)
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3·5
3
2·5
2
1·5
1
0·5
0
9
15
17
34
20
18
15
15
0
1
2
3
4
5
6
7
8
9
Number of sub-cultures

Figure 5.2. Analysis of genomic DNA isolated from C0 and Cd-tolerant lines (C1.4, C1.8, C2.6 and C3.2) after 1, 2, 3 and 4 subcultures. Equivalent amounts of genomic DNA isolated from: lane 1, C0; and Cd-tolerant lines - lane 2, C1.4; lane 3, C1.8; lane 4, C2.6 and lane 5, C3.2; was restricted with *Sal*I. Panel *A*: hybridisation to *smtA*, panel *B*: hybridisation to *psaE*.

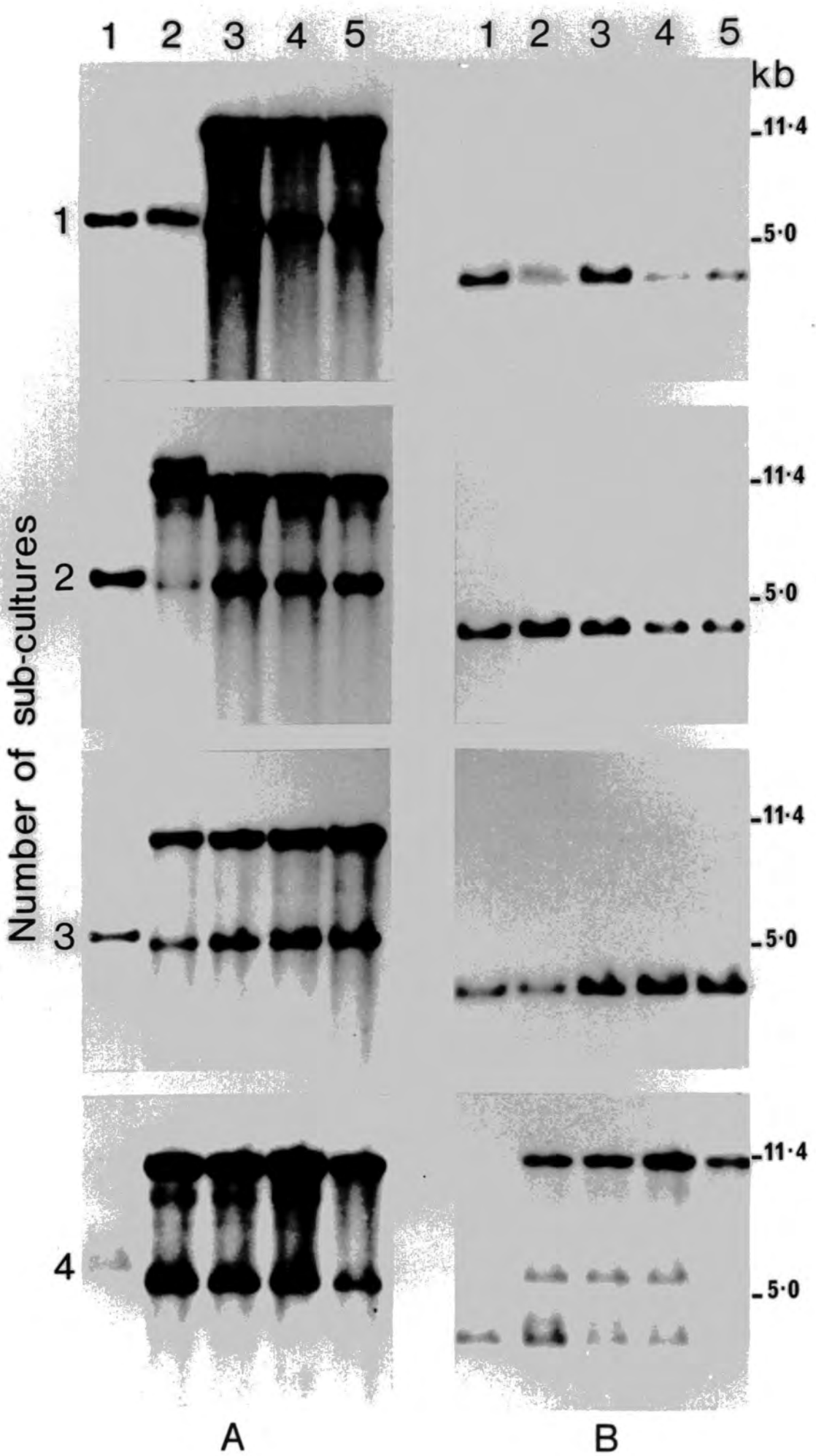

1 2 3 4 5
1 2 3 4 5
kb
11·4
5·0
11·4
5·0
11·4
5·0
11·4
5·0
Number of sub-cultures
1
2
3
4
A
B

Figure 5.3. Analysis of *in vitro* and *in vivo* effects of Cd on restriction of genomic DNA with *Sal*I. Panel *A*: *In vitro* effects of Cd on restriction: hybridisation with *smtA* and *psaE* after *Sal*I restriction of genomic DNA in the presence of different Cd concentrations - lane 1, 0 μM; lane 2, 10^{-10} μM; lane 3, 10^{-9} μM; lane 4, 10^{-8} μM; lane 5, 10^{-7} μM; lane 6, 10^{-6} μM; lane 7, 10^{-5} μM; lane 8, 10^{-4} μM; lane 9, 10^{-3} μM; lane 10, 10^{-2} μM; lane 11, 10^{-1} μM. Panel *B*: Short-term *in vivo* effects of Cd on restriction: hybridisation with *smtA* after *Sal*I restriction of genomic DNA isolated from non-exposed (lane 1) and 3.2 μM Cd exposed (2 h) culture (lane 2).

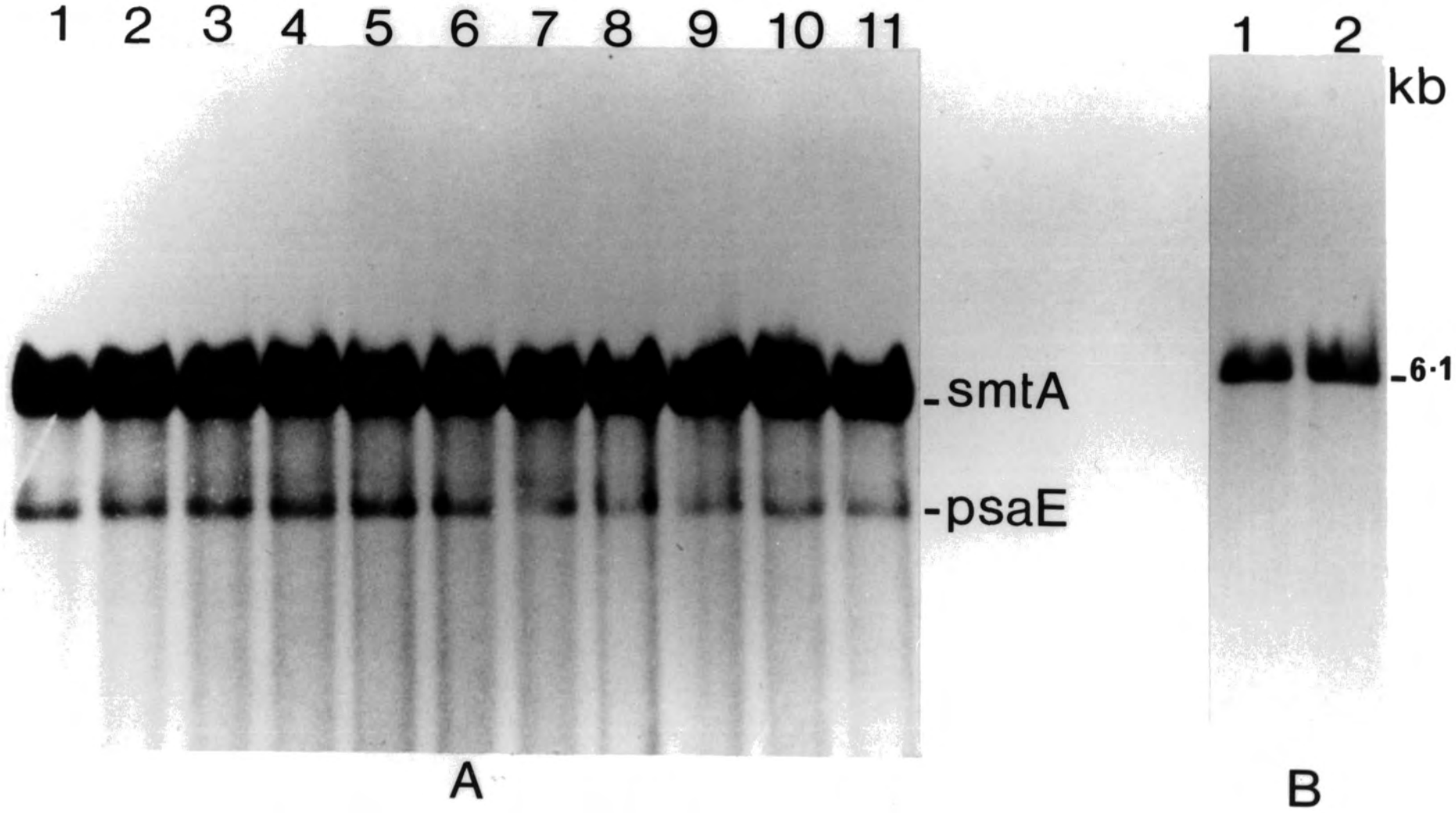
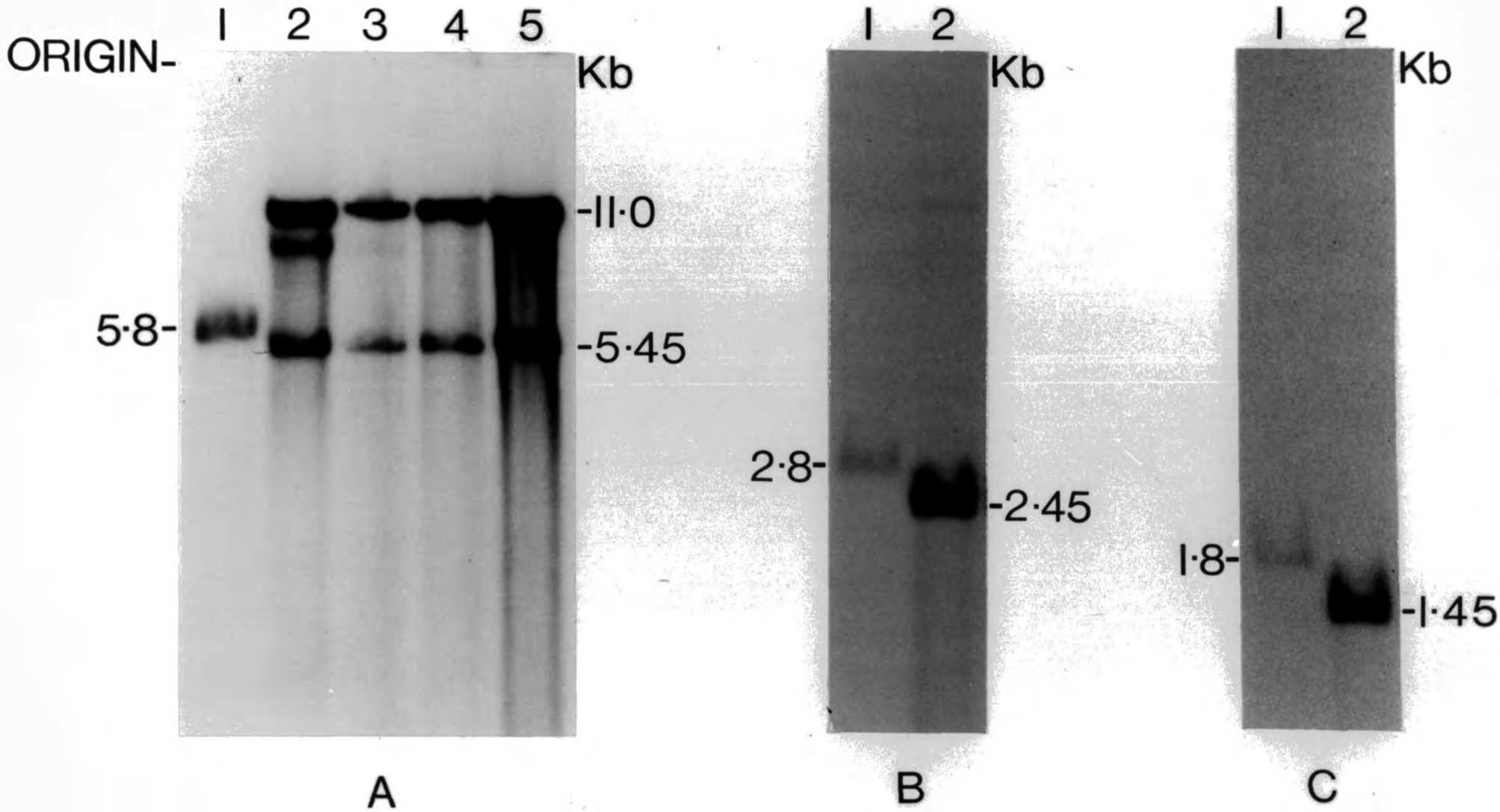

1 2 3 4 5 6 7 8 9 10 11
-smtA
-psaE
A
1 2
kb
-6·1
B

Figure 5.4. Analysis of *Sal*I restricted genomic DNA isolated from: Panel *A*: lane 1, non-selected line C0; and Cd-tolerant lines: lane 2, C1.4; lane 3, C1.8; lane 4, C2.6 and lane 5, C3.2, after 5 subcultures in presence of the respective Cd concentrations. Panel *B*: *Hin*dIII, and panel *C*: *Hin*dIII-*Sal*I restricted genomic DNA from: lane 1, C0 and lane 2, C3.2.


ORIGIN-
1 2 3 4 5
Kb
-11·0
5·8-
-5·45
A
1 2
Kb
2·8-
-2·45
B
1 2
Kb
1·8-
-1·45
C

Chapter 6

CHARACTERISATION OF REARRANGEMENT

6.1 Introduction

The prokaryotic metallothionein gene, *smtA*, of *Synechococcus* PCC 7942 was isolated by Huckle *et al.* (in press) from a size-fractionated genomic library and its sequence determined at an intermediate stage of the research presented here. The sequence contains a divergently transcribed open reading frame upstream of *smtA*, and has been designated *smtB*. *smtB* encodes a protein of 122 amino acids and has similarity to certain known regulators (Section 1.7). The data presented so far (Chapters 4, 5) have provided evidence for amplification of the prokaryotic metallothionein gene, *smtA*, and rearrangement of the *smt* locus. The smaller and larger restriction fragments observed could be attributed either to deletion in the flanking regions of *smtA*, integration of *smtA* into another region of the chromosome or point mutations leading to loss, or acquisition of novel restriction sites. The observed rearrangement (resulting in restriction fragments smaller by ca. 350 bp in Cd-tolerant line C3.2) has subsequently been observed on a small *Hin*dIII-*Sal*I restriction fragment of ca. 1.45 kb (Section 5.34). Characterisation of the observed rearrangement may provide further insight into its relationship to Cd-tolerance.

6.2 Materials and methods

6.21 Cloning of the altered *smt* locus from Cd-tolerant cell line C3.2

A size-fractionated genomic DNA library from the Cd-tolerant cell line C3.2, was screened for transformants containing the *smt* locus. Ten micrograms of genomic DNA isolated from C3.2 was restricted with *Sal*I, and separated (two tracks) on an agarose gel. One track was cut from the gel, DNA transferred to Nylon+ membrane and hybridised, to identify *smtA* restriction fragments. Equivalent sized DNA (corresponding to the two restriction fragments) was recovered separately from the duplicate track. The recovered DNA was restricted with *Hin*dIII, ligated to *Sal*I-*Hin*dIII restricted plasmid pGEM4Z, and

used to transform *E. coli* JM101 competent cells. Transformants containing the plasmids were detected by colony hybridisation techniques and DNA recovered by alkaline lysis (Section 2.322). Plasmid sequencing was performed as described in Section 2.3214.

6.22 Localisation of the altered region by PCR

The altered region in the *smt* locus was localised by analysing the sequences flanking the *smtA* gene(s) in the two plasmid (corresponding to the DNA from the two *Sal*I fragments in C3.2). Universal M13- forward and reverse primers were used in conjunction with *smtA* N-terminal primer

(primer N, 5'GGCGGATCCCCATGACCTCAACAACCTTGGTC 3'),

directing synthesis from the 5' end of *smtA* into the 3' region, and C-terminal primer

(primer C, 5'GGCGAATTCACTACAGTCGCAGCCGGTGTGGCC 3'),

directing synthesis from the 3' end of *smtA* into the 5' region (Robinson *et al.*, 1990). The reactions were subjected to 20 cycles of the following series of temperatures and times: denaturation 94°C for 1 min, annealing 55°C for 1 min, extension 72°C for 5 min, followed by 1 cycle of the following: denaturation 94°C for 1 min, annealing 55°C for 1 min, extension 72°C for 9 min (using a Hybaid Intelligent Heating block).

6.3 Results

6.31 Production of size-fractionated genomic library

Genomic DNA from the two *Sal*I restriction fragments was isolated, restricted, ligated to pGEM4Z and used to transform *E. coli* JM101 cells (Section 6.21). The transformants containing plasmids pAGNR12a (for the ca. 5.4 kb fragment) and pAGNR13a (for the ca. 11.0 kb fragment) were selected by *in situ* hybridisation. The plasmids were recovered by alkaline lysis and analysed as given in Sections 6.32 and 6.33.

6.32 Localisation of rearrangement

6.321 Localisation of rearrangement using PCR

The plasmid DNA recovered from pAGNR12a, pAGNR13a and pJHNR49 was analysed for sequences flanking the *smtA* gene(s), by PCR. *In vitro* amplification products would only be obtained when using M13-forward primer in conjunction with the 5' primer of *smtA*, and M13-reverse primer with the 3' primer of *smtA*. This would facilitate the localisation of the altered region within the flanking regions of *smtA*, since an alteration in either of the flanking sequences would give a different banding pattern.

Analysis of the PCR products using sets of primers mentioned above, showed that in comparison to products generated from pJHNR49 (used as control), both pAGNR12a and pAGNR13a gave identical molecular weight products with M13-forward primer and primer N (5' *smtA* primer) (Fig. 6.1). However, both pAGNR12a and pAGNR13a gave lower molecular weight products when amplified using M13-reverse primer and primer C (3' *smtA* primer) (Fig. 6.1). The amplification products in the two plasmids (pAGNR12a and pAGNR13a) were however identical in size. The rearrangement was thus localised to a 600 bp region in the 5' flanking region of *smtA*, between the *Hin*dIII site and the primer N binding site in *smtA*, in both pAGNR12a and pAGNR13a.

6.322 Localisation of rearrangement by restriction mapping

The presence or absence of certain restriction endonuclease recognition sites, was used to identify the region of rearrangement. The plasmids from pJHNR49, pAGNR12a and pAGNR13a were restricted with *Hin*dIII-*Sal*I, *Pst*I, *Sac*I and *Acc*I (Fig. 6.2).

Restriction of the plasmids with *Hin*dIII-*Sal*I released the entire cloned fragment. The cloned fragment in pAGNR12a and pAGNR13a was observed to be ca. 350 bp smaller than the fragment released from pJHNR49 (Fig. 6.2B). *Pst*I has a recognition site within the *smtA* coding region and another site upstream of *smtA*, thus releasing a fragment of 213 bp. Upon restriction with *Pst*I, the sizes of released fragments was similar, but the

remaining plasmid from pAGNR12a and pAGNR13a was smaller than pJHNR49, suggesting rearrangement within the ca. 500 bp region between the *Hin*dIII and *Pst*I sites (Fig. 6.2B).

The *Sac*I restriction site is unique at position 337 within the *Hin*dIII-*Sal*I cloned fragment, but an additional site is present in the vector. A fragment from pJHNR49 is released upon restriction with *Sac*I, whilst a linear DNA (no release of fragment) is observed on restriction of pAGNR12a and pAGNR13a (Fig. 6.2A, B). Similarly, *Acc*I has two recognition sites at positions 220 and 1776. A lack of fragment release from pAGNR12a and pAGNR13a, contrary to pJHNR49, was also observed upon restriction with *Acc*I (Fig. 6.2A, B). The high molecular weight products observed in DNA from pAGNR12a and pAGNR13a restricted with *Acc*I are possibly anomalous incomplete restriction products. The *Sac*I and *Acc*I recognition sites at positions 337 and 220 lie within the coding region of *smtB*. Loss of these recognition sites in both clones suggests that the observed rearrangement involves *smtB* and flanks the two restriction endonuclease recognition sites.

6.33 Nucleotide sequence analysis

The nucleotide sequence presented in Fig. 6.3 is the first 660 bp from the *Hin*dIII site of the *Hin*dIII-*Sal*I fragment from pJHNR49: the *smt* locus from *Synechococcus* PCC 7942 (Huckle *et al.*, in press). The nucleotide sequence of the 5' flanking region of pAGNR12a and pAGNR13a was determined, and found to be identical in both clones. However, a fragment of 352 bp was missing from within a 360 bp region between nucleotides 100-459 inclusive (positions marked in Fig. 6.3). The *smtA* 3' flanking sequence and the sequence flanking the excised fragment from the two clones, pAGNR12a and pAGNR13a, was identical to that determined for the *Synechococcus* PCC 7942 *smt* locus (Huckle *et al.*, in press). A deletion within this region causes a disruption within the *smtB* coding region, retaining the first 20 of the 122 codons and leaving the putative terminator intact.

6.34 Genomic DNA analysis

The results described above have demonstrated a deletion within *smtB*. Genomic DNA isolated from A0, A0.8, A1.3 and A1.7 after 1, 2, 4, 7 and 12 subcultures, and C0, C1.4, C1.8, C2.6 and C3.2 after 1, 2, 3, 4 and 5 subcultures was checked for the presence or absence of the excised element. Southern blots of genomic DNA previously hybridised to *smtA* and *psaE* were stripped of radioactivity and re-hybridised to a radiolabelled 187 bp *Acc*I-*Nhe*I fragment released from pJHNR49 (Fig. 6.4).

Hybridisation to genomic DNA isolated from A0, A0.8, A1.3 and A1.7, and on comparison with the observations for *smtA* probing (Fig. 4.2, 4.3), the excised element is evidently present in all the restriction fragments observed in DNA isolated from the Cd-tolerant cell lines (Fig. 6.4A-E). However, upon hybridisation to DNA isolated from lines C0, C1.4, C1.8, C2.6 and C3.2, weak hybridisation signals were observed in DNA isolated C1.8, C2.6 and C3.2 after 1, 2, 3, 4 and 5 subcultures (Fig. 6.4F-J). In DNA isolated from C1.4, the line tolerant to the lowest concentration of Cd, strong hybridisation signals were observed. After the first subculture strong hybridisation was observed in C1.4 to a restriction fragment which was the same size as that observed in DNA from C0 (Fig. 6.4F). DNA isolated after 2 subcultures showed very strong hybridisation to a restriction fragment of ca. 11 kb (Fig. 6.4G). In DNA isolated after subsequent subcultures, restriction fragments of lower molecular weight were more apparent (Fig. 6.4H-J). These results suggest that in the DNA isolated from the Cd-tolerant line C1.4, copies of the excised element are still retained, whilst, in DNA isolated from C1.8, C2.6 and C3.2, copies of the excised element are not retained. The weak hybridisation observed in these lines is probably background hybridisation due to the presence of inverted repeats/palindromic sequences.

To investigate the possibility that the background hybridisation was not due to presence of a single copy of the excised element, the DNA fragment used to probe Southern blots, was used to hybridise *Hin*dIII-*Sal*I fragments released from pAGNR12a, pAGNR13a and pJHNR49. Extremely weak hybridisation signals were again observed in pAGNR12a and pAGNR13a (Fig. 6.5), whilst strong hybridisation is observed in

pJHNR49 (Fig. 6.5), suggesting that the background hybridisation is indeed due to hybridisation to elements other than the excised element, since sequencing of plasmid DNA from pAGNR12a and pAGNR13a has demonstrated the excision of the 352 bp fragment (Section 6.33).

6.35 Analysis of genomic DNA from C0 and C3.2

The weak hybridisation observed to a radiolabelled fragment of the excised element, does not unequivocally rule out the possibility of the presence of a single copy of the excised element. It is therefore possible that reversion of the observed excision might be observed upon growth of the Cd-tolerant cell line C3.2 in media with no added Cd. Cd-tolerant cell line C3.2 and non-selected line C0 were grown in media containing no added Cd. Genomic DNA was isolated and analysed after one and three subcultures in the absence of Cd (Fig. 6.6).

Genomic DNA isolated from the two lines (C0 and C3.2) was restricted with *Sal*I and *Hin*dIII-*Sal*I restriction endonucleases, and hybridised to radiolabelled *smtA*. In DNA isolated from C3.2 after the first subculture, two restriction fragments (larger and smaller than C0) were observed on *Sal*I restriction (Fig. 6.6A), and again only a single but smaller, than that in C0, was observed on restriction with *Hin*dIII-*Sal*I (Fig. 6.6B). Additionally, isolation of genomic DNA from C3.2 after three subcultures, showed a similar pattern of restriction. Both larger and smaller restriction fragments, relative to C0, were obtained on *Sal*I restriction (Fig. 6.6C). *Hin*dIII restriction yielded a single restriction fragment which was again ca. 350 bp smaller than the fragment observed in C0 (Fig. 6.6D).

6.36 Analysis of total RNA isolated from C0 and C3.2

6.361 Expression of *smtA* following growth in the presence, absence, and following exposure to Cd

Total RNA was isolated from the non-selected line C0, and C3.2 growing in the presence of 3.2 μM Cd and after one subculture in the absence of Cd, and analysed by hybridisation to radiolabelled *smtA*.

In RNA isolated from C0, no hybridisation to *smtA* was detectable, but in RNA isolated from C3.2 a strong hybridisation signal was observed (Fig. 6.7B). The hybridisation intensity in RNA isolated from C3.2 after growth in the absence of Cd was slightly less than in RNA isolated from C3.2 growing in the presence of supplemented Cd. However, rRNA bands in ethidium-bromide stained gel (Fig. 6.7A) suggest that similar amounts of RNA from C0 and C3.2 grown in the presence of Cd, but slightly less RNA from C3.2 grown in the absence of Cd, was present (Fig. 6.7A).

Total RNA was also extracted from C0 and Cd-tolerant cell line C3.2, grown in Cd-free medium, after three and five subcultures. The cultures of C0 and C3.2 after 5 subcultures in the absence of Cd were exposed to 1.4 μM Cd for 2 h. Total RNA was extracted from C0 and C3.2 after 2 h exposure. An aliquot of the culture following 2 h exposure to Cd, was washed and transferred to fresh media containing no added Cd. Total RNA was extracted after one and two subcultures in the absence of Cd following exposure. Isolated RNA was analysed by hybridisation to radiolabelled *smtA* (Fig. 6.8).

Hybridisation to *smtA* was not detectable in RNA isolated from C0 after three and five subcultures in the absence of Cd, but strong hybridisation was observed in RNA isolated from C3.2 after three and five subcultures in the absence of Cd (Fig. 6.8B). Upon exposure to 1.4 μM Cd for 2 h, an increase in transcript abundance was detectable in RNA isolated from C0, and a greater increase, relative to that of C0, was observed in RNA isolated from C3.2 (Fig. 6.8B). Upon return of cell lines to conditions of growth with no added Cd, transcript was not detectable in RNA isolated from C0 immediately after the

first subculture, whilst transcript was detectable in C3.2 even after the second subculture (Fig. 6.8B). However, the transcript abundance in RNA isolated from C3.2 after the first and second subculture following exposure to Cd for 2 h, was less than that observed prior to Cd exposure. This effect could be attributable to slightly less amount of RNA present (Fig. 6.8A).

Visualisation of rRNA bands in ethidium-bromide bromide stained gel (Fig. 6.8A) suggests that similar amounts of RNA was loaded for C0 and C3.2 after third and fifth subculture and following exposure to Cd. However, slightly less RNA from the two lines isolated after the first and second subculture following exposure to Cd, was present (Fig. 6.8A).

6.37 Study on tolerance of C3.2 to other metals

The capacity of cell line C3.2 (initially selected for tolerance to elevated concentrations of Cd) for co-tolerance to other metals and increased tolerance to Cd was investigated. Cell line C3.2 and C0 was inoculated at an initial cell density of 1×10^6 cells ml^{-1}, into different concentrations of Cd, Zn and Cu. Cd concentrations were in the range of 0.5 to 10 μM, whilst that for Zn and Cu were in the range of 2 to 20 μM.

On incubation for 14 days increased tolerance to Cd and also capacity to tolerate elevated concentrations of Zn and Cu, relative to the non-selected cell line C0, was observed in the Cd-tolerant cell line C3.2.

	After 14 d incubation		
	Cd (μM)	Zn (μM)	Cu (μM)
C0	3.5	2.5	<2.0
C3.2	6.5	6.0	2.0

The results provide preliminary evidence that the mechanism of Cd tolerance may also be involved in conferring tolerance to elevated concentrations of Zn and Cu.

6.4 Summary

The results presented in this chapter can be summarised as follows:

1) An apparent rearrangement of the *smt* locus was isolated on a *Hin*dIII-*Sal*I fragment from size-fractionated libraries, and designated pAGNR12a (for the ca. 5.4 kb fragment) and pAGNR13a (for the ca. 11.0 kb fragment).

2) The altered region in pAGNR12a and pAGNR13a was localised by PCR and restriction mapping to a 600 bp region in the 5' flank of *smtA*.

3) Nucleotide sequence from pAGNR12a and pAGNR13a was identical and demonstrated a loss of 352 bp from within a region of 360 bp between nucleotides 100 and 459 inclusive. The *smtA* 3' flanking sequence and the sequence flanking the excised fragment from the two clones, pAGNR12a and pAGNR13a was identical to that determined for the *Synechococcus* PCC 7942 *smt* locus.

4) The excised element encoded the C terminal end of *smtB*. The excision disrupts *smtB*, but retains the first 20 of the 122 codons and leaves the putative terminator intact.

5) Strong hybridisation of genomic DNA to the excised fragment was observed in all the Cd-tolerant cell lines except lines C1.8, C2.6 and C3.2 where very weak hybridisation was apparent.

6) Weak hybridisation of the excised fragment was also observed to DNA from pAGNR12a and pAGNR13a, where nucleotide sequence has shown absence of the fragment.

7) An elevated basal level of *smtA* transcripts was observed in C3.2 grown in the absence of Cd.

8) Greater increase in *smtA* transcript abundance was observed in C3.2 following exposure to Cd, as compared to C0. The *smtA* transcript abundance rapidly reduced to near basal values in C0 following growth in absence of Cd, whilst a high basal level was observed in C3.2.

9) A rearranged pattern of restriction fragments was observed in C3.2 following growth in the absence of Cd.

10) An increased tolerance to Cd, Cu and Zn, relative to C0, was observed in C3.2.

In conclusion, by using PCR and restriction mapping it was possible to identify the region of alteration within the *smt* locus. Nucleotide sequence analysis of the genomic clones pAGNR12a and pAGNR13a further suggests that the prokaryotic MT locus, *smt*, is identical in the two rearranged fragments observed after restriction of genomic DNA from C3.2 with *Sal*I restriction endonuclease. However, comparison of the nucleotide sequence of pAGNR12a and pAGNR13a with that from pJHNR49 shows a deletion of 352 bp in the 5' flanking region of *smtA*. The excised fragment encodes the C-terminal of *smtB* (subsequently shown to be a regulator of *smtA*; Huckle *et al.*, In press). It can therefore be concluded that the deletion disrupts *smtB*, retaining the first 20 codons and the putative terminator intact. The disruption of *smtB* facilitates derepressed expression of *smtA*, and an increased basal level of *smtA* transcripts is observed.

Figure 6.1. Localisation of the altered region by PCR. Gel photograph of PCR products using: lanes 1-3, M13-reverse primer and primer C; lanes 4-6, M13-forward primer and primer N. The template was plasmid DNA isolated from: lanes 1 and 4, clone pAGNR12a from Cd-tolerant line C3.2; lanes 2 and 5, clone pJHNR49; lanes 3 and 6, clone pAGNR13a from Cd-tolerant line C3.2.

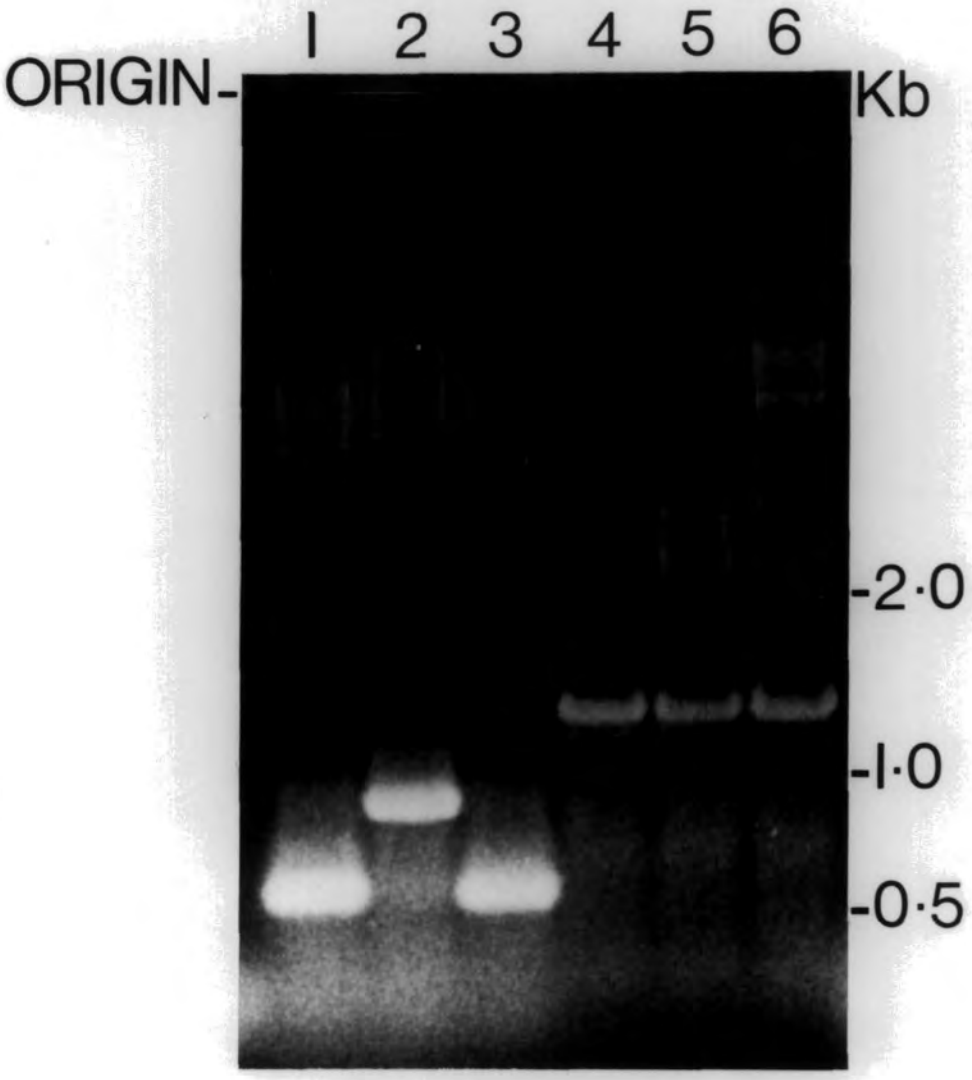

1 2 3 4 5 6
ORIGIN-
Kb
-2·0
-1·0
-0·5

Figure 6.2. Localisation of the altered region by restriction mapping. Panel *A*: visualisation of plasmid DNA isolated from: lanes 1, 4, 7 and 10, clone pAGNR12a from Cd-tolerant line C3.2; lanes 2,5,8 and 11, clone pJHNR49 and lanes 3, 6, 9 and 12, clone pAGNR13a from Cd-tolerant line C3.2; was restricted with: lanes 1-3, *Hin*dIII-*Sal*I; lanes 4-6, *Pst*I; lanes 7-9, *Sac*I and lanes 10-12, *Acc*I. Panel *B*: hybridisation to *smtA*.

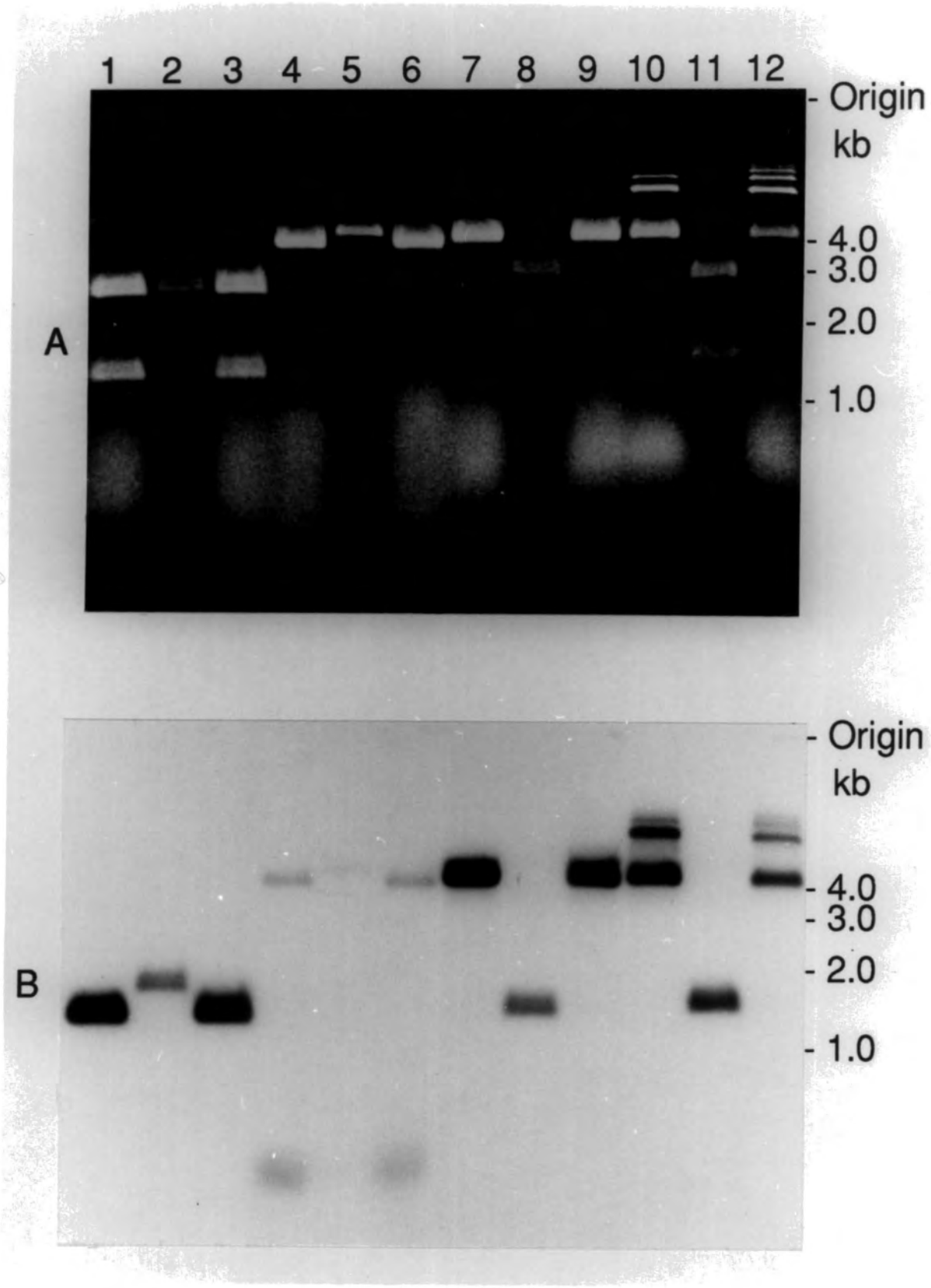

1 2 3 4 5 6 7 8 9 10 11 12
Origin
kb
4.0
3.0
2.0
1.0
A
Origin
kb
4.0
3.0
2.0
1.0
B

Figure 6.3. Nucleotide sequence of part of the *smt* locus from non-selected *Synechococcus* PCC 7942 (reproduced from Huckle *et al.*, in press) and Cd-tolerant cell line C3.2 (denoted by C3.2). The sequence from C3.2 has been aligned against the sequence from *Synechococcus* PCC 7942. In the nucleotide sequence of the Cd-tolerant cell line C3.2, 352 nucleotides from within a 360 bp region between nucleotides 100-149 inclusive, are missing. The left and right borders of excision are marked by double underline. Other features of interest are marked and labelled.

```
AAGCTTTACTACAACGAGCGCCGCTATCTACAGCAACTCGATCAAGAACGCTGCCTGAAT  C3.2
AAGCTTTACTACAACGAGCGCCGCTATCTACAGCAACTCGATCAAGAACGCTGCCTGAAT  60

CCCCAAGCATTCTTGGGCATGACAGAGCACGATGCTACTGCGATCGC               C3.2
CCCCAAGCATTCTTGGGCATGACAGAGCACGATGCTACTGCGATCGCCCCGACCACTCCC  120
Putative terminator                      Left border of deletion

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

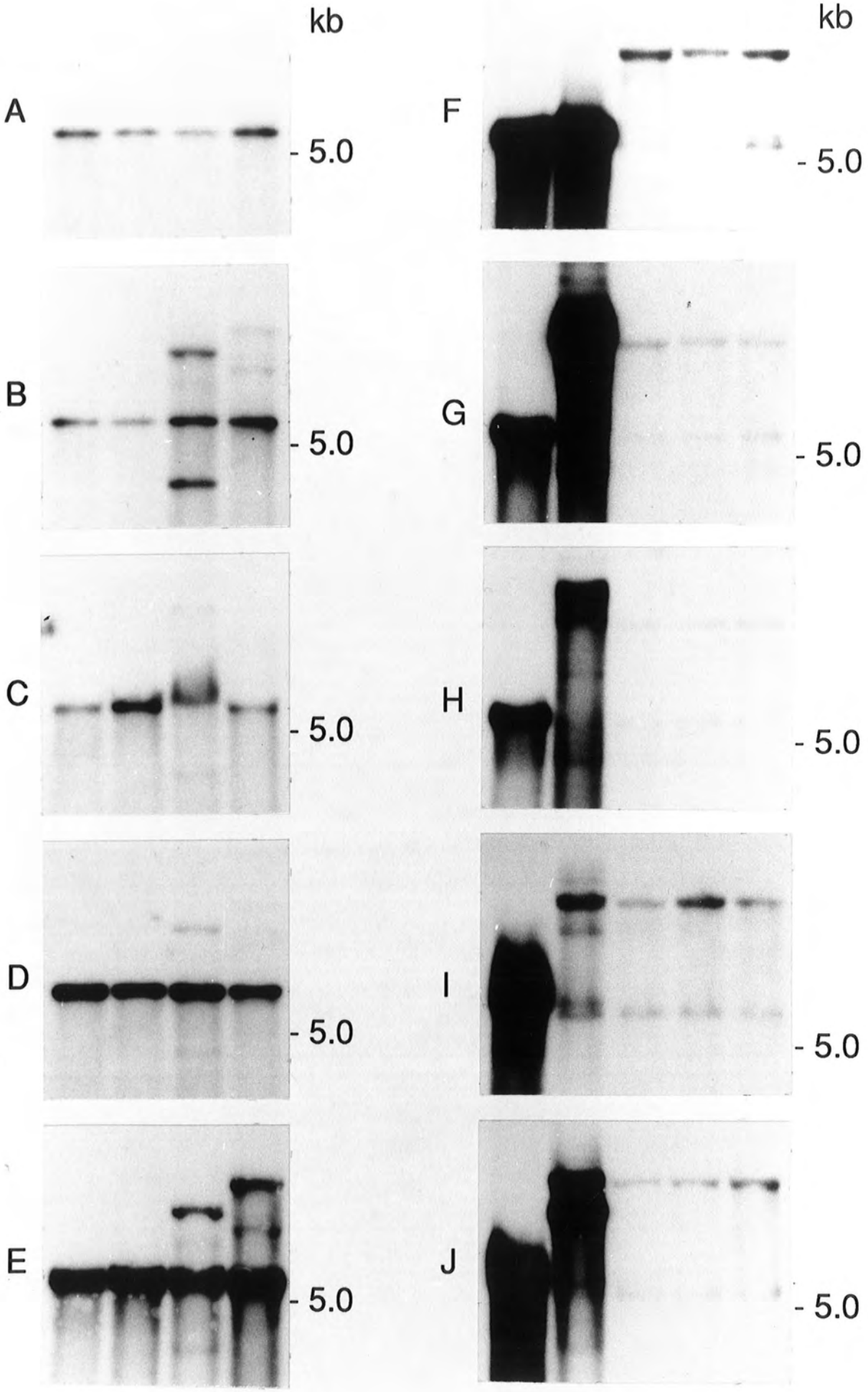
                                          CGCATGAGTCCCTTGGCAGAC  C3.2
AACTTCGGGTGCGATCGCTTGAAGCTCCGAGGCGATCGCCGCATGAGTCCCTTGGCAGAC  480
 V  E  P  A  I  A  Q  L  E  S  A  I  A  A  H  T  G  Q  C  V
                                Right border of deletion

                                smtB Transcript start  |
TACCGTCTCTCCGTCCTGCAGCACTGGTTTTGTCATGAGCCAATCACGGTTTGTCCACCC  C3.2
TACCGTCTCTCCGTCCTGCAGCACTGGTTTTGTCATGAGCCAATCACGGTTTGTCCACCC  540
 V  T  E  G  D  Q  L  V  P  K  T  M        S.D.

ACCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGATATT  C3.2
ACCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATTCAGATATT  600
  -10                       -10        |  smtA Transcript start

   S.D.          M  T  S  T  T  L  V  K  C  A  C  E  P  C  L
CAAAGGAGTTGCTGTCATGACCTCAACAACGTTGGTCAAATGCGCTTGTGAGCCCTGTCT  C3.2
CAAAGGAGTTGCTGTCATGACCTCAACAACGTTGGTCAAATGCGCTTGTGAGCCCTGTCT  660
```

Figure 6.4. Analysis of genomic DNA isolated from Panels *A-E*: non-selected line A0 and Cd-tolerant line A0.8-A1.7 after 1, 2, 4, 7 and 12 sub-cultures, and panels *F-J*: non-selected line C0 and Cd-tolerant lines C1.4, C1.8, C2.6 and C3.2 after 1, 2, 3, 4 and 5 sub-cultures. Equivalent amounts of genomic DNA transferred to nylon membranes (from figures 4, 5, 8 and 10A) were stripped of previous radioactivity and hybridised to 187 bp *Acc*I-*Nhe*I fragment (corresponding to the excised element in C3.2) released from pJHNR49.


kb
A
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B
- 5.0
C
- 5.0
D
- 5.0
E
- 5.0
kb
F
- 5.0
G
- 5.0
H
- 5.0
I
- 5.0
J
- 5.0

Figure 6.5. Analysis of plasmid DNA isolated from: lane 1, pAGNR12a; lane 2, pJHNR49; lane 3, pAGNR13a. Panel *A*: visualisation of ethidium-bromide stained DNA restriction fragments from plasmid DNA restricted with *Hin*dIII-*Sal*I. Panel *B*: hybridisation to 187 bp *Acc*I-*Nhe*I fragment (corresponding to the excised element in C3.2) released from pJHNR49.

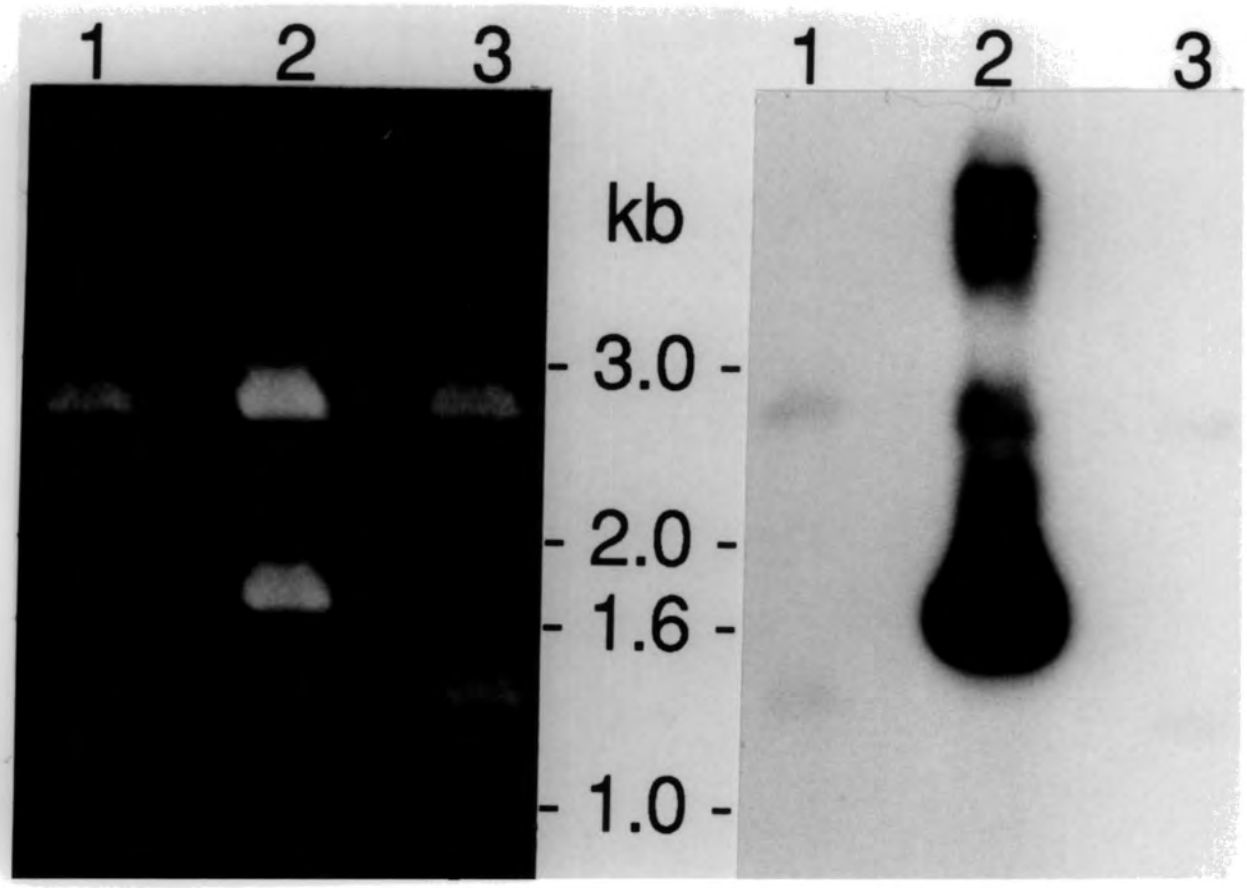
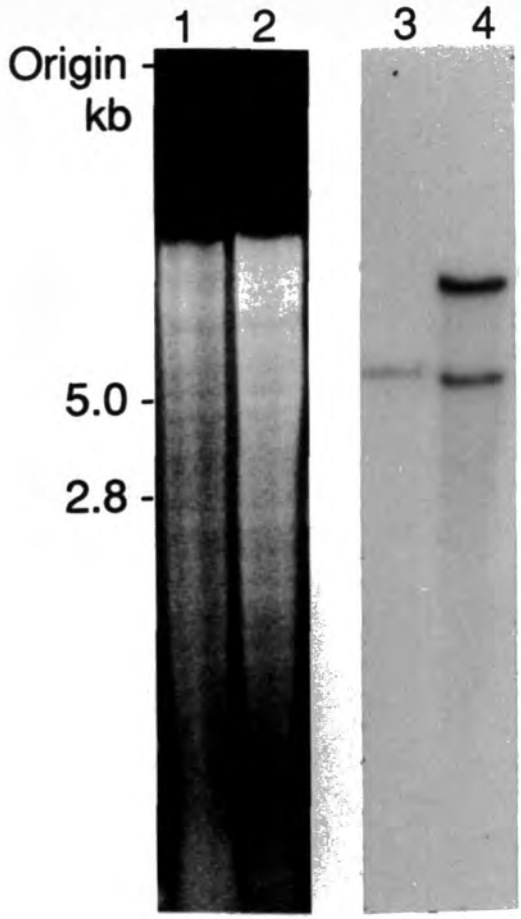

1
2
3
kb
3.0
2.0
1.6
1.0
1
2
3

Figure 6.6. Analysis of genomic DNA isolated from non-selected line C0 and Cd-tolerant line C3.2 after: Panels *A* and *B*, 1; and panels *C* and *D*, 3 subcultures in absence of Cd. Lanes 1 and 2 show visualisation of ethidium-bromide stained genomic DNA isolated from: lane 1, C0; and lane 2, C3.2. Lanes 3 and 4 show Southern hybridisation of genomic DNA isolated from: lane 3, C0; and lane 4, C3.2, and restricted with: panels *A* and *C*: *Sal*I; Panels *B* and *D*: *Hin*dIII-*Sal*I.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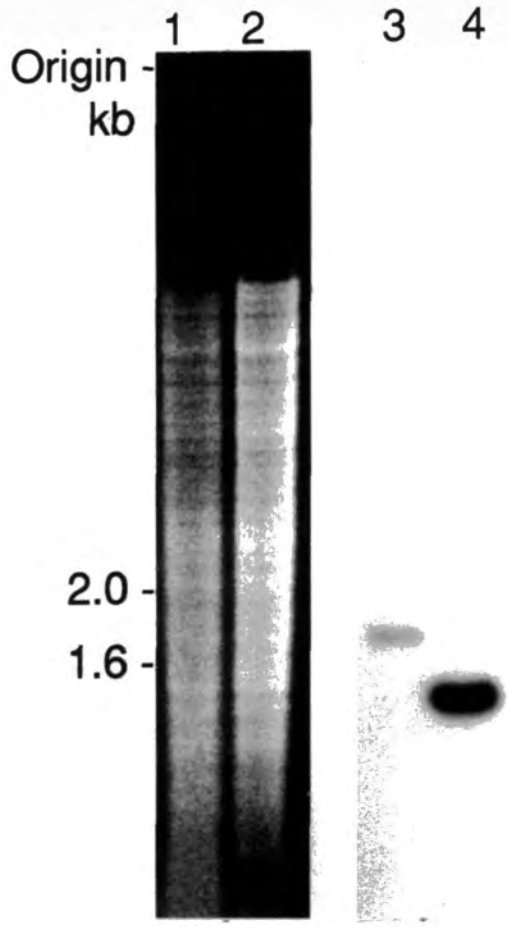
2
3
4
Origin
kb
5.0
2.8

A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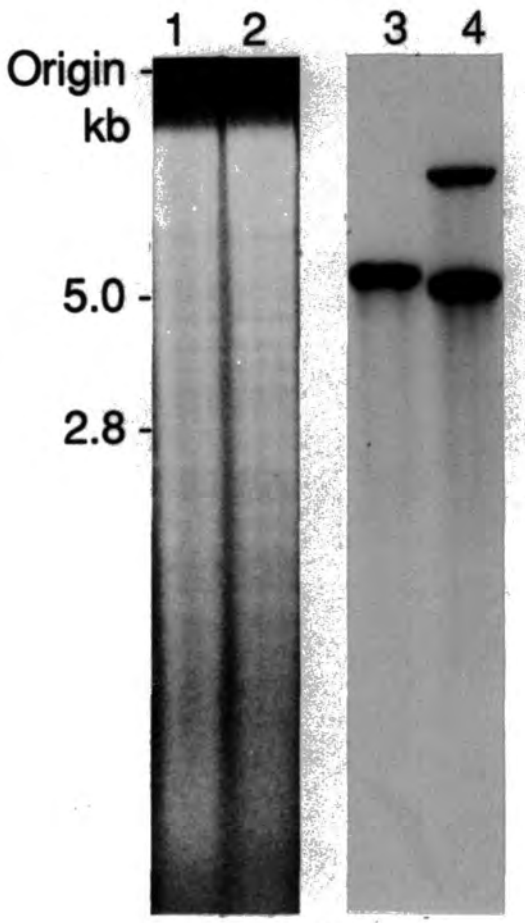
2
3
4
Origin
kb
2.0
1.6

B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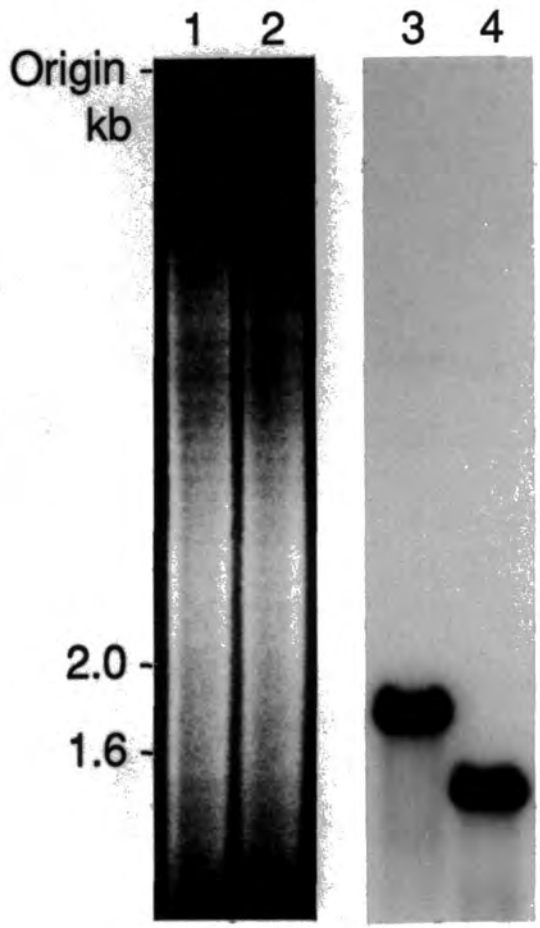
C


1
2
3
4
Origin
kb
2.0
1.6

D

Figure 6.7. Northern blot analysis of RNA isolated from: lane 1, non-selected line C0; lane 2, Cd-tolerant line C3.2 growing in the presence of Cd; lane 3, Cd-tolerant line C3.2 grown for 1 subculture in the absence of Cd. Equivalent amounts of RNA were loaded onto each track as visualised by ethidium-bromide stained rRNA bands (panel *A*), and hybridised to *smtA* (panel *B*).

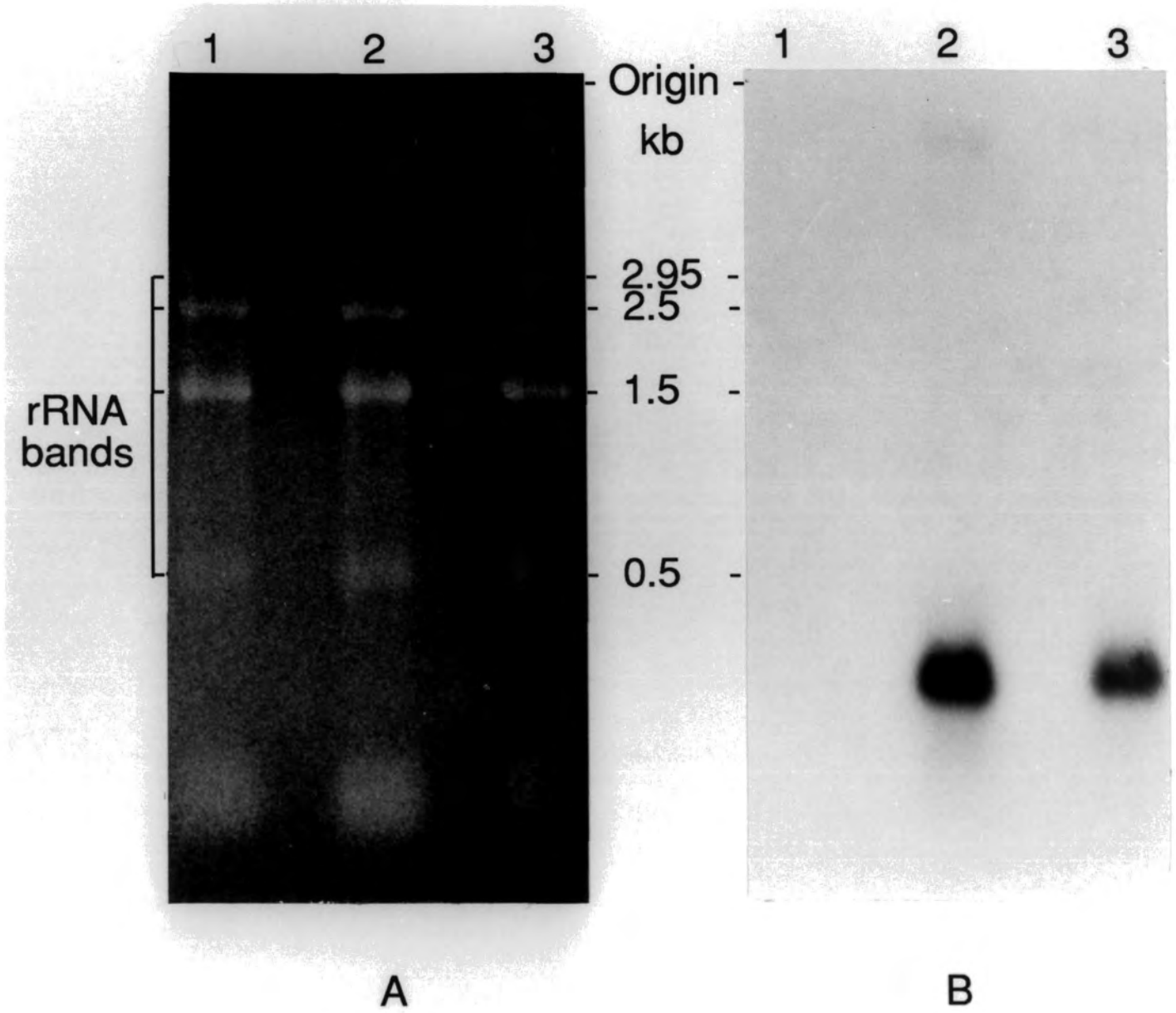
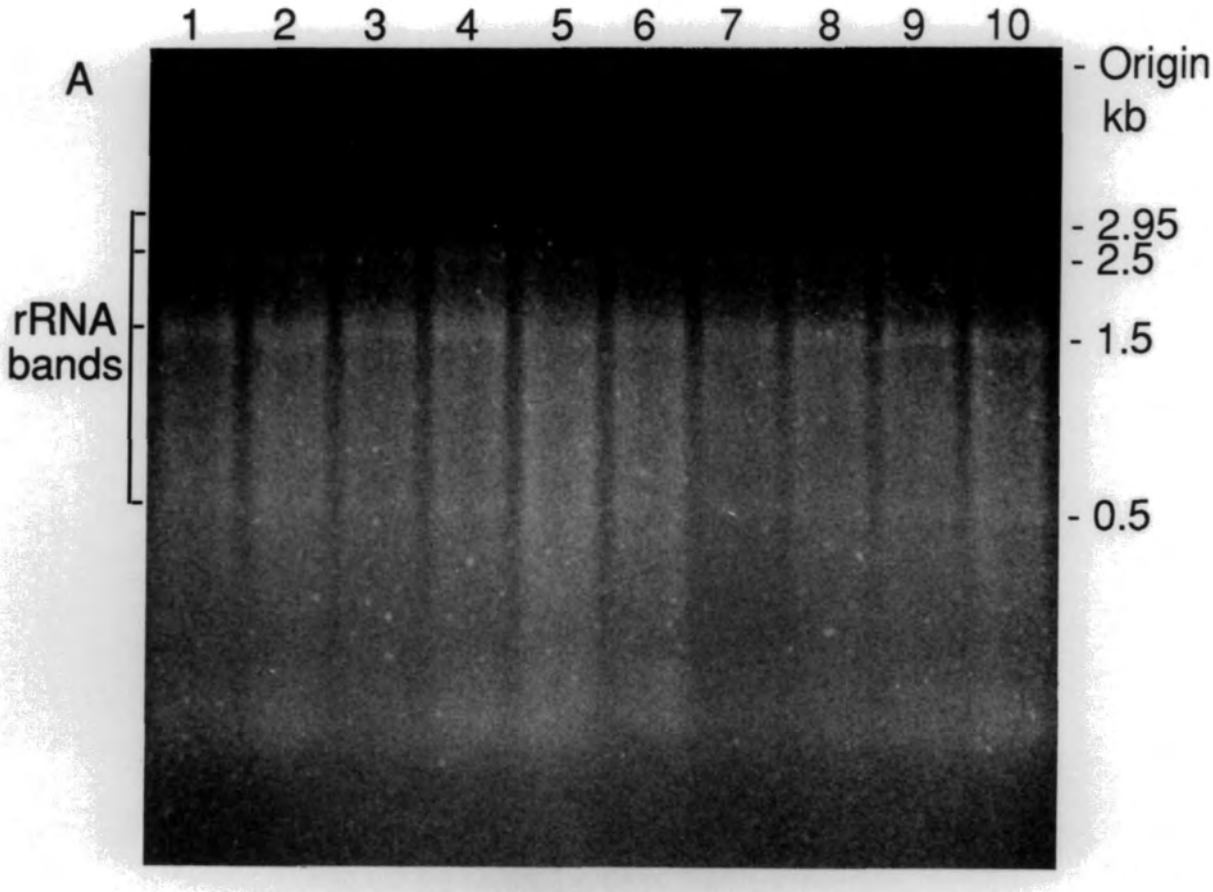

1
2
3
Origin
kb
2.95
2.5
1.5
0.5
rRNA
bands
A
1
2
3
B

Figure 6.8. Northern blot analysis of total RNA isolated from: lanes 1 and 3, non-selected line C0; lanes 2 and 4, Cd-tolerant line C3.2 grown in the absence of Cd for 3 and 5 subcultures, respectively; lane 5, C0 exposed to 1.4 μM Cd for 2 h; lane 6, C3.2 exposed to 1.4 μM Cd for 2 h; lanes 7 and 9, C0 grown in absence of Cd following exposure, for 1 and 2 subcultures, respectively; lanes 8 and 10, C3.2 grown in the absence of Cd following exposure, for 1 and 2 subcultures, respectively. Equivalent amounts of RNA was loaded onto each track as visualised by ethidium-bromide stained rRNA bands (panel *A*), and hybridised to *smtA* (panel *B*).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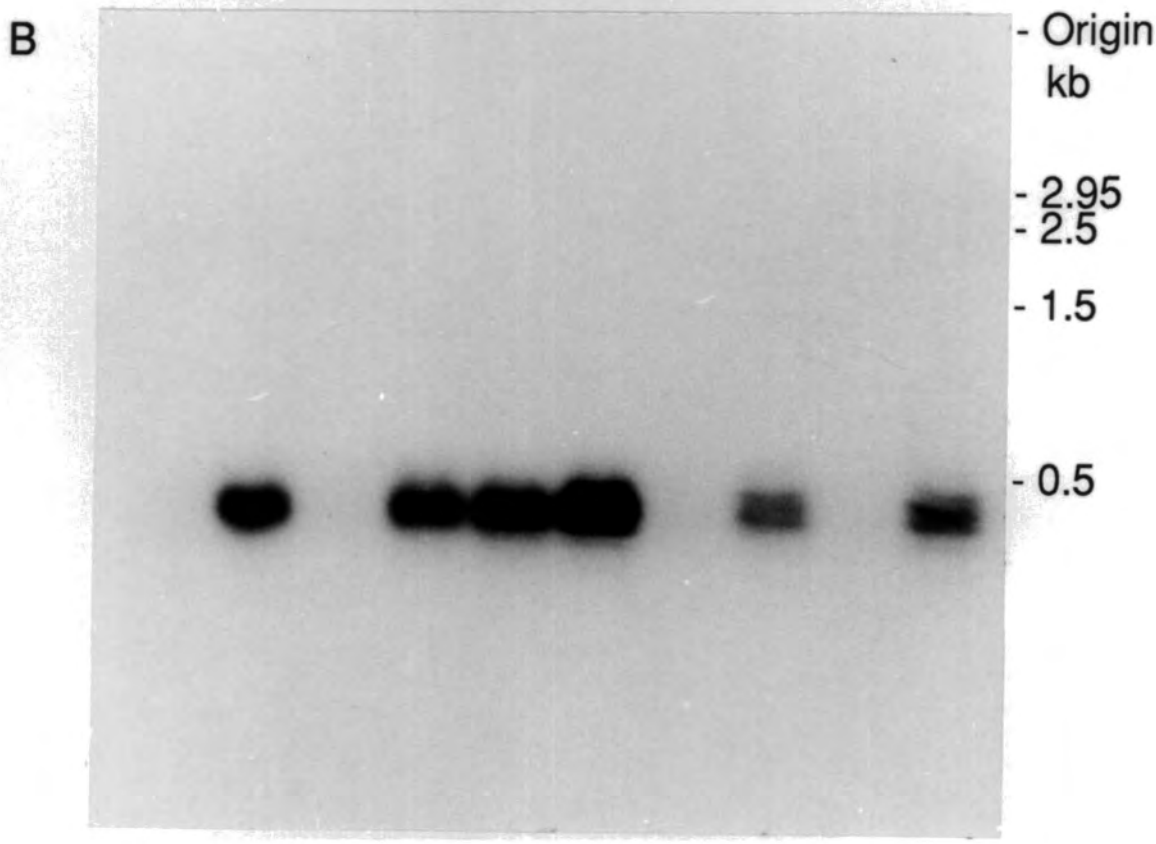
- Origin
kb
- 2.95
- 2.5
- 1.5
- 0.5
rRNA
bands


B
- Origin
kb
- 2.95
- 2.5
- 1.5
- 0.5

Chapter 7

IDENTIFICATION OF HOMOLOGUES

7.1 Introduction

Metallothioneins have been isolated and characterised from a wide range of eukaryotes, and are often considered to be ubiquitous. Following the isolation and characterisation of the metallothionein locus, *smt*, from *Synechococcus* PCC 7942 (Robinson *et al.*, 1990; Huckle *et al.*, in press) (Section 1.7), during an intermediate stage of this research, it was possible to investigate other cyanobacterial strains for the presence of homologous systems. The results presented below deal with the attempts to identify homologues of *smtA* in other cyanobacteria, in particular, isolates from metal polluted environments.

7.2 Results

In vitro amplification of DNA using primers corresponding to the coding region of *smtA*, was attempted as a diagnostic tool for identification of *smtA* homologues in other cyanobacteria. Repeatedly, cross contamination of test DNA with the positive control (*smtA*), despite attempts to eliminate contamination (for e.g. by UV irradiation), negated the attempts to use PCR as a diagnostic tool for identification of homologues. However, by PCR, a homologue of *smtA* from *Synechococcus* PCC 6301 was identified in a closely related *Synechococcus* PCC 7942 (Fig. 7.1A). The PCR amplification product from *Synechococcus* PCC 7942 was the same size (144 bp) as that from *Synechococcus* PCC 6301. The identification of a homologue of *Synechococcus* PCC 6301 *smtA* in *Synechococcus* PCC 7942 was further analysed by Southern hybridisation of *Sal*I restricted genomic DNA from the two organisms, to radiolabelled *smtA*. The *Sal*I *smtA* restriction fragment from *Synechococcus* PCC 7942 was identical in size to that from *Synechococcus* PCC 6301 (Fig. 7.1B).

7.21 Screening of other cyanobacteria for homologues of *smtA*

Genomic DNA isolated from different cyanobacterial strains, isolated from different environmental conditions, was screened for homologues of *smtA*. Genomic DNA was isolated from *Synechococcus* D562, *Synechocystis* PCC 6803, *Oscillatoria* D813, *Oscillatoria* D814, *Microchaete* D578 and *Calothrix* D184 (all axenic except D813). Genomic DNA from *Synechococcus* PCC 6301 was used as a control.

Southern hybridisation of genomic DNA restricted with *Eco*RI, to radiolabelled 144 bp fragment of *smtA* did not yield any evidence for the presence of homologues in any of the strains tested. Therefore, the blot was stripped of radioactivity and re-hybridised to radiolabelled 1.8 kb *Hin*dIII-*Sal*I fragment carrying the entire *smt* locus (released from clone pJHNR49). Following hybridisation, the Southern blots were washed at a very low stringency (2X SSC, 0.1% SDS, room temperature, 5 min), to remove excess radioactivity.

A very strong hybridisation signal was observed in the control DNA from *Synechococcus* PCC 6301. Additionally, weak hybridisation signals were also observed in genomic DNA isolated from D562, PCC 6803, D813 and a relatively stronger signal in D814 (Fig. 7.2B). However, no evidence of any hybridisation was observed in DNA isolated from D184 and D578.

7.22 Analysis of homologues of *smtA* in other cyanobacterial strains

Genomic DNA isolated from D562, D814 and PCC 6803 (selected from amongst the identified homologues because of the axenic nature of their culture) was further analysed by using different restriction endonucleases.

DNA isolated from D562, D814, PCC 6803 and *Synechococcus* PCC 6301 was restricted with *Eco*RI, followed by *Hin*dIII. Hybridisation of restricted DNA to radiolabelled 1.8 kb *smt* fragment identified a 2.8 kb *smtA* fragment in *Synechococcus* PCC 6301 (Fig. 7.3B). Two restriction fragments (ca. 1.8 kb and 1.0 kb) were observed in DNA from D562, whilst only a single hybridising fragment, same size as the low molecular

weight fragment in D562, was observed in D814 and PCC 6803. The intensity of hybridisation signals in D814 and PCC 6803 was very weak, even in comparison to the signal observed in D562 (Fig. 7.3B).

Restriction of genomic DNA from the four strains with *Sal*I, followed by *Hin*dIII, and subsequent hybridisation revealed a restriction fragment in D562 which was the same size as observed in PCC 6301 (1.8 kb) (Fig. 7.3B). Additionally, a weakly hybridising second fragment of ca. 1.0 kb was also observed in D562. Single restriction fragments, similar in size to the low molecular weight (ca. 1.0 kb) fragment of D562, were also observed in DNA from D814 and PCC 6803 (Fig. 7.3B).

Genomic DNA isolated from the four strains was also analysed by restriction with single endonuclease (*Sal*I, *Hin*dIII) restrictions, followed by Southern hybridisation. In DNA restricted with *Sal*I, again two restriction fragments were observed. The higher molecular weight fragment was more abundant than the low molecular fragment (Fig. 7.4B). However, the size of the fragments was smaller than that observed in PCC 6301. Only single restriction fragments were observed in DNA from D814 and PCC 6803. These fragments were again similar in size to the lower molecular weight fragment observed in D562.

*Hin*dIII restriction of DNA and subsequent hybridisation identified a fragment of 2.8 kb in DNA from PCC 6301. However, in DNA from D562, two fragments were identified. The higher molecular weight fragment was more abundant and significantly larger than that observed in PCC 6301. The lower molecular weight and less abundant fragment was smaller than the fragment observed in PCC 6301 (Fig. 7.4B). In DNA isolated from D814, two fragments were detected. The higher molecular weight fragment of D814 was the same size as the lower molecular weight fragment observed in D562. However, the lower fragment was significantly smaller. A single fragment of the same size as those observed in D562 and D814 was also observed in DNA from PCC 6803 (Fig. 7.4B).

7.3 Summary

The results can be summarised as follows:

1) A homologue of *smtA* from *Synechococcus* PCC 6301 was identified by PCR in *Synechococcus* PCC 7942.

2) The homologue in PCC 7942 was also identified by Southern hybridisation of genomic DNA on a restriction fragment which was the same size as that in *Synechococcus* PCC 6301.

3) No hybridisation of *smtA* was detected in genomic DNA from *Synechococcus* D562, *Synechocystis* PCC 6803, *Oscillatoria* D813, *Oscillatoria* D814, *Microchaete* D578 and *Calothrix* D184.

4) Weak hybridisation to *smtA Hin*dIII-*Sal*I fragment was observed only in genomic DNA from D562, D813, D814 and PCC 6803.

5) Hybridisation of genomic DNA from D562, D814 and PCC 6803 after restriction with different endonucleases showed relatively strong hybridisation to a fragment only in D562, in addition to a weakly hybridising fragment.

6) Restriction fragments hybridised very weakly to *smt* in genomic DNA from D814 and PCC 6803.

7) The sizes of fragments from D562, D814 and PCC 6803 exhibiting weak hybridisation to *smt* were similar in size.

It can be concluded that a homologue of *smtA* from *Synechococcus* PCC 6301 was detected in *Synechococcus* PCC 7942 by PCR and Southern hybridisation of genomic DNA. This gene was subsequently cloned from *Synechococcus* PCC 7942 (Huckle *et al.*, in press). Additionally, sequences showing homology to the 1.8 kb fragment carrying the *smt* locus were identified in several other cyanobacterial isolates by Southern hybridisation to genomic DNA. However, the fragments remain to be cloned and therefore their identities are unknown at this time. No homologous sequences were identified in genomic DNA isolated from D184 and D578.

Figure 7.1. Identification of *smtA* homologue in *Synechococcus* PCC 7942. Lanes 1 and 2 are visualisation of PCR amplified *smtA* fragments on an agarose gel using template DNA from: lane 1, *Synechococcus* PCC 6301; lane 2, *Synechococcus* PCC 7942. Lanes 3 and 4 are a Southern blot of *Sal*I restricted genomic DNA isolated from: lane 3, *Synechococcus* PCC 6301; lane 4, *Synechococcus* PCC 7942 and hybridised to *smtA*.

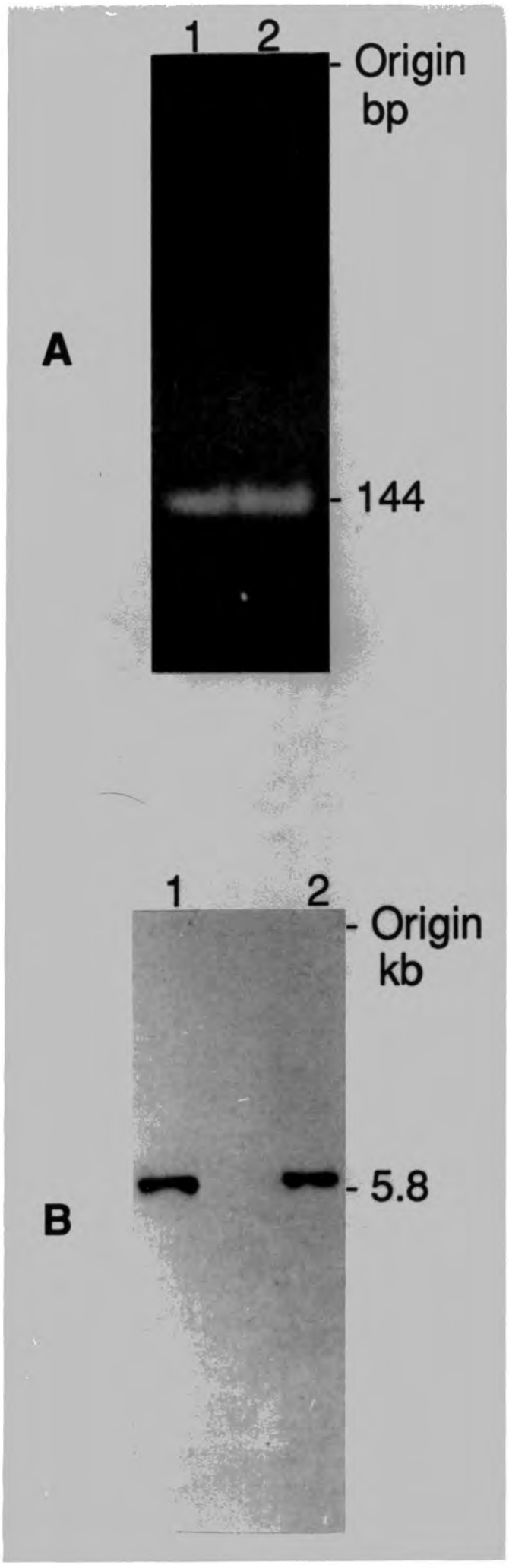
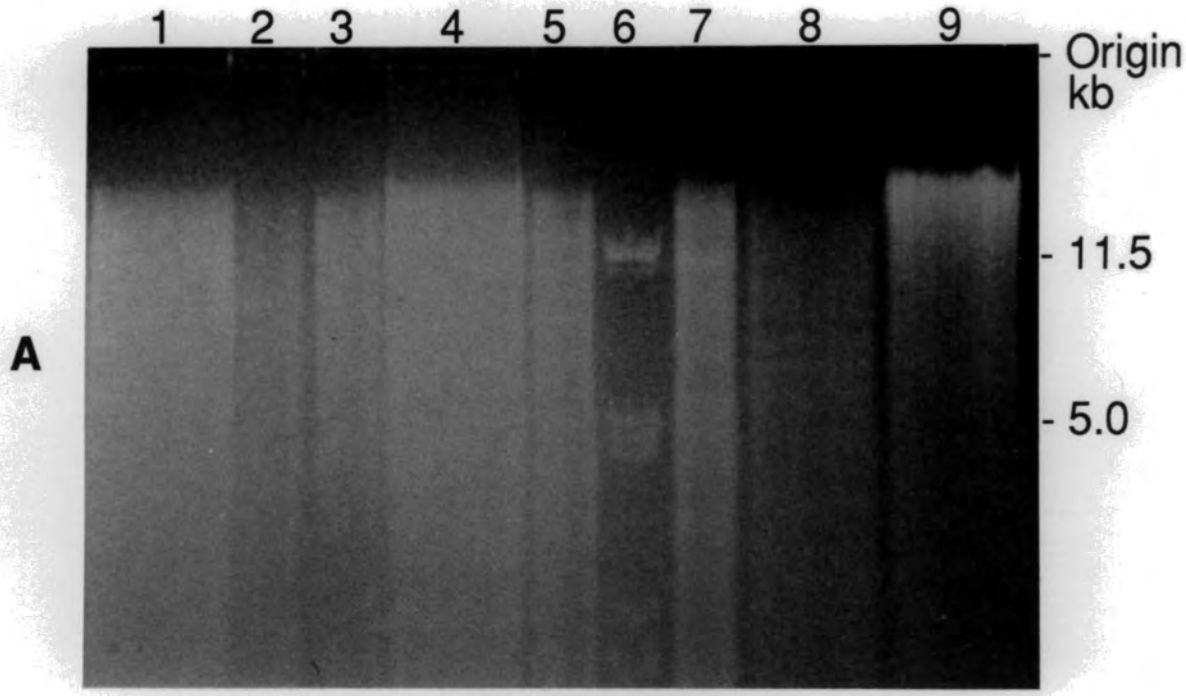

1
2
Origin
bp
A
144
1
2
Origin
kb
5.8
B

Figure 7.2. Identification of *smtA* homologues in different cyanobacterial strains. Panel *A*: visualisation of *Eco*RI restricted genomic DNA. Panel *B*: Southern hybridisation of genomic DNA isolated from: lanes 1 (100 μg) and 2 (10 μg), *Synechococcus* D562; lane 3, *Synechocystis* PCC 6803; lane 4 (100 μg) and 5 (10 μg), *Oscillatoria* D813; lane 7, *Oscillatoria* D814; lane 8, *Microchaete* D578; lane 9, *Calothrix parietina* D184; to 1.8 kb *Hin*dIII-*Sal*I fragment carrying the *smt* locus.


1 2 3 4 5 6 7 8 9
Origin
kb
11.5
5.0
A

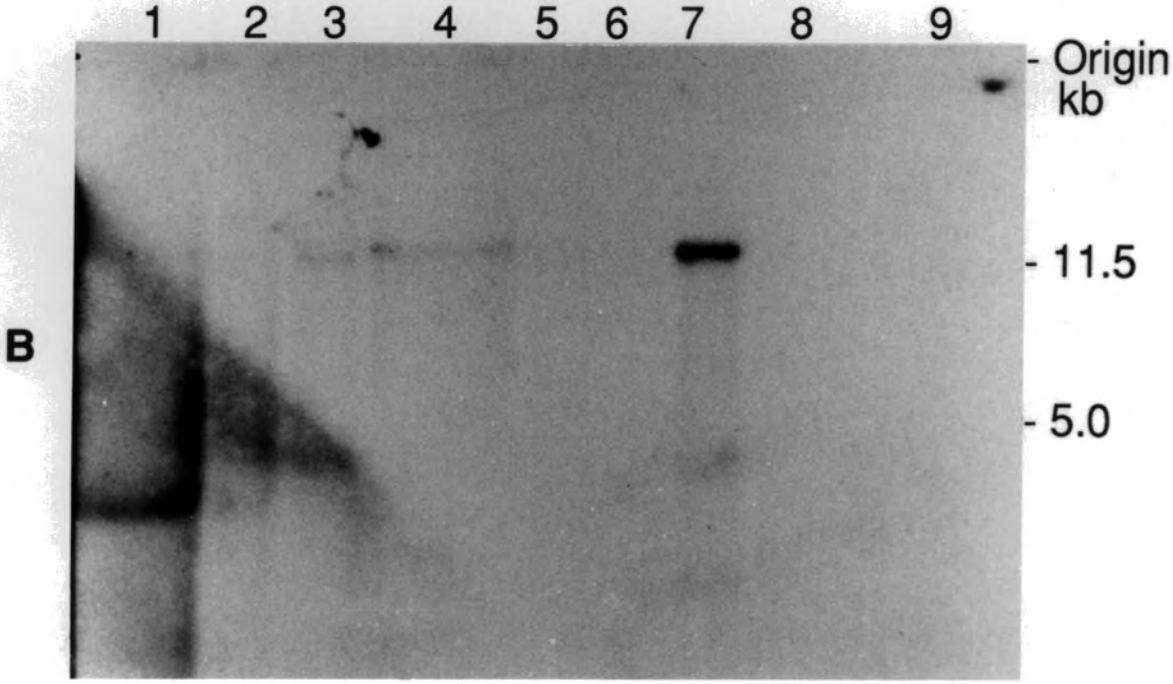

1 2 3 4 5 6 7 8 9
Origin
kb
11.5
5.0
B

Figure 7.3. Restriction analysis of *smtA* homologues identified in different cyanobacterial strains. Ten μg of genomic DNA isolated from: lanes 1 and 8, *Synechococcus* PCC 6301; lanes 2 and 7, *Synechococcus* D562; lanes 3 and 6, *Oscillatoria* D814; lanes 4 and 5, *Synechocystis* PCC 6803; was restricted with: lanes 1 to 4, *Eco*RI-*Hin*dIII; lanes 5 to 8, *Hin*dIII-*Sal*I. Panel *A* shows the visualisation of ethidium-bromide stained genomic DNA, and panel *B* shows hybridisation to 1.8 kb *Hin*dIII-*Sal*I fragment of the *smt* locus.

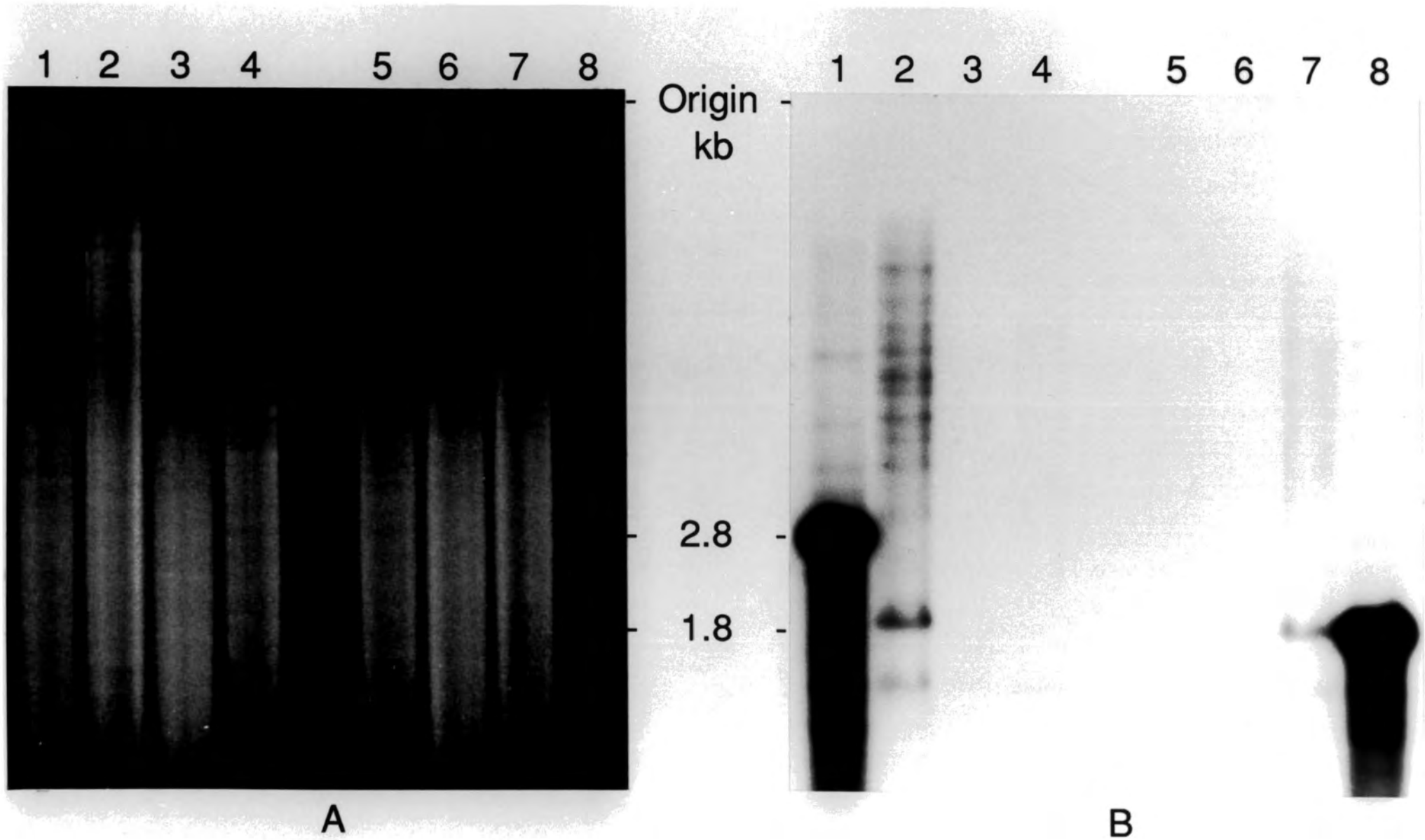
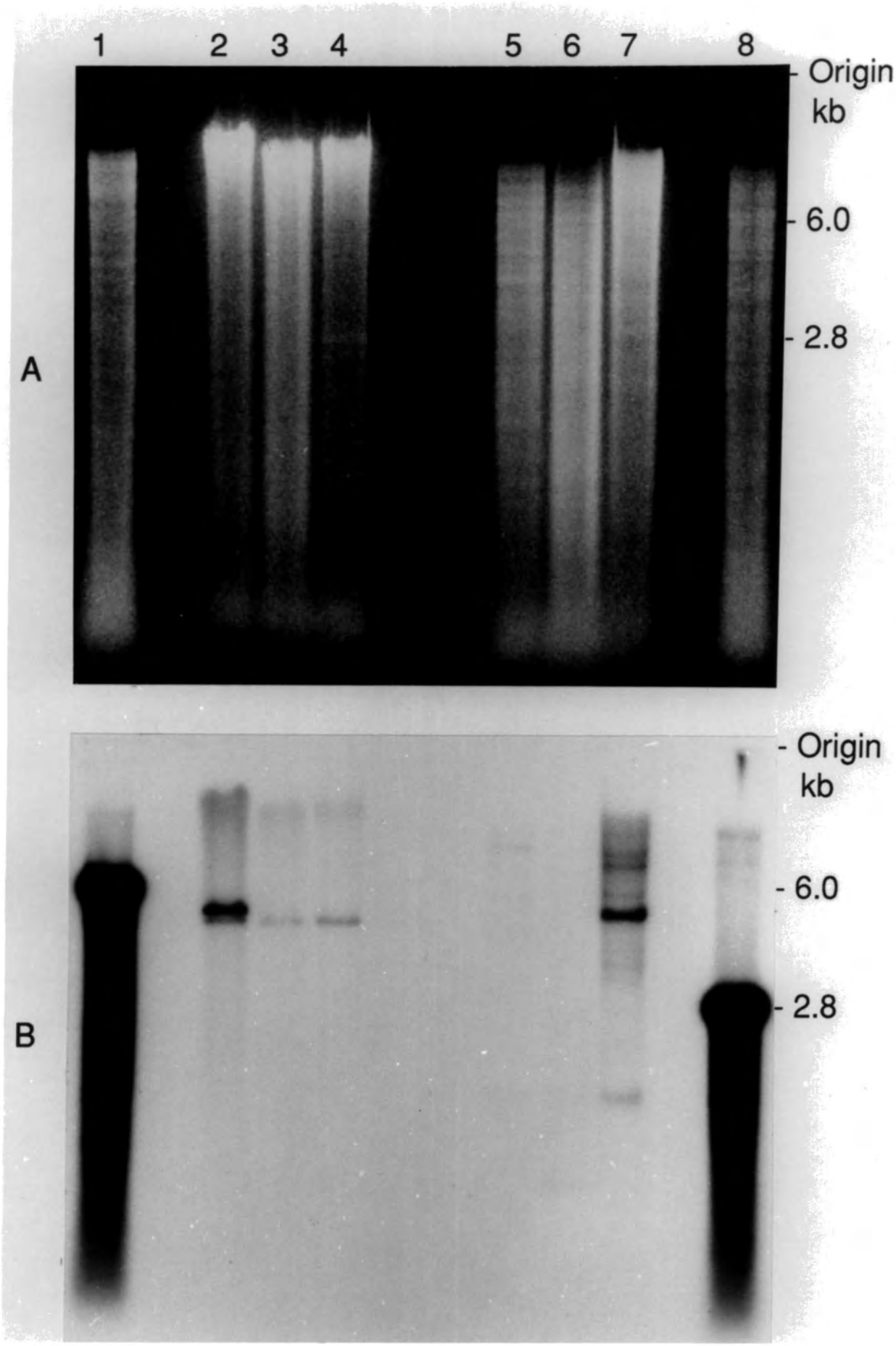

1 2 3 4 5 6 7 8
Origin
kb
2.8
1.8
A
1 2 3 4 5 6 7 8
B

Figure 7.4. Restriction analysis of *smtA* homologues identified in different cyanobacterial strains. Ten μg of genomic DNA isolated from: lanes 1 and 8, *Synechococcus* PCC 6301; lanes 2 and 7, *Synechococcus* D562; lanes 3 and 6, *Oscillatoria* D814; lanes 4 and 5, *Synechocystis* PCC 6803; was restricted with: lanes 1 to 4, *Sal*I; lanes 5 to 8, *Hin*dIII. Panel *A* shows the visualisation of ethidium-bromide stained genomic DNA, and panel *B* shows hybridisation to 1.8 kb *Hin*dIII-*Sal*I fragment of the *smt* locus.


1
2
3
4
5
6
7
8
Origin
kb
6.0
2.8
A
Origin
kb
6.0
2.8
B

Chapter 8

DISCUSSION

The main aim of this research was to identify a molecular mechanism of metal tolerance in cyanobacteria. Cd-tolerant cell lines of *Synechococcus* PCC 6301 developed by step-wise adaptation were aimed to be used to examine the role of the prokaryotic metallothionein locus, *smt*, in the acquisition of Cd tolerance in cell lines of *Synechococcus* PCC 6301. The results presented and summarised in previous chapters have provided evidence for amplification of *smtA* and rearrangement of the *smt* locus in step-wise selected Cd-tolerant cell lines of *Synechococcus* PCC 6301. Additionally, putative homologues of *smtA* have been identified in cyanobacterial strains isolated from metal-polluted sites. The demonstration of amplification and characterisation of rearrangement has suggested a role for the prokaryotic metallothionein gene, *smtA*, in Cd-tolerance of step-wise selected Cd-tolerant cell lines. Similarly, the identification of putative *smtA* homologues indicates that analogous mechanisms of metal tolerance might operate in different cyanobacterial strains.

8.1 Localisation of *smtA*

In metal-tolerant cultured eukaryotic cell lines, amplification of MT genes (initially chromosomal) have been demonstrated (Sections 1.631, 8.2), and in *Synechococcus* TX-20 (Section 1.8) Cd tolerance was postulated to involve the amplification of MT gene localised on the plasmid (Section 1.7). Additionally, as summarised in Chapters 4 and 5, amplification of *smtA* and rearrangement of the *smt* locus has been observed. Therefore, an understanding of the chromosomal or extrachromosomal localisation of *smtA* in *Synechococcus* PCC 6301 was considered essential, since amplification of *smtA* would be easily explained if *smtA* were to be localised on an extrachromosomal element. However, the potential presence of *smtA* on the small plasmid of *Synechococcus* has been eliminated by the hybridisation of *smtA* to DNA isolated from a small plasmid cured strain (Turner *et al.*, 1992). Similarly, the lack of correlation between the sizes of *Bam*HI, *Sal*I and *Hin*dIII restriction fragments of genomic DNA from *Synechococcus* PCC 6301 and the 48.5 kb

plasmid suggest against the extrachromosomal localisation of *smtA*. However, from Southern hybridisation there is an indication that weak homology exists between *smtA* and sequences within the 10.6 kb *Bam*HI fragment of the 48.5 kb plasmid (pPLAN Ba2). The weak hybridisation of pPLAN Ba2 to *smtA* (Fig. 3.2B), and a lack of correlation between the fragment sizes of pJHNR49 and pPLAN Ba2 strongly suggests that *smtA* is not present on the 48.5 kb plasmid, but is chromosomally localised. The extent of homology in pPLAN Ba2 can only be determined after complete DNA sequence analysis of the region exhibiting homology.

Although it is suggested that plasmids in cyanobacteria have functions comparable to those of analogous elements found in bacteria, such as resistance to heavy metals and antibiotics, sexuality etc., no plasmid-encoded functions have so far been identified in cyanobacteria (Ciferri *et al.*, 1989, also Section 1.7). Naturally occurring plasmids from cyanobacteria appear to be phenotypically cryptic (Kuhlemeier *et al.*, 1981), but plasmids in cyanobacteria have been speculated to play a role in lateral gene transfer (Cifferri *et al.*, 1989; Saunders, 1992). Furthermore, Van der Plas *et al.* (1992) have recently obtained complete nucleotide sequence of pUH24, the small plasmid (7.835 kb) of *Synechococcus* PCC 7942. From nucleotide sequence analysis and from the distribution of translation start and stop codons, they have identified 36 open reading frames that could potentially encode polypeptides of 50 or more amino acids, but suggest that only eight of these open reading frames are actual coding sequences. Additionally, a region putatively involved in the segregational stability of the plasmid has been identified and designated as *pmaA* and *pmaB*. Similarly, a region occupied by two overlapping genes (*repA* and *repB*) and thought to encode essential replication proteins has also been identified (Van der Plas *et al.*, 1992). However, the data described in Section 8.5 and the nucleotide sequence of pUH24 would help in understanding the functional significance of cyanobacterial plasmids and their potential role in cellular adaptation.

8.2 Amplification in step-wise selected *Synechococcus* PCC 6301

An increase in hybridisation intensity, relative to that in the non-selected line A0, was observed in the Cd-tolerant cell line and has been summarised in Chapters 4 (Section 4.4) and 5 (Section 5.4). To obtain quantitative data on amplification (i.e. increase in gene copy number), the *smtA* hybridisation of *Sal*I restricted genomic DNA isolated from non-selected line A0 and Cd-tolerant lines (A0.8, A1.3, A1.7) was compared to standard amounts of plasmid DNA containing *smtA* (calculated from gene copy number equivalents: Section 2.3212). Additionally, the hybridisation intensity of DNA isolated from A0 (Fig. 4.2A) and observations summarised in Chapter 3 (Section 3.3) suggest that *smtA* occurs at a low copy number (probably one) on the cyanobacterial chromosome.

Cell lines of *Synechococcus* PCC 6301 selected to elevated concentrations of Cd by step-wise selection, exhibited increased tolerance to Cd and were phenotypically distinct from the non-selected cell line A0 (Fig. 4.1). Additionally, in cell lines selected from a culture of *Synechococcus* PCC 6301, amplification (increase in the 'relative' amount of a gene or DNA sequence within a cell) was observed in Cd-tolerant cell line A1.7 (Fig. 4.2A). Subsequently, amplification and additional restriction fragments, suggestive of rearrangement, were also observed in other Cd-tolerant cell lines (Fig. 4.3A). However, no evidence of amplification or generation of additional restriction fragments was ever observed in the non-selected cell line A0. Three independent restrictions of genomic DNA isolated from C1.3 after 2 subcultures showed a similar pattern of restriction, suggesting that the additional restriction fragments observed in Cd-tolerant cell lines are genuine and not artefacts due to anomalous restriction by *Sal*I endonuclease.

Cd-tolerant cell lines of *Synechococcus* PCC 6301 were re-selected from a culture developed from a single plated colony (clonal culture). This repetition was necessary since the Cd-tolerant cell lines selected initially (cell lines A0.8, A1.3, A1.7) could result from selection of particular variants from the genetic diversity occurring within the culture of *Synechococcus* PCC 6301 after prolonged maintenance in liquid medium.

Genomic DNA isolated from the new set of non-selected (C0) and Cd-tolerant cell lines (C1.4, C1.8, C2.6, C3.2) was analysed by hybridisation to radiolabelled *smtA* (Fig. 5.2). Amplification of *smtA*, relative to C0, was again observed in Cd-tolerant cell lines. Additionally, smaller and larger restriction fragments were also observed in Cd-tolerant lines. There was no evidence of any amplification or rearrangement in DNA isolated from C0 during any stage of selection or subsequent maintenance.

The apparently unique restriction fragments might result from some effect of Cd on restriction, since Cd-tolerant lines are maintained in the presence of Cd. However, addition of Cd *in vitro* did not directly affect restriction of genomic DNA with *Sal*I restriction endonuclease (Fig. 5.3A), considering that no unique restriction fragments were observed. In addition, there were no apparent rapid indirect effects of Cd under *in vivo* conditions (e.g. modification of restriction endonuclease recognition sites), following exposure of cells to Cd for 2 h (Fig. 5.3B), since the restriction pattern and *smtA* hybridisation intensities in genomic DNA from Cd-exposed and non-exposed cells was similar. Moreover, in DNA isolated from C1.4, after one subculture in the presence of Cd, no unique *smtA* restriction fragments were detectable (Fig. 5.2A, panel 1). Additional restriction fragments were detectable in DNA isolated from the same line (C1.4) after two, three and four subcultures. Similarly, in DNA from A1.3 unique restriction fragments were only detectable after the second subculture, and three independent restriction reactions of genomic DNA from A1.3 revealed a similar restriction pattern (Fig. 4.4). The additional unique larger and smaller *smtA* restriction fragments observed in genomic DNA isolated from Cd-tolerant cell lines (A0.8, A1.3, A1.7, C1.4, C1.8, C2.6, C3.2) are therefore ascribed to rearrangements within the *smt* locus.

Hybridisation of DNA isolated from the non-selected and Cd-tolerant lines to a control gene, *psaE*, did not show any unequivocal evidence for amplification of the *psaE* gene (Fig. 4.2B, 4.3B, 5.2B). Furthermore, by contrast to *smtA*, no evidence of unique restriction fragments (larger or smaller) of *psaE*, either during selection or subsequent maintenance of these lines, were observed. However, it is noted that hybridisation of *psaE* to DNA from all lines, including A0/C0, identifies two restriction fragments, the larger

fragment being more apparent upon prolonged exposure to X-ray sensitive film (e.g. Fig. 4.3B, panels 2, 4). In some blots hybridised to *psaE,* additional restriction fragments corresponding to *smtA* were also observed, since the blots were first hybridised to *smtA* and then washed before hybridisation to *psaE* (Fig. 4.3B, panel 7; 5.2B, panel 4). Additionally, in some blots slight variability in *psaE* hybridisation was observed. Upon visualisation of the corresponding ethidium-bromide stained genomic DNA, these small changes in *psaE* hybridisation were found to correlate with slight variations in the amounts of genomic DNA loaded on the agarose gel. Moreover, the magnitude of variation in hybridisation to *psaE* was much less than that observed for increases in hybridisation of *smtA* to DNA isolated from Cd-tolerant lines (Fig. 4.3B, panel 4; 5.2B, panels 1, 3). The variations in *smtA* hybridisation did not correlate with variations in loading of genomic DNA. For example, in DNA isolated from A1.7 (Fig. 4.2A) an increase in hybridisation to *smtA* is observed, but a slight decrease in *psaE* hybridisation is evident. The data presented clearly suggest that unlike *smtA,* there is no amplification or rearrangement of the *psaE* gene in genomic DNA isolated from any of the cell lines, during any stage of selection or subsequent maintenance.

The rearrangement in C3.2 has been further characterised. In all the Cd-tolerant cell lines (C1.4, C1.8, C2.6, C3.2) two restriction fragments of ca. 5.4 and 11.0 kb are apparent on restriction with *Sal*I (Fig. 5.2A). However, only a single restriction fragment is obtained on restriction of DNA from C3.2 with *Hin*dIII and *Hin*dIII-*Sal*I. The single hybridising fragments were consistently ca. 350 bp smaller in C3.2 than in C0. This indicates rearrangement within the previously isolated and characterised 1.8 kb *Hin*dIII-*Sal*I fragment corresponding to the *smt* region from *Synechococcus* PCC 7942 (Huckle *et al.*, in press). The variation in sizes of restriction fragments from those observed in C0 can be attributed either to deletion in the flanking regions of *smtA* or integration of *smtA* into another region of the chromosome.

Exposure of a culture growing in the absence of Cd resulted in a growth lag, and resumption of growth occurred coincident with an increase in cellular levels of MT (Olafson, 1986). Furthermore, transfer of these Cd-resistant cells to media containing no

added Cd, resulted in reduction of MT to near basal values. However, these cells grew immediately without the predicted growth lag upon re-transfer to Cd-containing media (Olafson, 1986). The rapid increase in Cd tolerance was proposed to be due to the amplification of an extrachromosomal MT gene. Furthermore, the results described in Chapter 3 suggest that *smtA* is localised on the chromosome rather than the ca. 8.0 kb or 48.5 kb plasmids of *Synechococcus* PCC 6301. The results in Chapters 4, 5 and 6 have established that in *Synechococcus* PCC 6301 development of tolerance to Cd (in lines A0.8, A1.3, A1.7, C1.4, C1.8, C2.6, C3.2) is associated with the amplification of a chromosomally localised metallothionein gene, *smtA*, and rearrangement of the *smt* locus.

The amplification of MT gene, *smtA*, in Cd-tolerant cell lines of *Synechococcus* PCC 6301 may be analogous to the observed amplification of MT genes in metal-tolerant eukaryotic cell lines. Stable Cd-resistant lines of cultured eukaryotic cells have been selected by continuous exposure of cells to step-wise increases in Cd concentrations (Durnam & Palmiter, 1987). During selection, the levels of MT mRNA greatly exceeds the maximum that can be induced in unselected cells (Durnam & Palmiter, 1987). Although a number of mechanisms could account for increases in MT mRNA production, the increase has invariably been associated with MT gene amplification (reviewed by Hamer, 1986; Palmiter, 1987). Some examples of observed MT gene amplification in eukaryotic cell lines are discussed.

In optimally induced, Cd-resistant mouse Friend leukaemia cells, a 14-fold more MT-I mRNA, 6-fold higher rate of MT-I gene transcription and 6-fold more MT-I genes, than non-resistant cells, was observed (Beach & Palmiter, 1981). The increase in the relative rate of MT-I gene transcription in Cd-resistant cells correlated with the relative amplification of MT-I genes. The largest amplified MT-I DNA fragment was 55 kb and thus included the closely linked MT-II gene (Searle *et al.*, 1984). Similarly, in Cu-resistant hepatoma cells, increased steady state mRNA levels for MT-1 (11-fold) and MT-2 (15-fold) genes was observed. Additionally, Cu-resistant hepatoma cells maintained in normal concentrations of Cu for prolonged periods showed an increase in MT-1 (8-fold) and MT-2 (10-fold) mRNA levels. The increased levels of metallothionein in Cd-resistant lines

involved a stable amplification of MT genes (Czaja *et al.*, 1991). MT gene amplification has also been studied in Chinese hamster ovary (CHO) cells. The Cd resistance in Cd-resistant variants of CHO cells was ascribed to the specific amplification of MT-I gene (Gick & McCarty, 1982; Hayashi *et al.*, 1983). Subsequently, Crawford *et al.* (1985) have shown chromosomal localisation of the MT-I and MT-II genes. Furthermore, in the lines resistant to high Cd-concentrations, 3- to 60-fold amplification of both the MT-I and MT-II genes, increased accumulation of MT-I and MT-II mRNA and polypeptides was demonstrated (Gick & McCarty, 1982; Crawford *et al.*, 1985). The stable Cd resistance correlated with the co-ordinate amplification and expression of both MT-I and MT-II genes in CHO Cd-resistant variants (Crawford *et al.*, 1985).

In addition to higher eukaryotes, MT gene systems have also been explored in metal-resistant lines of lower eukaryotes. Copper resistance in the yeast *Saccharomyces cerevisiae* is controlled by the *CUP1* locus located on the chromosome. An increase in the copy number of the Cu-MT, *CUP1*, from *S. cerevisiae,* was observed (Fogel & Welch, 1982; Karin *et al.*, 1984). Cu-sensitive strains ($cup1^S$) contain a single copy of the *CUP1* locus, whereas Cu-resistant strains ($CUP1^r$) contain 10 or more tandemly iterated copies of *CUP1*. The presence of different copy numbers of the *CUP1* locus have differential effects on Cu resistance (Jeyaprakash *et al.*, 1991). The *CUP1* gene amplification and increased mRNA transcription enables synthesis of sufficient gene product to generate resistance to Cd and Cu. Recent studies have shown that the transcription of *CUP1* gene in a Cd-resistant strain of *Saccharomyces cerevisiae* 301N was constitutive (occurs in the absence of added metal ions), and the rate of transcription is further increased by exposure to Cd or Cu ions (Tohoyama *et al.*, 1992). In *Candida glabrata* MT constitute a multigene family, and two genes MT-I and MT-II have been characterised (Mehra *et al.*, 1990). In *C. glabrata* strains selected for increased Cu resistance, the MT-I gene is present as a single copy, but stable chromosomal amplification (>30 copies) of the MT-II gene is exhibited. The amplified copies of the MT-II gene were arranged tandemly and there is increased accumulation of the MT-II mRNA than MT-I mRNA, and a higher concentration of MT-II protein than MT-I protein (Mehra *et al.*, 1988; 1990). Despite the

wide-spread occurrence of amplification in MT gene systems, the possible mechanisms of MT gene amplification are not clear and several postulations have been reviewed by Hamer (1986).

8.3 Characterisation of rearrangement in *Synechococcus* PCC 6301

In addition to amplification of the *smtA* gene in Cd-tolerant lines of *Synechococcus* PCC 6301 developed by step-wise selection, unique restriction fragments were also observed. Previously, a 1.8 kb *Hin*dIII-*Sal*I fragment carrying the *smt* locus, which includes the *smtA* gene and a divergently transcribed *smtB* gene, has been isolated and characterised from a *Synechococcus* PCC 7942 genomic library (pJHNR49: Huckle *et al.*, in press). During this study, the *Hin*dIII-*Sal*I fragment, carrying the *smt* locus, has been isolated from a size-fractionated genomic library of C3.2, a cell line of *Synechococcus* PCC 6301, tolerant to 3.2 μM Cd (pAGNR12a, pAGNR13a). Southern analysis of *Sal*I restricted genomic DNA from C3.2 had shown larger and smaller restriction fragments, relative to C0, and no fragment corresponding to the size observed in C0 was detectable in C3.2. The observed rearrangement was identified on a minimal *Hin*dIII-*Sal*I fragment, and only a single restriction fragment was detected in genomic DNA from C3.2 (Fig. 5.4C). The single hybridising fragment in C3.2 (ca. 1.45 kb) was ca. 350 bp smaller than that observed in C0 (1.8 kb). This suggested a rearrangement within the *smt* locus.

The rearrangement was localised by restriction mapping and PCR to the 5' region of *smtA* and involving *smtB*. The nucleotide sequence of DNA from pAGNR12a and pAGNR13a was identical. On comparison to nucleotide sequence from pJHNR49, a deletion within the *smtB* coding region, in DNA from pAGNR12a and pAGNR13a was identified. The deleted sequence was 352 bp within a total length of 360 bp. The excised sequence encoded the C-terminus of SmtB, thus retaining the first 20 amino acids and the putative terminator intact (Fig. 6.3). The functional deletion of *smtB* in Cd-tolerant cells argues against a direct function of this gene in the management of supraoptimal concentrations of Cd, but is consistent with the proposal that SmtB might act as a repressor of *smtA* transcription. The deduced polypeptide product of the *smtB* gene has

similarity to a known transcriptional repressor, ArsR, and recently *smtB* has been shown to be a *trans*-acting transcriptional repressor of *smtA* (Huckle *et al.*, In press). Furthermore, SmtB has recently been shown to be required for the formation of a Zn-responsive complex with the *smt* operator-promoter, and from the predicted structure of SmtB it has been proposed that there is a direct SmtB-DNA interaction exerting metal-ion inducible negative control (Morby *et al.*, 1992).

The deletion of a fragment encoding the C-terminal end of *smtB* may be considered analogous to a terminal differentiation event. This event could have possibly arisen from extreme culture conditions. However, in mutant cell lines devoid of *smtB* an increased basal level of *smtA* is achieved, and the transcription is metal independent (Huckle *et al.*, in press). Therefore, the functional deletion of *smtB* may be of advantage to cells cultured under conditions of elevated metal concentrations. Furthermore, the deletion within *smtB* may also be considered analogous to the deletion of an 11 kb (*xisA*) fragment from within a 55 kb region of the *nif* operon in *Anabaena* PCC 7120. The terminal differentiation event involving the excision of *xisA* gene contained on a 11 kb fragment from within the *nif* operon in *Anabaena* PCC 7120 provides a positive advantage to the cell and facilitates the synthesis of nitrogenase (reviewed by Haselkorn, 1989). However, no similarity was found between the excision points within the *nif* operon and the excision points within the *smt* locus.

Southern hybridisation of genomic DNA isolated from cell line C3.2 to radiolabelled DNA corresponding to the excised element suggested that all copies of the deleted element are lost from the cell (Fig. 6.4, panels F-J). Additionally, evidence suggesting that the event of amplification precedes the event of deletion is also obtained. This is judged from the hybridisation observed in the cell line C1.4 after 2, 3, 4 and 5 subcultures. An increase in hybridisation is observed, followed by loss of fragment in the region of 5.8 kb, but detectable in the region of 11.0 kb, and subsequent regeneration of fragment in the 5.45 kb region, which is predominant with *smtA* hybridisation but not when hybridised to the excised element (Fig. 6.4, panels F-J).

Cd-tolerant line C3.2, maintained in 3.2 μM Cd, was re-transferred to media containing no added Cd. Hybridisation of genomic DNA isolated from C3.2 growing in conditions of no added Cd for 1 and 3 subcultures, to radiolabelled *smtA* suggests that stable amplification of the *smt* locus and functional deletion of the *smtB* gene is achieved in the Cd-tolerant cell line C3.2, since amplification of *smtA* and rearrangement of the *smt* locus were observed in C3.2 even after three subcultures in the absence of Cd.

The functional deletion of *smtB* would provide a state of de-repressed *smtA* expression which may be beneficial for cells continuously exposed to Cd. Total RNA isolated from C0, and C3.2 growing in the presence of Cd and in the absence of Cd for 1, 3 and 5 subcultures, and hybridised to radiolabelled *smtA,* suggested that whilst no transcript is detectable in the non-selected cells C0, an elevated level of basal *smtA* expression is observed in C3.2 (Fig. 6.7, 6.8). Furthermore, C0 cells and C3.2 cells grown for 5 subcultures in the absence of Cd were exposed for 2 h to 1.4 μM Cd, and transferred to fresh media with no added Cd for 1 and 2 subcultures. Northern blot analysis of RNA isolated from these cells showed that *smtA* transcript abundance increased in C0 and C3.2, the transcript abundance in C0 being less than that in C3.2 (Fig. 6.8). Furthermore, the decay of *smtA* transcripts in C0 was rapid as compared to that in C3.2. No transcripts were detected in C0 after 1 subculture, whilst a high level of transcript abundance was detectable in C3.2 even after 2 subcultures in the absence of added Cd (Fig. 6.8) following exposure to 1.4 μM Cd for 2 h. Increased basal expression (ca. 20-fold) from an *smtA* operator-promoter has recently been reported in cells devoid of *smtB* (Huckle *et al.*, in press). The level of basal expression in these cells (*smtB* devoid) was ca. 4-fold greater than maximal expression observed in metal-induced cells which contain *smtB* (Huckle *et al.*, in press). The increase in levels of mRNA following amplification may be analogous to that observed in eukaryotic cell lines, but the true impact of the observed *smtB* deletion on metal tolerance remains to be investigated. However, the results discussed above have established a role for the prokaryotic metallothionein gene, *smtA*, in the acquisition of Cd tolerance in Cd-tolerant cell lines of *Synechococcus* PCC 6301 selected by step-wise adaptation.

8.4 Homologues of *smtA*

The first prokaryotic metallothionein was characterised from the cyanobacterium *Synechococcus* TX-20 (Olafson, 1986), whilst similar proteins have been proposed in other cyanobacteria (Section 1.7). Results obtained during this study have provided evidence for the presence of homologues of *smtA* in different strains of cyanobacteria, isolated from metal polluted environments. However, these putative homologues remain to be cloned and characterised. Recently, the DNA sequence for a MT gene has been described for *S. vulcanus*, and has been designated *mtnA* (Data currently available in the database: Accession No. X53839). This MT gene, has ca. 50% identity to *smtA* at the level of DNA and protein (A.P. Morby, personal communication). The identification of *mtnA*, and putative *smtA* homologues in cyanobacterial strains isolated from metal-polluted sites suggests that systems analogous to *smtA* might operate in a wide range of cyanobacterial species. This is of particular significance since metallothioneins are known to be involved in metal metabolism (Section 1.6) and as observed in Cd-tolerant cell lines of *Synechococcus* PCC 6301, *smtA* is also involved as a mechanism of metal tolerance. It would be of interest to look at cyanobacterial strains isolated from metal-polluted sites for similar rearrangements as observed in the *smt* locus during this research,. However, characterisation of such rearrangements would be difficult in view of the lack of a proper control. Additionally, it would be relatively difficult to establish whether such rearrangements, if any, occurred in the original environment or during subsequent culture in the laboratory. Furthermore, conditions where an organism can be isolated from a metal-polluted site and site upstream of the metal-polluted site would be most suited.

In view of the results described in previous chapters and as discussed above, it is proposed that increased internal metal ion sequestration by the SmtA protein promotes Cd tolerance in Cd-tolerant cells of *Synechococcus* PCC 6301. Cyanobacteria have been isolated from a wide range of environmental conditions, and are often the predominant forms in metal polluted waters. Different effects of Cd-toxicity have been demonstrated in

cyanobacterial strains isolated from non-metal and metal polluted waters, but no genes involved in metal tolerance have previously been identified (Section 1.52).

Cyanobacterial isolates, from metal polluted environments, show increased tolerance to the polluting metal and other metals (Section 1.52). It is worth noting at this stage that under natural conditions various factors could affect the toxicity of metals (Sections 1.3, 1.4). However, return of isolates to non-metal supplemented conditions results in a loss of tolerance, which is regained by subsequent sub-culturing in intermittent concentrations of the metal. These observations may be attributable to analogous systems (such as amplification and/or rearrangement) as described during this research, and could suggest a state of continuous genome flux within the organism in the natural environment.

8.5 Nucleotide sequence and database analysis: Implications for genome plasticity and adaptation of cyanobacteria to environmental change

Subsequent to the experimental work described in this thesis, analysis of the nucleotide sequence from pAGNR12a and pAGNR13a (by A.P. Morby & N.J. Robinson) revealed that a twelve nucleotide inverted repeat, in which 11 of 12 bases are identical (5'-GCGATCGCC[C/T]CG-3') at positions 100-111 and 459-448, inclusive, traverses the borders of the excised fragment. A palindrome 5'-GCGATCGC-3' lies within the inverted repeat and flanks the borders of the excision termini (Fig. 8.1). Additionally, this palindrome is highly abundant in the *smt* locus (occurring seven times within the 1326 nucleotides) and has thus been designated HIP1 for highly iterated palindrome.

A search of the GenBank (version 70.0) database (by A.P. Morby & N.J. Robinson) for sequences identical to HIP1 in other prokaryotes, and calculation of the total number of nucleotides of sequence represented in the database for different organisms, has allowed frequencies of HIP1 occurrence to be calculated. The frequency of multiple HIP1 occurrence in the sequence entries of *Synechococcus* and other cyanobacterial genera is greater than that seen for other prokaryotes (Figs. 8.2, 8.3). Of the 12 genera with the greatest HIP1 frequency, 10 are cyanobacteria, and the other two are *Thermoactinomyces* and *Achromobacter*, following which is a marked reduction in the frequency of HIP1

occurrence. The highest frequency of HIP1 iteration is in *Synechococcus* (15.1 x 10^{-4} HIP1/nucleotide), whilst, *E. coli*, *Bordetella*, *Bacillus* and *Paracoccus* have the lowest values (0.3 x 10^{-4} HIP1/nucleotide). The frequency of HIP1 iteration in the small plasmid pUH24 (7.835 kb) of *Synechococcus* PCC 7942 was calculated from the total nucleotide sequence (Van der Plas *et al.*, 1992). The frequency was calculated to be once every 435 nucleotides. The accuracy by which the frequency of HIP1 can be used to reflect the genome of the organism clearly will depend on the extent to which the sequences present in the database are representative of the remaining genome. Since the nucleotide information for some genera in the database is very low, the calculated HIP1 frequency may not be a true reflection of the entire genome. If a minimum threshold for sequence information is established at 10,000 nucleotides, of the 12 genera with the greatest HIP1 frequencies, *Achromobacter, Plectonema, Pseudanabaena, Spirulina* and *Thermoactinomyces* would be eliminated. The resulting seven genera with the highest HIP1 frequencies are all representatives of cyanobacteria, followed by a marked decline in the HIP1 frequencies. Certain genera, for example *Mycoplasma,* has a database representation of ca. 75 kb containing no HIP1 sites. However, the representation of 75 kb might not be sufficient to predict a deviation from the expected frequency (from its G+C content, which ranges from 23-40%).

The HIP1 sequence is high in G+C content (75%) and its occurrence might therefore be expected to be affected by the G+C content of the organism. Figure 8.4 shows the HIP1 frequencies, estimated from database analyses and also calculated from genomic G+C contents, for individual organisms (with >10 kb of sequence entry) within the genera, with the highest (*Synechococcus*) and lowest (*E. coli*) calculated HIP1 frequencies. The observed HIP1 frequencies (from database analysis) for *Synechococcus* PCC 6301 (55.1% G+C), PCC 7942 (55.2% G+C) and PCC 7002 (49.1% G+C) were calculated to be one HIP1 sequence for every 555, 851 and 944 nucleotides, respectively. Moreover, from first principles the HIP1 frequency was calculated to be one HIP1 sequence for every 45,703 (PCC 6301), 45,368 (PCC 7942) and 71,113 (PCC 7002) nucleotides. However, the observed frequency for *E. coli* (51.7% G+C) was one HIP1 sequence for every 30,835

nucleotides, whilst the expected frequency was calculated to be once every 58,715 nucleotides. This suggests that the estimated (from database analysis) HIP1 frequencies are not a direct function of chromosomal G+C content (Fig. 8.4).

8.51 Characteristics of HIP1

Database analysis has shown that HIP1 is especially frequent in cyanobacterial genomes. In sequence entries of *Synechococcus* HIP1 is present both within the coding region and in intergenic regions. HIP1 sequences occurring within the protein coding regions are found in all three possible reading frames; 5'-GCG ATC GC-3' (Ala-Ile-Ala), 5'-GC GAT CGC-3' (Xaa-Asp-Arg) and 5'-G CGA TCG C-3' (Xaa-Arg-Ser-Xaa). The central motif (5'-GATC-3') of HIP1 is a Dam methylase recognition site, and the central 6 bp of the HIP1 sequence also represent the recognition site for *Pvu*I restriction endonuclease. The HIP1 sequences present in the *smtB* gene can be considered as short homologous direct repeats.

Repeat sequences have been identified in a wide range of organisms. The first of such repetitive sequences were initially identified in *E. coli* and *Salmonella typhimurium* and are described as REP (Repetitive Extragenic Palindromic) or PU (Palindromic unit) sequences (Higgins *et al.*, 1982; Gilson *et al.*, 1984). The REP sequence structure consists of a 38 bp concensus sequence which is a palindrome and can form a stable stem-loop structure with a 5 bp variable loop in the central region of the concensus (Stern *et al.*, 1984).

```
   G          G  C   G  C        C
GCC GATG.CG CG ..... CG CTTATC GGCCTAC
   T          A  T   A  T        A
```

Concensus REP sequence (reproduced from Stern *et al.*, 1984).

The REP sequences can occur singly or in tandem (up to four copies); the tandem copies are always inverted with respect to each other. Between 500 and 1000 copies of the REP sequence are found on the chromosomes of *E. coli* and *Salmonella typhimurium*, thus occupying about 1% of the genome (Stern *et al.*, 1984; Higgins *et al.*, 1988). Extrapolation of the HIP1 frequency values observed in *Synechococcus* database sequence

entries suggest an overall occurrence of ca. 4500 HIP1 sequences per genome, which would represent about 1% of the *Synechococcus* genome. During database analysis no evidence has so far been obtained for the tandem occurrence of HIP1 in bacterial genomes.

In contrast to HIP1 sequences, no example of the REP sequence has been found within the coding sequence for a protein. In sequences identified so far, they are present in extragenic, non-translated regions, either between two genes that are co-transcribed as part of a single operon or, alternatively, within the 3' untranslated region at the end of an operon (reviewed by Higgins *et al.*, 1988). Recently it has been reported that clusters of REP sequence on the *E. coli* chromosome could also contain other repeated elements in specific arrangements. These elements were termed as BIME (Bacterial Interspersed Mosaic Element: Gilson *et al.*, 1991)(reviewed by Lupski & Weinstock, 1992). The BIME structures are always found in extragenic locations and the *E. coli* chromosome is estimated to have about 500 BIME structures.

Another group of interspersed repetitive DNA sequences identified in *E. coli*, *Salmonella typhimurium* and other enterobacteriaceae were designated as IRU (Intergenic Repeat Unit: Sharples & Lloyd, 1990) or ERIC (Enterobacterial Repetitive Intergenic Concensus: Hulton *et al.*, 1991). The ERIC or IRU sequences are approximately 126 bp in length and are located in non-coding transcribed regions of the chromosome, and includes a conserved inverted repeat. Recently, three distinct families of repeated sequences have been identified in the cyanobacterium *Calothrix* PCC 7601 (Mazel *et al.*, 1990). These repeated sequences are present at a level of about 100 copies per *Calothrix* genome, consist of tandemly amplified heptanucleotides and were thus named STRR (Short Tandemly Repeated Repetitive) sequences. The concensus sequences for the three STRR sequences are quite distinct from each other and are as follows:

STRR1	CCCCA(A/G)T
STRR2	TT(G/T)GTCA
STRR3	CAACAGT

Furthermore, the heptanucleotide sequences of STRR are not particularly abundant but, when found, are often amplified. Additionally, a heptanucleotide sequence

(CGATCGC; which is also identical to the last 7 nucleotides of HIP1) was mentioned to be amongst the most abundant heptanucleotide sequences, was not a component of STRR and was never found to be tandemly amplified. The STRR sequences were absent from *E. coli* and *Bacillus subtilis*, whilst in the 24 cyanobacterial strains tested, they were present only in filamentous heterocystous forms (Mazel *et al.*, 1990).

8.52 Functions and uses of repeat sequences

Various functions of REP and other repeat sequences have been widely reviewed (Higgins *et al.*, 1988; Lupski & Weinstock, 1992). REP sequences present at the 3' end of a gene or operon have been shown to function as mRNA stability determinants. Specific mRNA species extending from the promoter to the REP sequence are detected, and the accumulation of mRNA upstream from REP sequence is due to an increase in the mRNA half-life (Newbury *et al.*, 1987); however, studies have shown that REP sequences are not normally signals for transcription termination (Stern *et al.*, 1984). In addition to mRNA stability, REP sequences are also implicated in differential expression within polycistronic operons. Despite the above mentioned functions, it has been suggested that this is unlikely to be the primary reason for the high degree of sequence conservation between REP sequences. It has also been proposed that REP sequences may play a role in the organisation of the prokaryotic chromosome. Recombination has been known to occur between REP sequences and REP is involved in the formation of chromosomal rearrangements such as duplications (Shyamala *et al.*, 1990). REP has also been shown to bind DNA gyrase (Yang & Ames, 1988), DNA polymerase I (Gilson *et al.*, 1990). In *Haemophilus influenzae*, cellular uptake of DNA depends on the presence of a repetitive 11 bp sequence which occurs on average once every 4 kb (Sisco & Smith, 1979). In the case of ERIC sequences, their chromosomal locations differ in different species. No evidence has so far been obtained in support of a classic transposition mechanism for dispersion of these repetitive sequences (Lupski & Weinstock, 1992).

Recent studies suggest that STRR sequences are not involved in regulation of gene expression in the heterocyst, despite their presence specifically in heterocystous strains of

cyanobacteria (Mazel *et al.*, 1990). By analogy to REP and ERIC sequences, it has been suggested that STRR might be the target sites for specific DNA-binding proteins responsible for chromosome condensation and/or their possible involvement in the control of chromosome distribution, or of chromosome replication during heterocyst differentiation (Mazel *et al.*, 1990). This may be of particular relevance since heterocysts differ from vegetative cells, in the expression of some genes, in the physical organisation of the genome and in the condensation state of the chromosome (Wolk, 1982).

The HIP1 sequence is distinct from previously reported repetitive sequences such as REP, ERIC and STRR in its small size, perfect palindromic structure, distribution and projected frequency of iteration (ca. 4-fold greater than REP and ca. 100-fold greater than STRR). Unlike REP, ERIC and STRR, HIP1 would allow for intra-genic rearrangements (as observed within *smtB*), and also inter-genic rearrangements. Additionally, it could facilitate recombination between functional protein domains and/or the deletion and amplification of individual genes.

The precise function of repetitive sequences, how they have dispersed throughout the genomes, and how their high degree of sequence similarity is maintained, is not known. However, the presence and widespread distribution of repetitive sequences strongly suggests that they are important to the structure and evolution of genomes. High representation of HIP1 in the small plasmid of *Synechococcus* (once every 435 nucleotides), and high representation of this motif in the genomes of a number of cyanobacteria suggests the possibility of a widespread role for HIP1 in genome plasticity and cellular adaptation in these organisms.

Recently, synthetic oligonucleotides corresponding to REP and ERIC sequences with the base inosine placed at the non-conserved positions have been used as primers in PCR using prokaryotic genomic DNA as template. This technique is known as rep-PCR and reveal a specific pattern of genomic DNA fingerprint, which appears to be species and strain specific (Versalovic *et al.*, 1991). This may have multiple applications in epidemiological studies of micro-organisms as well as for quality control of micro-organisms used in medical, agricultural and industrial applications. Additionally, rep-PCR

may be useful for mapping of insertion sequences, such as mutations caused by transposon insertions. Furthermore, because of the presence of STRR sequences in a large number of cyanobacterial strains, they constitute a powerful tool for taxonomic studies. Indeed, the use of STRR sequences as probes for hybridisation of DNA, would facilitate researchers to determine whether different isolates are members of the same genus or even the same species. In addition they may also be important in monitoring putative DNA rearrangement events occurring during the differentiation process of hormogonia, akinetes or heterocysts. Analogous to the usefulness of STRR sequences, HIP1 may provide a more efficient tool than STRR for similar purposes, due to a more diverse distribution and a high frequency of iteration. Use of HIP1 in a similar manner as REP and ERIC for PCR may facilitate the development of new taxonomic positions for organisms, based on their genetic make-up, since the distribution of HIP1 has been observed to be independent of the G+C content of an organism.

In conclusion, acquisition of Cd-tolerance in *Synechococcus* PCC 6301 has been shown to involve amplification of the prokaryotic metallothionein gene, *smtA*. Additionally, loss of *smtB* has been observed in Cd-tolerant cell lines selected by step-wise adaptation. This represents the first characterisation of a molecular mechanism for metal-tolerance in cyanobacteria. The loss of a functional *smtB* provides enhanced expression of *smtA* and suggests increased internal metal ion sequestration in Cd-tolerant cell lines. However, the possibility that the SmtA protein has a more dynamic role in metal tolerance, possibly via enhanced metal efflux, has not yet been tested. Since, metal-tolerant cyanobacteria have been isolated from metal-polluted environments and putative homologues of *smtA* have been identified in several strains of cyanobacteria, it is postulated that similar systems of metal tolerance might operate in cyanobacteria selected for metal tolerance in metal polluted sites. Furthermore, deletion within the *smt* locus is bordered by a palindromic sequence HIP1. The high frequency of HIP1 iteration in cyanobacteria suggests more general role for this palindrome (not merely at the *smt* locus) in genome plasticity and hence adaptation of cyanobacteria to environmental change. It

will be of interest to look for evidence of HIP1 mediated rearrangements in other cyanobacterial genes following adaptation to other environmental changes.

Figure 8.1. Representation of the *smt* locus. A 1326 bp *Hin*dIII-*Nae*I fragment containing the *smt* locus from non-selected cells is represented, showing the *smtA* and *smtB* genes. Vertical lines represent HIP1 sequences. The expanded region shows the sequences traversing the deletion end-points, the HIP1 sequences are in bold. The horizontal arrow delineate an inverted repeat which includes, and extends beyond, the two HIP1 sites.

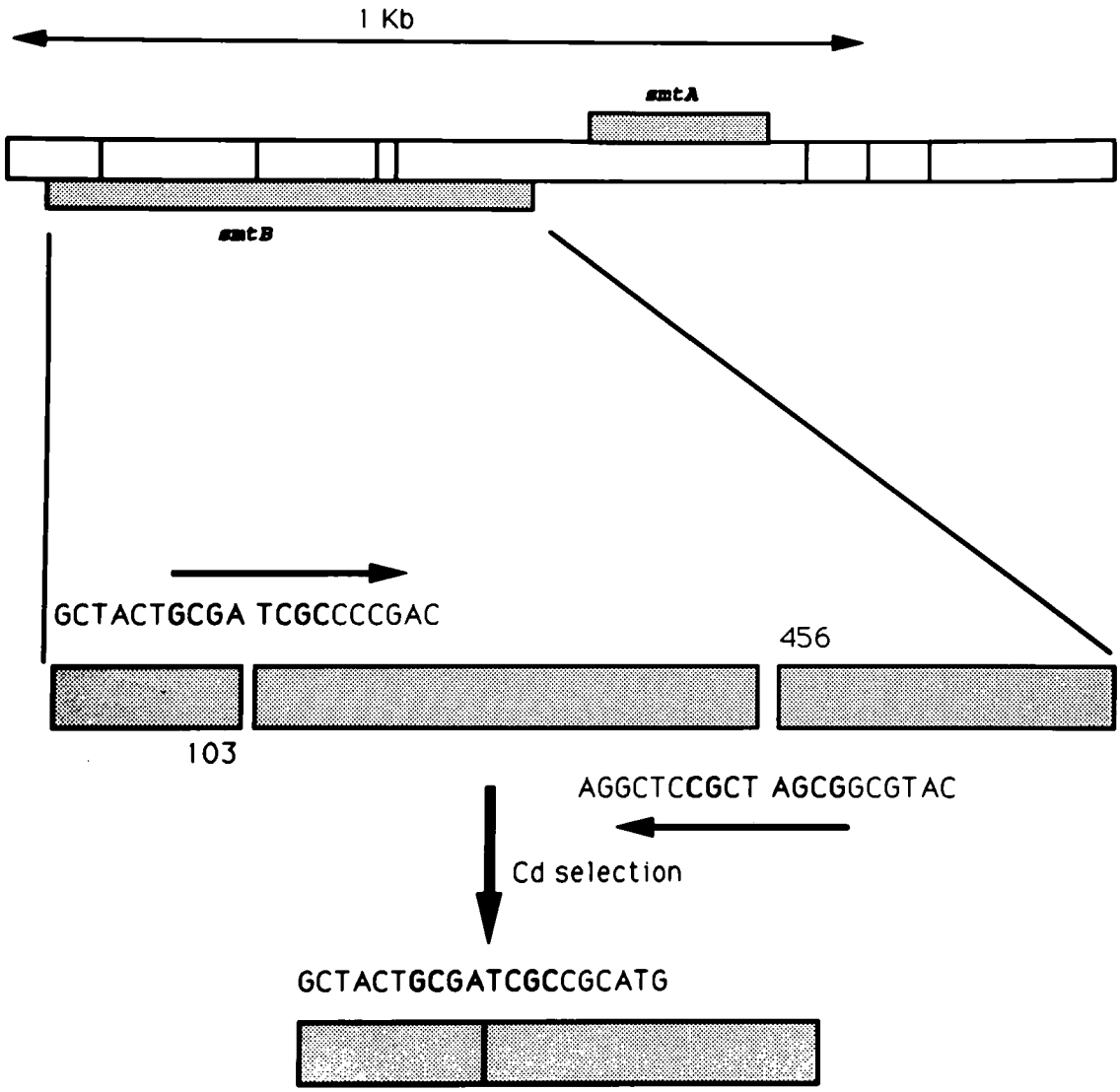

1 Kb
smtA
smtB
GCTACTGCGA TCGCCCCGAC
456
103
AGGCTCCGCT AGCGGCGTAC
Cd selection
GCTACTGCGATCGCCGCATG

Figure 8.2. Multiple HIP1 sequences per database entry per genera in GenBank 70.0. The number of sequence entries for each genera containing 3 or more HIP1 sequences is shown and *E. coli* is shown for reference. "n" represents the number of HIP1 sequences within a given entry.

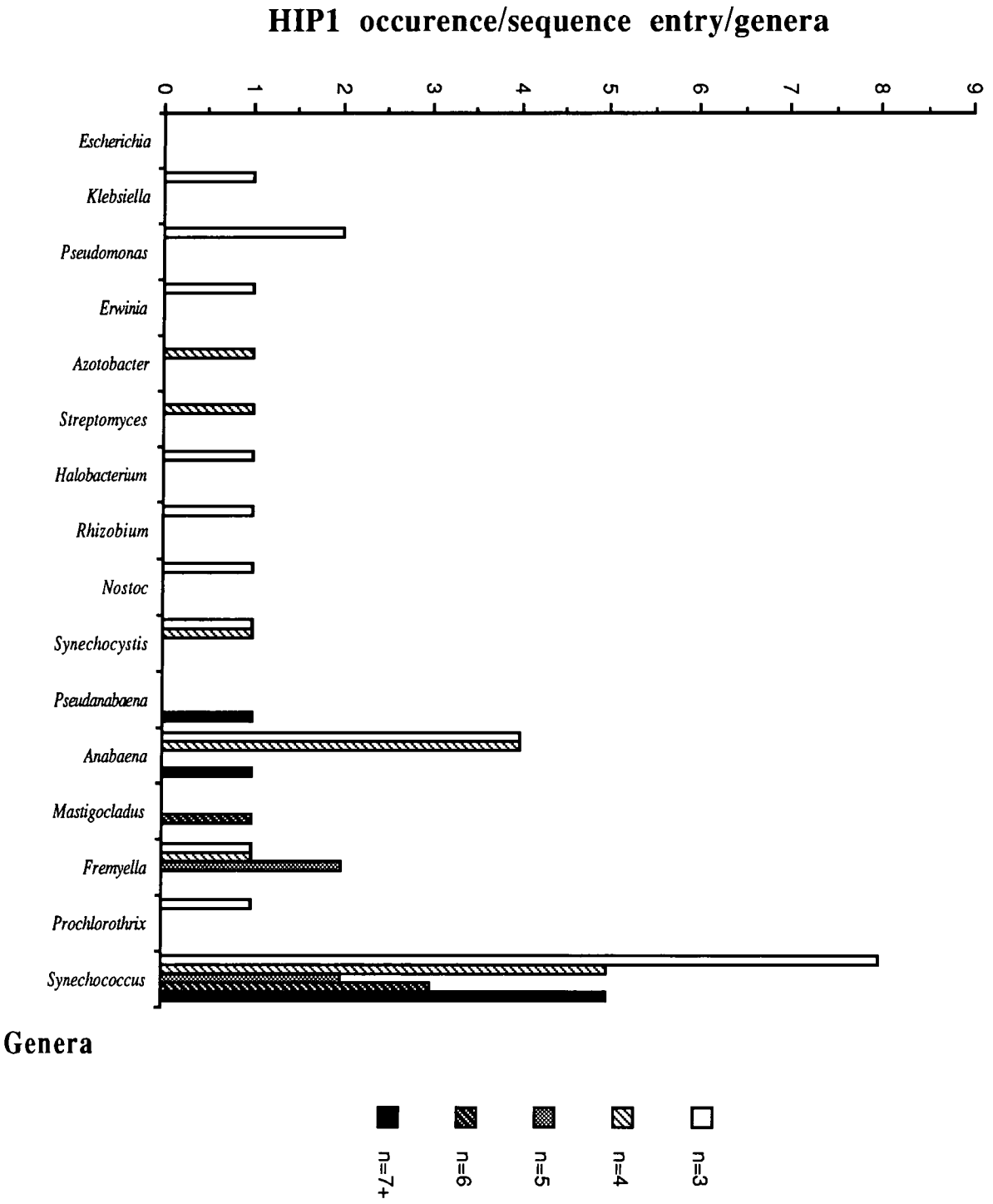

HIP1 occurence/sequence entry/genera
0
1
2
3
4
5
6
7
8
9
Escherichia
Klebsiella
Pseudomonas
Erwinia
Azotobacter
Streptomyces
Halobacterium
Rhizobium
Nostoc
Synechocystis
Pseudanabaena
Anabaena
Mastigocladus
Fremyella
Prochlorothrix
Synechococcus
Genera
n=7+
n=6
n=5
n=4
n=3

Figure 8.3. Estimated frequency of HIP1 in the genomes of different genera in the DNA sequence databank. The frequency of HIP1 per nucleotide of sequence information for a given genera represented in the databank, is shown for all genera for which the estimated value exceeds zero.

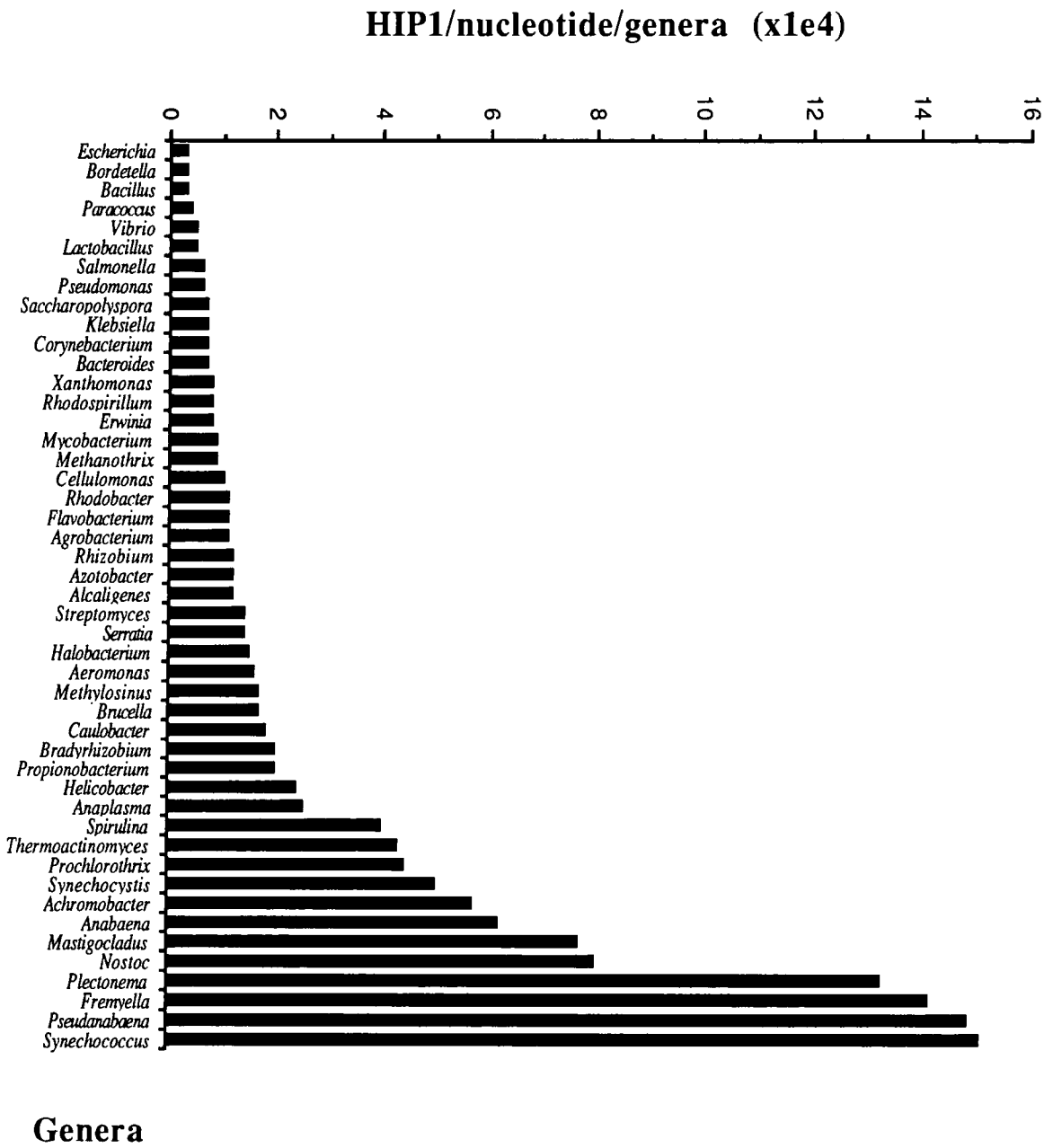
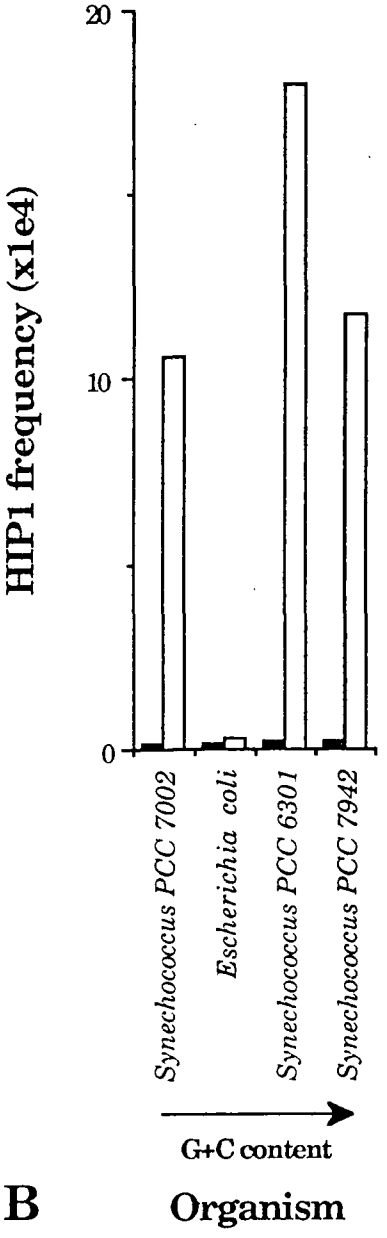

HIP1/nucleotide/genera (x1e4)
0
2
4
6
8
10
12
14
16
Escherichia
Bordetella
Bacillus
Paracoccus
Vibrio
Lactobacillus
Salmonella
Pseudomonas
Saccharopolyspora
Klebsiella
Corynebacterium
Bacteroides
Xanthomonas
Rhodospirillum
Erwinia
Mycobacterium
Methanothrix
Cellulomonas
Rhodobacter
Flavobacterium
Agrobacterium
Rhizobium
Azotobacter
Alcaligenes
Streptomyces
Serratia
Halobacterium
Aeromonas
Methylosinus
Brucella
Caulobacter
Bradyrhizobium
Propionobacterium
Helicobacter
Anaplasma
Spirulina
Thermoactinomyces
Prochlorothrix
Synechocystis
Achromobacter
Anabaena
Mastigocladus
Nostoc
Plectonema
Fremyella
Pseudanabaena
Synechococcus
Genera

Figure 8.4. The estimated frequency of HIP1 (open columns) in the genomes of individual species in the genera estimated to have the highest (*Synechococcus*) or lowest (*Escherichia*) HIP1 frequencies. The organisms are listed in order of increasing G+C content. Anticipated HIP1 frequencies predicted from the G+C content (closed columns) are shown for comparison.


HIP1 frequency (x1e4)
20
10
0
Synechococcus PCC 7002
Escherichia coli
Synechococcus PCC 6301
Synechococcus PCC 7942
G+C content
Organism

B

Chapter 9

SUMMARY

To investigate the role of *smtA* in Cd tolerance, and to establish an analogy to the functional roles of MT systems in eukaryotic cell lines, two sets of Cd-tolerant *Synechococcus* PCC 6301 cell lines were developed by step-wise selection to increasing Cd concentrations. The various results described in the previous chapters and the conclusions derived from them can be summarised as follows:

1) No hybridisation of DNA from pPLAN Ba1-Ba7 and pPLAN B2 was observed to the *Hin*dIII-*Sal*I fragment carrying the *smt* locus. Weak hybridisation of the *smtA* gene is observed to DNA from pPLAN Ba2. Sizes of the hybridising fragments in pPLAN Ba2 and the control do not correspond. Therefore, *smtA*, can be assigned to be located on the chromosome. The weak homology would be clarified only after nucleotide sequence analysis of the region of homology.

2) Cd-tolerant cell lines of *Synechococcus* PCC 6301 were developed by step-wise selection to increasing Cd concentrations. A culture of *Synechococcus* PCC 6301 that had undergone prolonged maintenance in liquid medium was used for step-wise selection. The Cd-tolerant cell lines showed phenotypic differences to the non-selected cell line.

3) Genomic DNA from Cd-tolerant cell lines and the non-selected cell line was analysed after maintenance in the respective Cd concentrations. An increase in hybridisation, and additional unique restriction fragments, relative to the non-selected line, were observed in genomic DNA from all the Cd-tolerant lines. No evidence for increase in hybridisation or appearance of unique restriction fragments was evident in genomic DNA from Cd-tolerant lines and non-selected line hybridised to a control gene, *psaE*. A similar pattern of restriction fragments was observed in three independent *Sal*I restrictions of genomic DNA isolated from Cd-tolerant line A1.3 after 2 subcultures.

4) Cd-tolerant cell lines of *Synechococcus* PCC 6301 were re-selected using a culture developed from a single plated colony. An increase in growth lag coincident with increase in tolerance was observed. The growth lag decreased following subsequent maintenance of Cd-tolerant cell lines in the presence of the respective Cd concentration.

5) Genomic DNA isolated from the Cd-tolerant cell lines and the non-selected cell line was analysed after 1, 2, 3, 4 and 5 subcultures in the presence of the respective Cd concentrations. An increase in hybridisation to *smtA*, and unique restriction fragments were observed in genomic DNA from all the Cd-tolerant cell lines after 1 subculture, except C1.4 where increase in hybridisation and additional restriction fragments were observed only after 2 subcultures.

6) No evidence for increase in hybridisation or appearance of unique restriction fragments was evident in genomic DNA from Cd-tolerant lines and non-selected line hybridised to a control gene, *psaE*. No evidence for any additional restriction fragments was observed in genomic DNA from the non-selected lines during selection or subsequent maintenance. The additional restriction fragments in Cd-tolerant cell lines were both larger and smaller, and the smaller restriction fragment in Cd-tolerant lines was ca. 350 bp smaller than that in the non-selected line. Restriction fragment equivalent in size to that in the non-selected line, was not apparent in DNA from the Cd-tolerant lines C1.8, C2.6 and C3.2.

7) The presence of Cd did not affect the restriction of genomic DNA with *Sal*I, under *in vitro* or short-term (2 h) *in vivo* conditions.

8) The apparent rearrangement was obtained on a *Hin*dIII-*Sal*I restriction as a single fragment. The single restriction fragment was ca. 350 bp smaller than that in DNA from the non-selected line. The rearrangement was isolated from size-fractionated genomic libraries. The rearrangement was localised to a 600 bp region in the 5' flank of *smtA* by PCR and restriction mapping.

9) Nucleotide sequence analysis demonstrated a loss of 352 bp from within a region of 360 bp between nucleotide positions 100-459 inclusive. The excised fragment encodes the C terminal end of the *smtB* gene, but retains the first 20 amino acids and the putative terminator intact.

10) The borders of the excised fragment are traversed by an octanucleotide palindromic sequence, 5'GCGATCGC 3'.

11) Southern hybridisation of DNA from the non-selected and Cd-tolerant cell lines suggests that all copies of the excised DNA fragment are almost certainly lost from the cell

in cell lines C1.8, C2.6 and C3.2. A rearranged pattern of restriction fragments is observed in C3.2 even after maintenance in absence of Cd for 3 subcultures.

12) An elevated basal level of *smtA* transcription was observed in C3.2 following maintenance in the absence of Cd. A greater increase in *smtA* transcript abundance, relative to the non-selected line, was observed in C3.2 following exposure to Cd after maintenance in the absence of Cd. Return of the non-selected line and Cd-tolerant line C3.2 following exposure to Cd, to medium containing no added Cd suggests a rapid degradation of the *smtA* transcript in the non-selected line (within one subculture). Whilst, in C3.2 an elevated basal expression was observed even after 3 subcultures.

The various results presented in previous chapters suggest that in Cd-tolerant lines of *Synechococcus* PCC 6301, developed by step-wise adaptation to increasing concentrations of Cd, increased internal metal ion sequestration by the SmtA protein promotes Cd tolerance. Derepressed expression of *smtA* in C3.2 may be beneficial for such cells continuously exposed to supra-optimal concentrations of Cd. Increase in *smtA* copy number may further enhance expression and facilitate Cd tolerance. Additionally, the identification of putative homologues of *smtA* in a number of cyanobacterial strains suggests that systems analogous to *smt* might operate in different cyanobacteria.

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

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SUMMARY

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

1. INTRODUCTION

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

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

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

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

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

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

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

2. MATERIALS AND METHODS

(*a*) *Materials and cyanobacterial culture*

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

(*b*) *Stepwise adaptation*

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

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

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

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

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

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

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

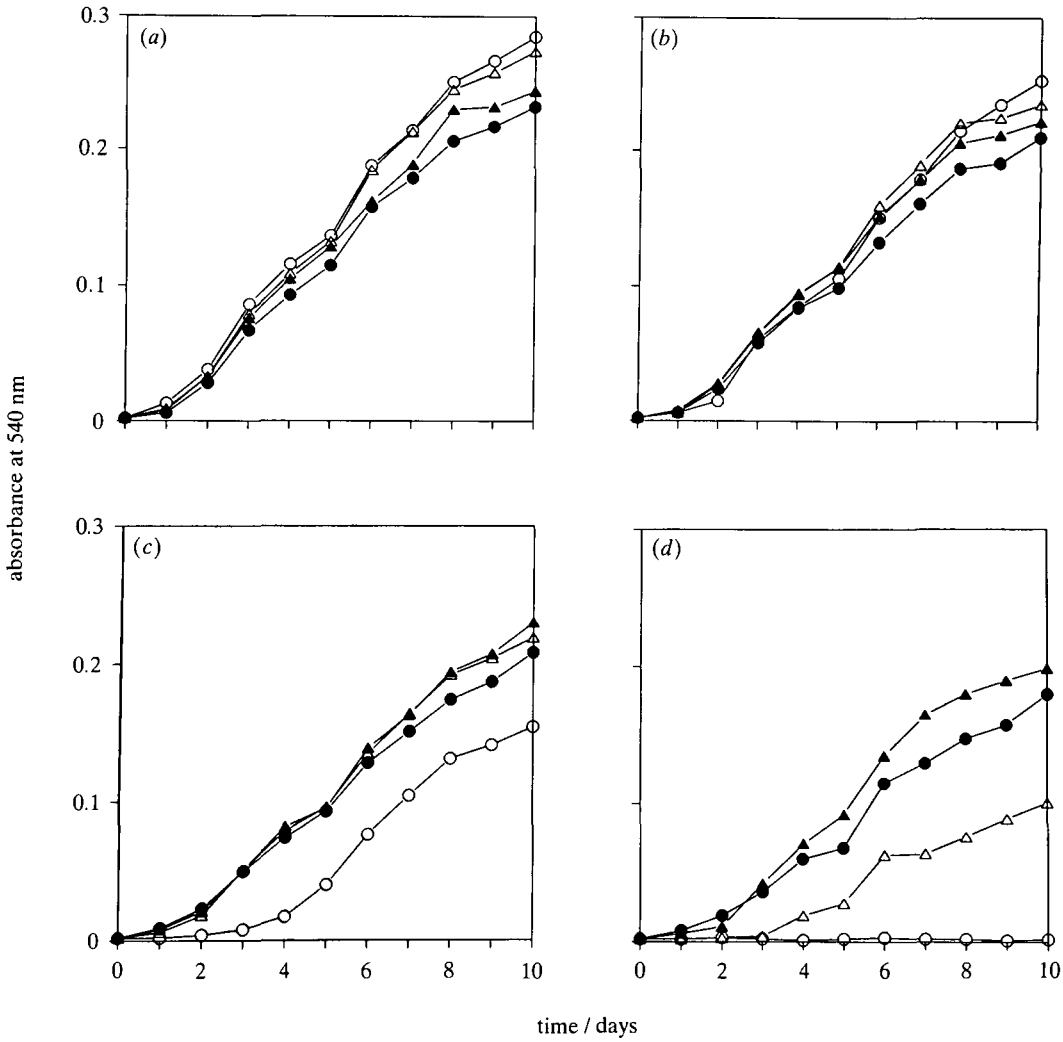

Figure 1. Growth of non-tolerant A0 (○) and Cd-tolerant lines (A0.8 (△), A1.3 (▲), and A1.7 (●)) in different concentrations of Cd. Growth in (*a*) 0 μM Cd, (*b*) 0.8 μM Cd, (*c*) 1.3 μM Cd, and (*d*) 1.7 μM Cd.

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

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

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

3. RESULTS

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

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

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

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

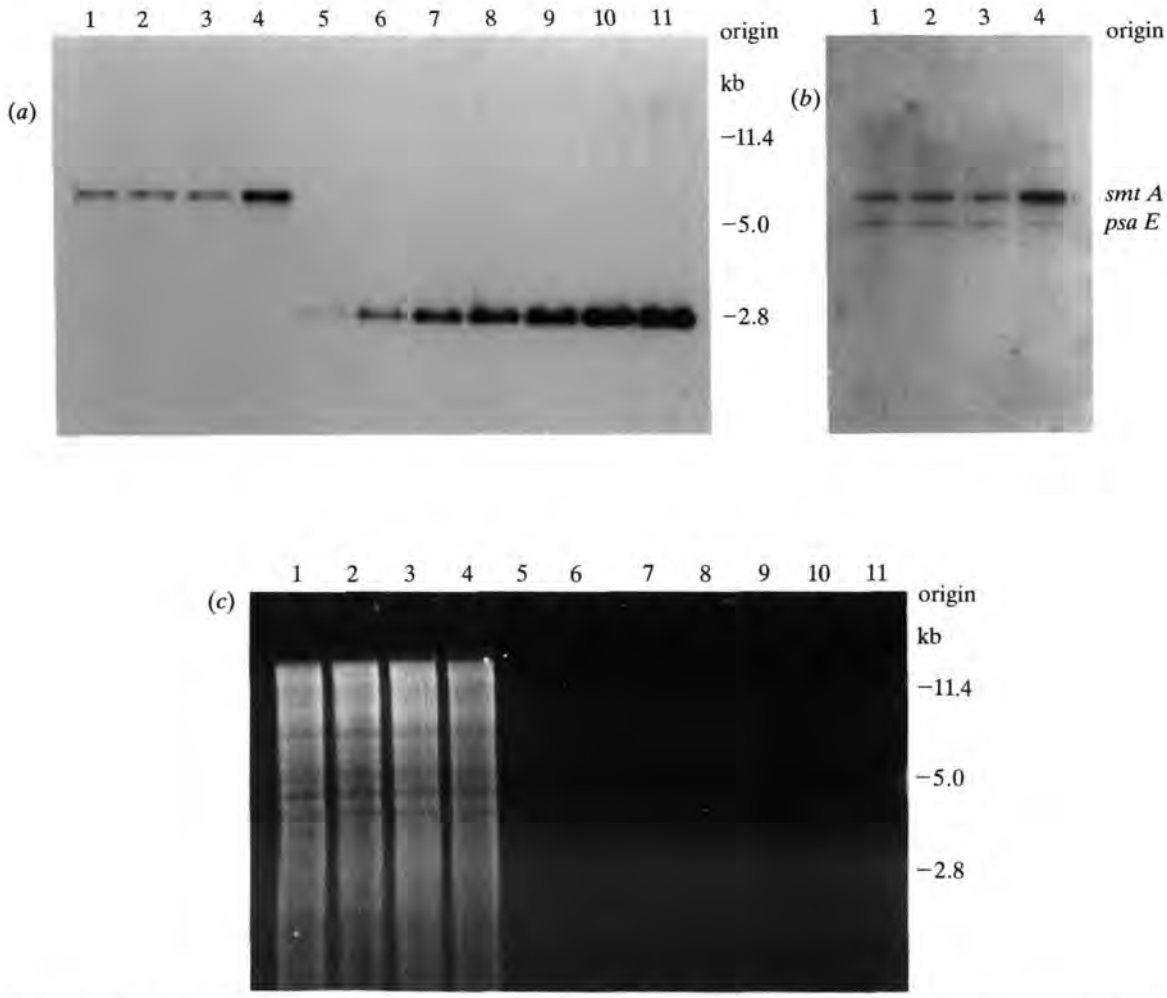

Figure 2. Analysis of genomic DNA isolated from A0 and Cd-tolerant lines. (*a*) Hybridization to *smtA*. (*b*) Hybridization to *psaE*. (*c*) Visualization of ethidium bromide-stained DNA. *Sal*I restricted genomic DNA was isolated from: lane 1, A0; lane 2, A0.8; lane 3, A1.3; and lane 4, A1.7. Lanes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

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

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

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

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

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

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

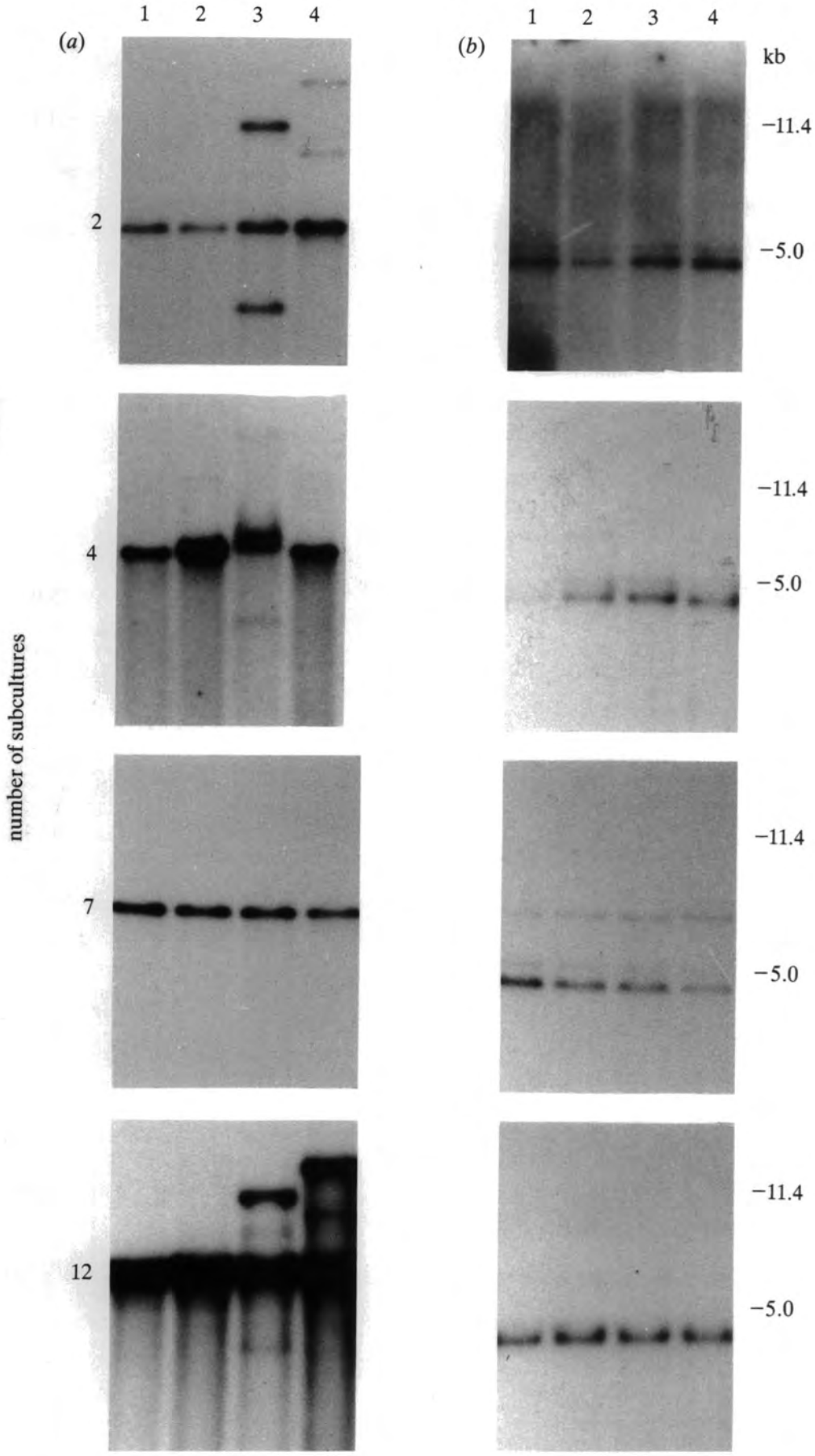

Figure 3. Analysis of genomic DNA isolated from A0 and Cd-tolerant lines (A0.8, A1.3 and A1.7) after two, four, seven and twelve subcultures. (*a*) Hybridization to *smtA*. (*b*) Hybridization to *psaE*. Equivalent amounts of DNA isolated from: lane 1, A0; and tolerant lines, lane 2, A0.8; lane 3, A1.3;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

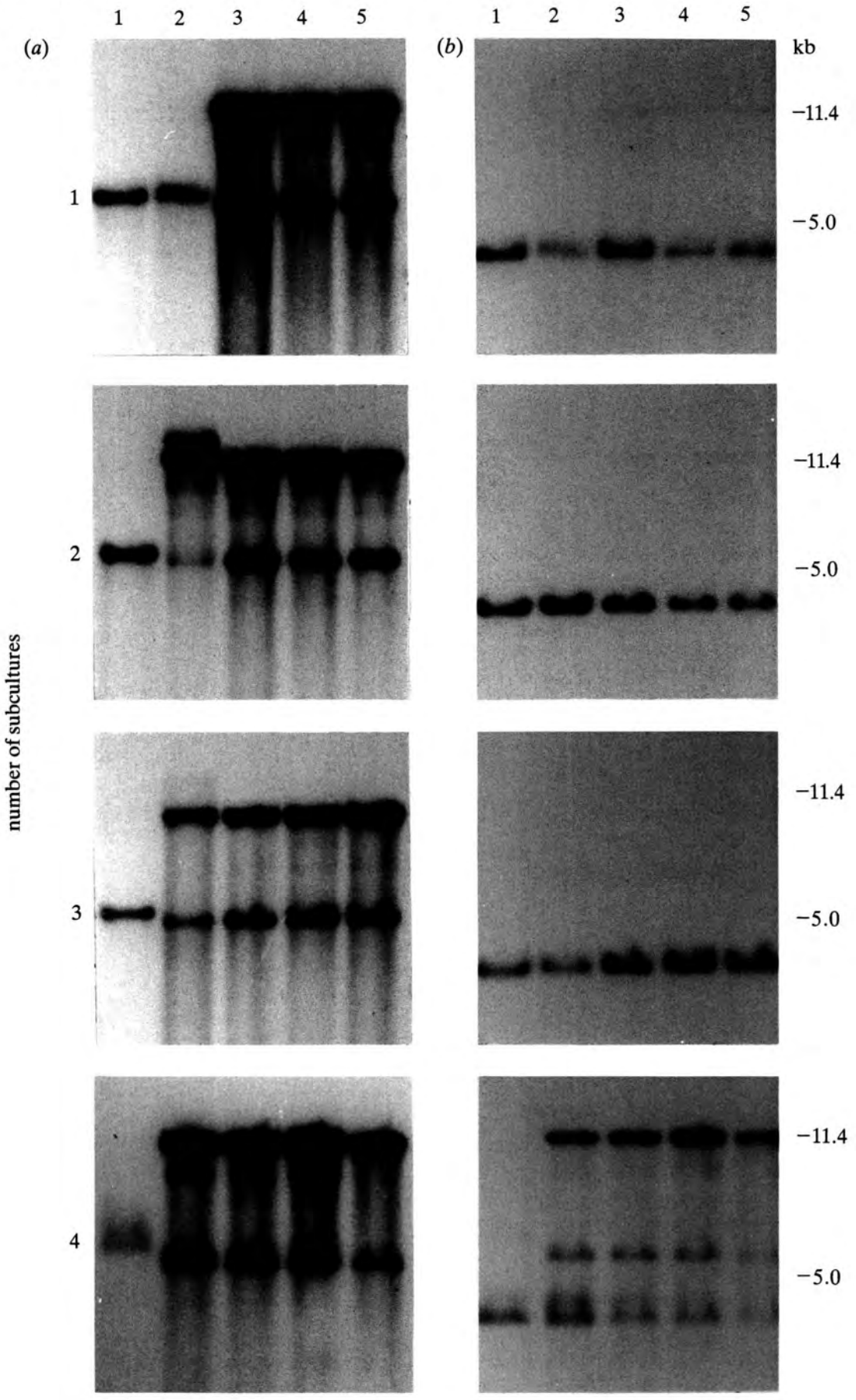

Figure 4. Analysis of genomic DNA isolated from C0 and Cd-tolerant lines (C1.4, C1.8, C2.6 and C3.2) after one, two, three and four subcultures. (*a*) Hybridization to *smtA*. (*b*) Hybridization to *psaE*. DNA isolated from: lane 1, C0; and tolerant lines, lane 2, C1.4; lane 3, C1.8; lane 4, C2.6; and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

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

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

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

4. DISCUSSION

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

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

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

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

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

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

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

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

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Deletion within the metallothionein locus of cadmium-tolerant *Synechococcus* PCC 6301 involving a highly iterated palindrome (HIP1)

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Summary

Genomic rearrangements involving amplification of metallothionein (MT) genes have been reported in metal-tolerant eukaryotes. Similarly, we have recently observed amplification and rearrangement of a prokaryotic MT locus, *smt*, in cells of *Synechococcus* PCC 6301 selected for Cd tolerance. Following the characterization of this locus, the altered *smt* region has now been isolated from a Cd-tolerant cell line, C3.2, and its nucleotide sequence determined. This has identified a deletion within *smtB*, which encodes a *trans*-acting repressor of *smt* transcription. Two identical palindromic octanucleotides (5'-GCGATCGC-3') traverse both borders of the excised element. This palindromic sequence is highly represented in the *smt* locus (7 occurrences in 1326 nucleotides) and analysis of the GenBank/EMBL/DDBJ DNA Nucleotide Sequence Data Libraries reveals that this is a highly iterated palindrome (HIP1) in other known sequences from *Synechococcus* species (estimated to occur at an average frequency of once every *c.* 664 bp). HIP1 is also abundant in the genomes of other cyanobacteria. The functional significance of *smtB* deletion and the possible role of HIP1 in genome plasticity and adaptation in cyanobacteria are discussed.

ntroduction

A transient increase in the metal tolerance of eukaryotic cells can be induced by prior treatment with sub-lethal concentrations of certain metal ions. This tolerance coincides with increased cellular metal-binding capacity due o transcriptional induction of metallothionein (MT) genes. A more stable tolerance can be selected by exposure of cell cultures to step-wise increases in metal ion concentrations. This tolerance is associated with an increase in MT gene copy number and a corresponding increase in MT mRNA and MT protein (Palmiter, 1987). Cu-resistant strains of *Saccharomyces cerevisiae* contain 10 or more tandemly amplified copies of a 2 kb genomic fragment carrying the *CUP1* gene, which encodes a Cu-binding protein, and an open reading frame (ORF) of unknown function. Strains containing only one copy of the amplifiable unit of DNA are relatively Cu-sensitive (Fogel and Welch, 1982; Karin *et al.*, 1984). Similarly, animal cell lines selected for Cd-resistance show amplification of MT genes and a corresponding increase in MT mRNA and polypeptides (Beach and Palmiter, 1981; Crawford *et al.*, 1985).

A gene corresponding to a previously reported cyanobacterial class-II MT (Olafson, 1984) has been isolated from *Synechococcus* PCC 7942 and designated *smtA* (Huckle *et al.*, 1992, accompanying paper). The MT locus also contains a divergently transcribed gene, *smtB*, which encodes a *trans*-acting repressor of transcription from the *smtA* operator-promoter (Huckle *et al.*, 1992, accompanying paper). Olafson (1986) observed a rapid adaptation to Cd tolerance in *Synechococcus* TX-20 (= PCC 6301) which coincided with an increase in cellular MT. This apparent acquisition of Cd tolerance was thought unlikely to be related to a chromosomal mutation since it would require a mutation frequency considered to be unreasonably high. It was therefore proposed that metal tolerance in these cells may be due to the amplification of an extrachromosomal gene (Olafson, 1984; 1986). Following the isolation of *smtA*, we have recently reported amplification (*c.* fourfold) and rearrangement of the *smt* locus in *Synechococcus* PCC 6301 cultures (PCC 6301 and PCC 7942 are considered to be of the same species: Wilmotte and Stam, 1984) selected for elevated tolerance to Cd (Gupta *et al.*, 1992). However, the *smtA* gene has been assigned to the chromosome (our unpublished observations), thereby raising additional questions concerning the swiftness of adaptation.

In order to characterize rearrangement of the *smt* locus occurring in a Cd-tolerant *Synechococcus* PCC 6301 cell line, C3.2, this paper reports the mapping, cloning and sequencing of the modified region followed by detailed analysis of an observed deletion within *smtB*.

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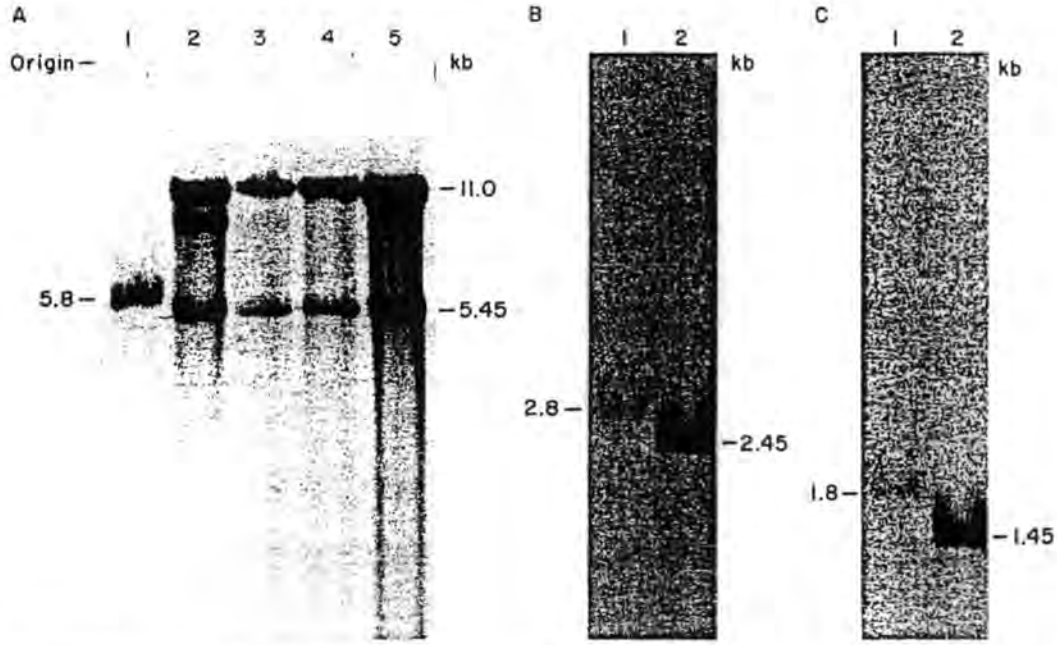

Fig. 1. Amplification and rearrangement of the *smt* locus.
A.Ten micrograms of *Sal*I-digested DNA from: non-selected *Synechococcus* PCC 6301 cells (lane 1) and cell lines tolerant to 1.4, 1.8, 2.6 and 3.2 μM Cd respectively (lanes 2–5). *Hin*dIII- (B) and *Hin*dIII–*Sal*I- (C) digested DNA from: a non-selected cell line (lane 1) and Cd-tolerant cell line C3.2 (lane 2).

Results

Mapping rearrangement of smt *in Cd-tolerant cells*

To identify a minimal fragment containing a previously observed rearrangement of the *smt* locus (Gupta *et al.*, 1992), *Sal*I-digested DNA from non-selected *Synechococcus* PCC 6301 (C0) and cell lines tolerant to, and maintained in, 1.4, 1.8, 2.6 and 3.2 μM Cd was probed with *smtA*. In all of these tolerant cells, two hybridizing fragments of *c.* 5.4 and 11 kb were apparent (Fig. 1A). In *Hin*dIII- and *Hin*dIII–*Sal*I-digested DNA from cell line C3.2, tolerant to 3.2 μM Cd, and also from (C0), single hybridizing fragments were detected but these were consistently *c.* 350 bp smaller in C3.2 than C0. This indicated rearrangement within the previously isolated and sequenced 1.8 kb *smt* region (Fig. 1, B and C).

Isolation of the altered smt *locus and localization of the modified region by PCR*

Two *Hin*dIII–*Sal*I genomic libraries of C3.2, derived from *Sal*I-digested genomic DNA of *c.* 5.4 and 11 kb, were screened for *smtA*. The *Hin*dIII–*Sal*I *smt* fragment was identical in size, *c.* 1.45 kb, when isolated from either the 5.4 or the 11 kb *Sal*I digested DNA. The clones were designated pAGNR12a (from the 5.4 kb *Sal*I fragment) and pAGNR13a (from the 11 kb *Sal*I fragment).

The altered region within the *smt* locus was localized by the polymerase chain reaction (PCR) with reactions performed using template DNA isolated from *Hin*dIII–*Sal*I genomic clone pJHNR49 of non-selected *Synechococcus* PCC 7942 (Huckle *et al.*, 1992, accompanying paper), pAGNR12a and pAGNR13a. M13 forward and reverse primers were used in conjunction with primers directing synthesis from the 3'-end of *smtA* into the 5' region (primer C) and from the 5'-end of *smtA* into the 3' region (primer N). Analysis of the PCR products (Fig. 2) showed that in comparison with products generated from pJHNR49 template DNA, both pAGNR12a and pAGNR13a gave lower molecular-weight products when generated with M13 reverse primer and *smtA* primer C, but identical molecular-weight products with M13 forward primer and primer N. The rearrangement was thus mapped to a 600 bp region in the 5' flank of *smtA* between the *Hin*dIII site and the primer N binding site in *smtA* in both pAGNR12a and pAGNR13a.

Nucleotide sequence analysis

The nucleotide sequence of the 5' *smtA* flanking region of pAGNR12a and pAGNR13a was determined and was identical in both. A fragment of 352 bp was missing from within a 360 bp region between nucleotides 100–459 (numbering refers to the complete sequence given by Huckle *et al.*, 1992, accompanying paper) inclusive (Fig. 3), while the remaining sequence was identical to that determined for the *Synechococcus* PCC 7942 *smt*

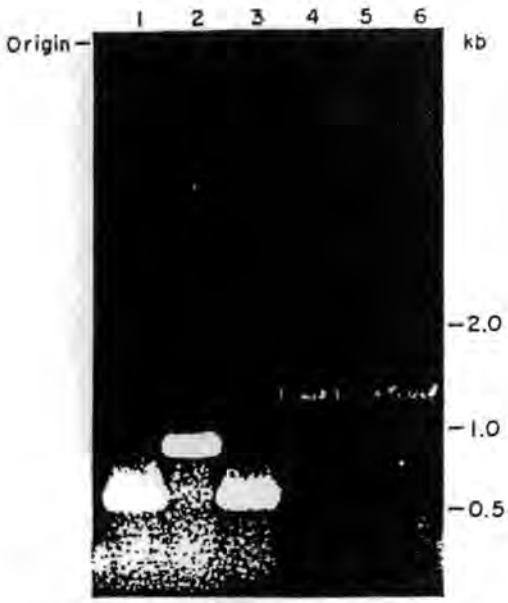

Fig. 2. Localization of the altered region by PCR. Gel photograph of PCR products using: M13-reverse primer and primer C (lanes 1-3); M13-forward primer (lanes 4–6), and primer N. The template was plasmid DNA from: Cd-tolerant cell line clone pAGNR12a (lanes 1 and 4); non-selected cell line clone pJHNR49 (lanes 2 and 5); and Cd-tolerant cell line clone pAGNR13a (lanes 3 and 6).

locus (Huckle *et al.*, 1992, accompanying paper). A deletion at this point disrupts the *smtB* coding region, but leaves the putative terminator intact.

Analysis of the borders of the excised fragment identified inverted repeat structures with 11 out of 12 nucleotides being identical (5'-GCGATCGCC[C/T]CG-3') at nucleotides 100–111 and 459–448 (numbering refers to the complete sequence given by Huckle *et al.*, 1992, accompanying paper) inclusive (Fig. 4). Within these inverted repeats is a palindromic sequence (5'-GCGATCGC-3'), the central motif of which is a Dam methylase recognition site (5'-GATC-3'). The palindromic sequence is highly represented in the *smt* locus, occurring seven times within 1.3 kb (Fig. 4) and has been designated HIP1 (for highly iterated palindromic sequence).

Databank library / analysis

More GenBank/EMBL/DDBJ DNA Nucleotide Sequence Data Library entries containing multiple HIP1 sequences were identified in *Synechococcus* than in any other prokaryotic genera, or group (data not shown). HIP1 was also prevalent in several other genera (or groups) of cyanobacteria.

Figure 5A shows the HIP1 frequencies for all genera (or groups) for which >10 kb of sequence is recorded in the data libraries and for which the estimated frequency is >0. *Achromobacter*, *Cellulomonas*, *Methylosinus*, *Plectonema*, *Propionibacterium*, *Pseudanabaena*, *Spirulina* and *Thermoactinomyces* have estimated HIP1 values of >0, but <10 kb of known sequence. The value shown on the graph equates to one HIP1 for every 664 bp in *Synechococcus*, which may be compared with one for every *c.* 31 kb in *Escherichia*. Extrapolation of the estimated HIP1 frequency in *Synechococcus* suggests *c.* 4500 HIP1 sequences per genome representing *c.* 1% of a *Synechococcus* genome.

HIP1 frequencies, estimated from data library analyses and also predicted from genomic G+C contents, are shown for the individual organisms (with >10 kb of known sequence) within the genera (or groups) with the highest (*Synechococcus*) or lowest (*Escherichia*) calculated HIP1 frequencies (Fig. 5B). The estimated HIP1 frequencies are not a direct function of chromosomal G+C content.

The frequency of HIP1 occurrence in the small plasmid pUH24 (7.835 kb) of *Synechococcus* PCC 7942 was calculated from the total nucleotide sequence (Van der Plas *et al.*, 1992) to be once every 435 bp. This raises interesting possibilities with respect to lateral gene transfer in cyanobacteria.

Discussion

We report the functional deletion of *smtB* in a Cd-tolerant cell line, C3.2, of *Synechococcus* PCC 6301. Increased (*c.* 20-fold) basal expression from an *smtA* operator-promoter has previously been reported in cells devoid of *smtB* (Huckle *et al.*, 1992, accompanying paper). The

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AAGCTTTACTACAACGAGCGCCGCTATCTACAGCAACTCGATCAAGAACGCTGCCTGAAT   60
CCCCAAGCATTCTTGGGCATGACAGAGCACGATGCTACTGCGATCGCCGCATGAGTCCCT  120
TGGCAGACTACCGTCTCTCCGTCCTGCAGCACTGGTTTTGTCATGAGCCAATCACGGTTT  180
GTCCACCCACCATACCTGAATCAAGATTCAGATGTTAGGCTAAACACATGAACAGTTATT  240
                Inverted repeat     -10      |
                                             smtA transcript start
          S.D           M  T  S  T  T  L  V  K  C  A  C  E
CAGATATTCAAAGGAGTTGCTGTCATGACCTCAACAACGTTGGTCAAATGCGCTTGTGAG  300
```

Fig. 3. Nucleotide sequence of part of the *smt* locus in Cd-tolerant cell line C3.2. 352 nucleotides from within a 360 bp region between nucleotides 100-459 inclusive, for the *smt* locus in non-selected cells (reported by Huckle *et al.*, 1992, accompanying paper), are missing in this sequence from Cd-tolerant cell line C3.2. The regenerated HIP1 sequence formed after excision is underlined. Other features of interest are marked and labelled. These sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide ~~Sequence Data Libra~~ries under the accession number 00000.

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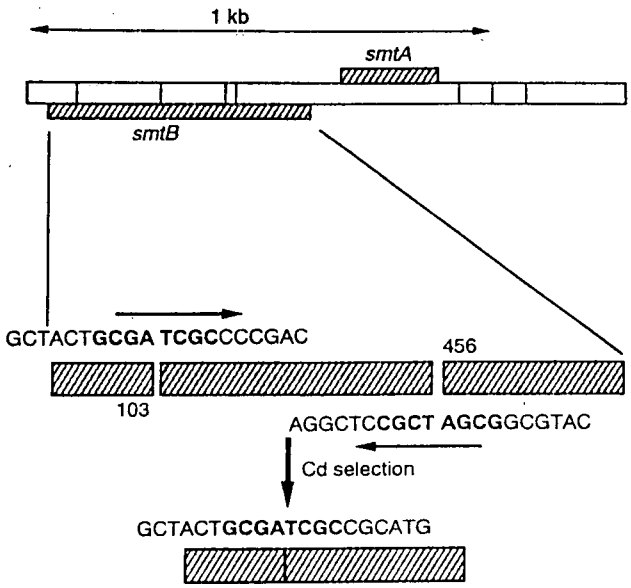
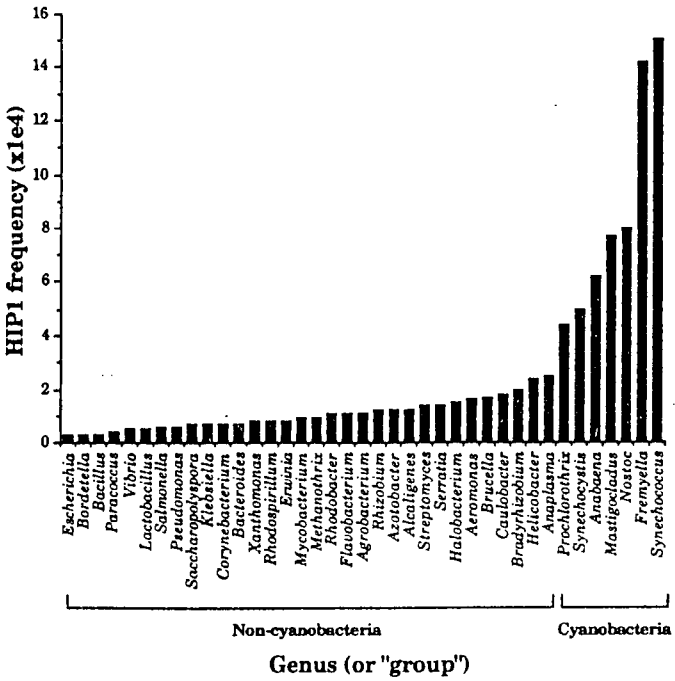


Fig. 4. Representation of the *smt* locus. A 1326 bp *Hin*dIII–*Nae*I fragment containing the *smt* locus from non-selected cells is represented, showing the *smtA* and *smtB* genes. Vertical lines represent HIP1 sequences. The expanded region shows the sequences traversing the deletion end-points, the HIP1 sequences are in bold. The horizontal arrows delineate an inverted repeat which includes, and extends beyond, the two HIP1 sites.

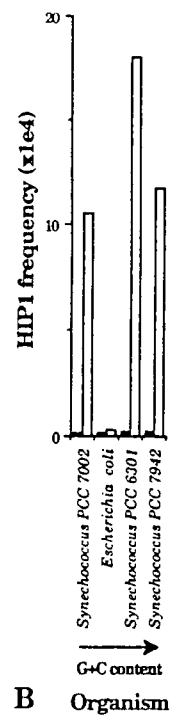
level of basal expression in these cells was *c.* fourfold greater than maximal expression observed in metal-induced cells which contain *smtB* (Huckle *et al.*, 1992, accompanying paper). Equivalent derepressed expression of *smtA* in C3.2 may be beneficial for such continuously metal-challenged cells. The increase in *smtA* copy number detected in C3.2 may further enhance expression.

Illegitimate recombination involving HIP1 sites could be explained by (i) replication slippage (copy-choice), (ii) DNA-breakage/reunion (nuclease/ligase activity) or (iii) a combination of replication and DNA-breakage. Within cyanobacteria, precise excision events associated with the terminal differentiation of an *Anabaena* PCC 7120 cell into a nitrogen-fixing heterocyst provides a well-documented example of protein-mediated (XisA) DNA-breakage-reunion between short defined regions (for a review, see Haselkorn, 1989). It is now necessary to define Cd exposure as either selective and/or stimulatory of *smtB* deletion.

Several genera (or groups) of cyanobacteria (and *Prochlorothrix*) contain HIP1 at a higher estimated frequency than other prokaryotes (Fig. 5). This may have significance for cyanobacterial taxonomy. In *Synechococcus* sp. sequence entries, HIP1 sequences are both intra- and intergenic. HIP1 sequences occurring within protein coding regions are found in all three possible reading frames; 5'-GCG ATC GC-3' (Ala–Ile–Ala), 5'-GC GAT CGC-3' (Xaa–Asp–Arg) and 5'-G CGA TCG C-3' (Xaa–Arg–Ser–Xaa). Previously described prokaryotic repetitive DNA sequences have not been found within protein coding regions and their greater size would restrict this. Known repetitive sequences include the *c.* 40 bp REP (repetitive extragenic palindromic) sequence (Higgins *et al.*, 1982;



A



B

Fig. 5. A. Estimated frequency of HIP1 in the genomes of different genera (or groups) in the DNA sequence databank. The frequency of HIP1 per nucleotide of sequence information for a given genus (or group) represented in the databank, is shown for all genera (or groups) for which there is at least 10 kb of recorded sequence and for which the estimated value exceeds zero.
B. The estimated frequency of HIP1 (open columns) in the genomes of individual species (with >10 kb of known sequence) in the genera (or groups) estimated to have the highest (*Synechococcus*) or lowest (*Escherichia*) HIP1 frequencies. The organisms are listed in order of increasing G+C content. Anticipated HIP1 frequencies predicted from the G+C content (closed columns) are shown for comparison.

Stern *et al.*, 1984) also called a PU (palindromic unit) (Gilson *et al.*, 1984; 1987) which is part of a larger element (BIME) (Gilson *et al.*, 1991); the *c.* 126 bp ERIC/IRU (enterobacterial repetitive intergenic consensus/intergenic repeat units) (Sharples and Lloyd, 1990; Hulton *et al.*, 1991); and STRR (short tandemly repeated repetitive) elements (Mazel *et al.*, 1990). In addition to known roles for REP/PU sequences in mRNA stability and gene expression, other functions have been suggested, including a possible involvement in chromosomal rearrangement (Stern *et al.*, 1984; for reviews see Higgins *et al.*, 1988; Lupski and Weinstock, 1992). REP/PU has been identified at the junction of tandem duplications (Shyamala *et al.*, 1990).

Repetitive genomic DNA has been reported in the heterocystous cyanobacterial strains *Anabaena, Calothrix* (*Fremyella*), *Nostoc* and *Fischerella*. The structure of the repeat is similar in all cases and consists of tandemly repeated heptanucleotide sequences which are clustered in intergenic regions (Alam *et al.*, 1986; Kallas *et al.*, 1988; McCarn *et al.*, 1988; Lang and Haselkorn, 1989; Mulligan and Haselkorn, 1989; Mazel *et al.*, 1990). Mazel *et al.* (1990) reported the presence of three classes of STRR sequences in the *Calothrix* (*Fremyella*) genome, all present at approximately 100 copies per genome. In addition, a highly iterated heptameric sequence detected in *Calothrix* (*Fremyella*) DNA by Mazel *et al.* (1990) is identical to the last seven nucleotides of HIP1.

The HIP1 sequence is distinct from previously reported, prokaryotic repetitive sequences such as REP/PU(BIME), ERIC/IRU and STRR in its small size its perfect palindromic structure; its phylogenetic distribution; its localizsation (also in protein coding regions); and its projected frequency of iteration (which is *c.* fourfold greater than REP/PU and 100-fold greater than STRR). The distribution of HIP1 could allow intragenic rearrangements, as observed here within *smtB*, and intergenic rearrangements. This may facilitate recombination between functional protein domains and/or the deletion and amplification of individual genes.

The deletion of a DNA fragment bordered by HIP1 sequences provides an example of its involvement in gene rearrangement. The over-representation of this motif in the genomes of a number of cyanobacteria suggests a widespread role for HIP1 in genome plasticity and cellular adaptation in these organisms.

Experimental procedures

Materials and cyanobacterial culture

Cultures of *Synechococcus* PCC 6301 tolerant to 1.4 μM (C1.4), 1.8 μM (C1.8), 2.6 μM (C2.6) and 3.2 μM (C3.2) Cd were selected by step-wise adaptation and cultured as previously described (Robinson *et al.*, 1990; Gupta *et al.*, 1992). Cultures were grown to mid-log phase in the presence of specified concentrations of added Cd before transfer to fresh media or isolation of genomic DNA. Restriction enzymes were supplied by Northumbria Biologicals Limited and Taq polymerase was supplied by Stratagene or Perkin-Elmer/Cetus. [a-^{32}P]-dCTP (14.8 TBq mmol^{-1}), nylon (Hybond N+) filters and Finebind DNA binding matrix were obtained from Amersham International.

Southern analysis, and cloning of the smt *locus from Cd-tolerant cell line C3.2*

Genomic DNA from non-selected and Cd-tolerant lines was analysed by Southern hybridization to *smtA* probe. The probe was prepared according to the procedure of Feinberg and Vogelstein (1983) using a fragment of the *smtA* gene released from pJHNR11 (Gupta *et al.*, 1992). To produce a size-fractionated genomic library, 10 μg of genomic DNA isolated from C3.2 was restricted with *Sal*I, and separated (two tracks) on a 0.7% agarose gel to allow identification of fragments hybridizing to *smtA* probe, and also for recovery of equivalently sized DNA (from the duplicate track). Recovered DNA was restricted with *Hin*dIII, ligated to *Sal*I–*Hin*dIII-restricted pGEM4Z (Promega) and used to transform *Escherichia coli* JM101 competent cells, prepared by the method of Alexander *et al.* (1984). Transformants containing the plasmids pAGNR12a and pAGNR13a were detected by standard colony hybridization techniques and DNA recovered by the standard alkaline-lysis protocol (Sambrook *et al.*, 1989). Sequences flanking the *smtA* gene(s) in pAGNR12a and pAGNR13a were also analysed by PCR using universal M13-forward and reverse primers, and *smtA N*-terminal and *C*-terminal primers as described by Robinson *et al.* (1990) (primer C, 5'-GGCGGATCCCCATGACCTCAACAACCTTGGTC-3' and primer N, 5'-GGCGAATTCACTACAGTCGCAGCCGGTGTGGCC-3'). Plasmid sequencing was performed by the dideoxy-sequencing method of Sanger *et al.* (1977) and reaction products analysed using an Applied Biosystems 370A DNA sequencer.

Computer analysis

All computer sequence analysis was performed on the SERC Daresbury facility DLVH using the UWGCG (Devereux *et al.*, 1984) and PIR programs. The number of HIP1 sequences per sequence entry was determined (in June 1992) by searching the bacterial entries within the 'GenBank' (version of the combined GenBank/EMBL/DDBJ Nucleotide Sequence Data Libraries) DNA sequence databank (Release 70.0) using the MATCH program within the PIR/NAQ package. The computer output was analysed to sum the total number of HIP1 sites in each genus (or group) and to identify entries containing multiple HIP1 sequences. Individual genera (or groups) were initially defined by the first name recorded in the databank under organism designation. It is noted that certain classifications, including *Synechococcus* and *Synechocystis*, have been described as groups and not genera (Waterbury and Rippka, 1989). Overlapping sequence entries within the databank for *Synechococcus* were subsequently eliminated, but this was not done for other genera (or groups). Databank entries under

the name *Anacystis nidulans* were pooled under *Synechococcus*. Similarly, *Fremyella* is used to denote the sequence entries of *Calothrix* and *Fremyella*, since *Fremyella* is most prevalent in the databank.

In order to estimate HIP1 frequencies, nucleotide counts were performed for all genera (or groups) containing HIP1 sites, defined by the previous search (bacteria not defined in the previous search have estimated HIP1 frequencies of zero), using the STRINGSEARCH program to list the size of all sequence entries for each genus. A program written and devised by Dr J. Parkhill (Dept of Biological Sciences, University of Birmingham, UK) was then used to sum the individual values for entry size to obtain an estimate of the total number of nucleotides in the databank for each genus (or group). Classifications containing less than 10 kb of sequence were eliminated.

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