Journal of Industrial Microbiology, (1995) 14, 119–125

Cyanobacterial metallothioneins: Biochemistry and molecular genetics

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(Received 4 April 1994; accepted 27 July 1994)

Key words: Zinc homeostasis; Cadmium tolerance; SmtB; SmtA; Metal-induced-transcription; Cyanobacteria; Metallothionein

SUMMARY

Metallothioneins have been extensively studied in many different eukaryotes where they sequester, and hence detoxify, excess amounts of certain metal ions. However, the precise functions of many of these molecules are not fully understood. This article reviews literature concerning their namesakes in prokaryotes.

INTRODUCTION

The name 'metallothionein' (MT) has become a generic term, applied to low-molecular-weight proteins or polypeptides which bind metal ions in metal-thiolate clusters and whose synthesis increases in response to elevated concentrations of certain metals [25]. Not surprisingly, this term now encompasses a collection of molecules with different primary structures, but all capable of forming such metal-thiolate clusters.

Proposed functions vary for some of the MTs occurring in different organisms. Nonetheless, class I MTs (see below for nomenclature) have similarity in amino acid sequence which suggests that they may share common role(s). In addition to metal detoxification (the spectrum of metals sequestered may vary for different MTs), several lines of evidence indicate other functions for some MTs including roles in Zn^{2+} homeostasis as it relates to the regulation of gene expression [64,68,69] and, increasingly, roles in protection against active oxygen species [2,57 and references cited therein].

Three classes of metallothionein

A protein associated with Cd^{2+}, and trace amounts of Zn^{2+} and Cu^{+}, isolated from the renal cortex of Cd^{2+}-intoxicated animals was the first molecule to be called a metallothionein [22,30]. The protein contained abundant cysteine residues, with characteristic cysteine-Xaa-cysteine motifs (where Xaa is an amino acid other than cysteine), no histidine and no aromatic amino acids [cited in 25]. Following the isolation of other molecules which meet the criteria to be called MT, three classes of MT were defined [9]:

Class I: Polypeptides with locations of cysteine closely related to those in equine renal MT. This includes most animal MTs [cited in 19], the Cu^{+}-MTs of *Neurospora crassa* [34] and *Agaricus bisporus* [35] and, subject to debate, certain polypeptides from higher plants [48].

Class II: Polypeptides with locations of cysteine only distantly related to those in equine renal MT. This includes polypeptides from sea urchin, a nematode, higher plants and the yeast *Saccharomyces cerevisiae* and *Candida glabrata* [cited in 19,48].

Class III: Atypical non-translationally synthesized metal-thiolate polypeptides. This includes secondary metabolites from higher plants, eukaryotic algae and certain fungi [for reviews refer to 17,44,47,48,55].

There are already a number of reviews describing eukaryotic MTs [13,17,20,21,23,42,44,46,48,55,60]. The aim of this article is to focus on the biochemistry and molecular genetics of MTs in prokaryotes.

EVIDENCE FOR METALLOTHIONEIN-LIKE PROTEINS IN PROKARYOTES AND THE ISOLATION OF CYANOBACTERIAL METALLOTHIONEIN GENES

Proteins termed 'MT-like' have only been described in two prokaryotic groups, *Synechococcus* sp. [29,36,38,40,56] and *Pseudomonas putida* [14]. Three low molecular weight cysteine rich metal-binding proteins were isolated from a Cd^{2+}-resistant strain of *P. putida* [14]. Metal-thiolate coordination, similar to eukaryotic MTs, was suggested by ^{113}Cd-NMR. These proteins remain to be sequenced.

Production of an MT-like protein was correlated with Cd^{2+} resistance in a cyanobacterium termed *Anacystis nidulans* [29]. Class II MTs have since been isolated from a marine cyanobacterium *Synechococcus* RRIMP NI [39,40], and freshwater strains *Synechococcus* UTEX-625 and *Synechococcus* TX-20 [36] (these strains, and very closely related strains, are also referred to as *Anacystis nidulans, Synechococcus* PCC 6301, *Synechococcus* PCC 7942 and *Synechococcus* R2). The MT from *Synechococcus* TX-20 was subsequently sequenced [41]. A metal-thiolate cluster, similar to that of eukaryotic MT but in a single domain, was suggested following spectroscopic studies [41].

Polymerase chain reaction products corresponding to part

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Abbreviations: MT, metallothionein; MRE, metal regulatory element.

of an MT gene were generated using template DNA from *Synechococcus* PCC 6301, the products sequenced and the gene called *smtA* [49]. These fragments were subsequently used as probes to isolate an MT divergon, *smt*, which includes *smtA*, and a divergently transcribed gene, *smtB* [16]. SmtA is identical to the polypeptide previously purified and sequenced by Olafson and co-workers [41], with the exception of a serine substitution for cysteine$_{32}$ and two additional amino acids at the carboxy-terminus (histidine and glycine). A gene from *Synechococcus vulcanus* encoding a polypeptide with similarity to SmtA, designed *mtnA*, has also been identified within the sequences flanking a previously characterized gene, *psaC* [52].

METAL-BINDING PROPERTIES OF CYANOBACTERIAL METALLOTHIONEIN

MT in *Synechococcus* sp. increased in abundance following exposure to elevated concentrations of Cd^{2+} or Zn^{2+}, but not Cu^{2+} [41]. The purified protein was associated with Cd^{2+} or Zn^{2+} (dependent upon the growth conditions) with copper as a minor component [41]. Takatera and Watanabe [56] similarly found that partly purified MT-like protein from Cd^{2+}-exposed *Anacystis nidulans* R2 (equivalent to *Synechococcus* PCC 7942) contained predominantly Cd^{2+} with lesser amounts of Zn^{2+} and copper ions. This may be contrasted with class II MTs in yeast which are 'normally' only synthesized in response to copper ions (and Ag^{+}) and associated *in vivo* with Cu^{+} rather than metals of the zinc triad [10,24].

SmtA has been expressed in *Escherichia coli* as a recombinant fusion protein [51]. The protein was associated with Zn^{2+}, Cd^{2+}, copper ions and Hg^{2+} (all metals examined) following purification from cells grown in metal-supplemented media. Enhanced accumulation of Zn^{2+} was also observed in these bacterial cells (in which production of SmtA is not metalloregulated) suggesting Zn^{2+}-binding *in vivo* [51]. Relative metal affinities were estimated from the pH at which 50% of the metal ions were displaced [51]. Compared to equine MT, recombinant SmtA had a greater affinity for Zn^{2+}, but lesser affinities for Cd^{2+} and copper ions.

Enhanced accumulation of Zn^{2+} in cyanobacteria (*Synechococcus* PCC 7942) with an intact *smt* divergon, as compared to mutants with an interrupted *smt* divergon, provides further evidence of Zn^{2+}-binding *in vivo* [63].

PHENOTYPIC ANALYSIS OF *smt*-DEFICIENT MUTANTS OF *SYNECHOCOCCUS* PCC 7942

Animal cell lines that lack MT gene expression, due to DNA methylation, are hypersensitive to Cd^{2+} [3]. Furthermore, transgenic animals in which genes encoding two MT isoforms had been disrupted, were unusually suspectible to hepatic poisoning by Cd^{2+} [31]. By contrast, mutants of *S. cerevisiae* deficient in the MT gene, *CUP1*, are hypersensitive to elevated concentrations of Cu^{2+} with no other phenotypic abnormality detected [58].

Cyanobacterial (*Synechococcus* PCC 7942) mutants with an interrupted *smt* divergon, *smt*$^{-}$, are sensitive (c. 5-fold reduction in tolerance) to Zn^{2+}, and show some reduction in tolerance to Cd^{2+} [62]. These cells retained normal tolerance to Cu^{2+} [62] and Hg^{2+} [61] indicating independence of Cu^{2+} and Hg^{2+} resistance from *smt*-mediated metal tolerance. Energy-dependent copper efflux has been proposed as a mechanism of Cu^{2+} resistance in *Synechococcus* sp. [37] and in another cyanobacterium *Nostoc calcicola* [65]. The sequence of a *Synechococcus* PCC 6301 gene encoding a putative P-type ATPase, started by Cozens and Walker [4], has recently been completed [cited in 54]. However, a 10-fold increase in copper resistance resulted from disruption of this gene [cited in 54], suggesting that this ATPase is involved in copper influx.

The *smt* divergon can be used as a marker to select for transformants derived from *smt*$^{-}$cells [62,63]. Cells containing re-introduced *smt* have been successfully isolated from *smt*$^{-}$ cells based upon restored tolerance to Zn^{2+} [62]. These cells also show restored tolerance to Cd^{2+}.

REGULATION OF SYNTHESIS OF *SYNECHOCOCCUS* METALLOTHIONEIN

Gene architecture and SmtB *protein sequence*

A 100-bp operator-promoter region lies between the *smtA* and *smtB* protein coding regions and contains divergent promoters (with similarity to *E. coli* −10 promoter consensus sequences) (Fig. 1) [16,33]. The deduced SmtB polypeptide shows sequence similarity to a number of bacterial proteins (Fig. 2), some of which are known to be transcriptional regulators and/or involved in metal metabolism. Furthermore, the deduced SmtB polypeptide contains a region that scores highly (5.5) on a prediction matrix [6] for a helix-turn-helix DNA binding motif (Fig. 2).

Within the sequences upstream (116 bp from the ATG) of *mtnA*, from *Synechococcus vulcanus* [52], is a divergently transcribed open reading frame (ORF) which has been partly sequenced. This encodes part of a protein with similarity to SmtB, which we will refer to hereafter as MtnB.

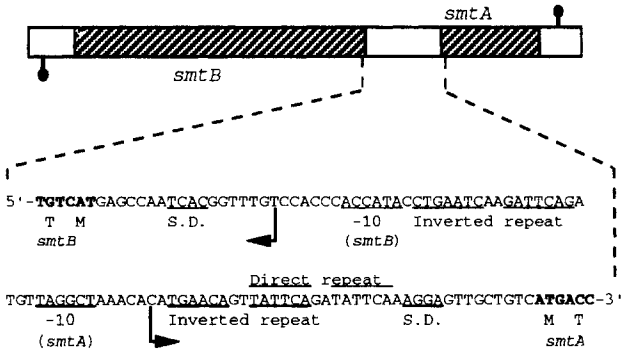

Fig. 1. Organization of the *smt* divergon. The protein coding regions of *smtA* and *smtB* (diagonal shading) are divergently transcribed and separated by a 100-bp operator-promoter region (sequence shown in full). Putative transcriptional terminators (circle), −10 consensus sequences (underlined), determined transcript start sites (bent arrows), Shine–Dalgarno sequences (S.D.) and inverted/direct repeats (under-/over-lined) are shown.

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        SmtB    MTKPVLQDGETVVCQGTHAAIASELQAIAPEVAQSLAEFFAVLADPNRLRLLSLL.ARSELCVGDLAQAIGVSE
R773    ArsR    ..............................MLQLTPLQLFKNLSDETRLGIVLLLREMGELCVCDLCMALDQSQ
pSX267  ArsR    ..............................MSYKELSTILKVLSDPSRLEILDLL.SCGELCACDLLEHFQFSQ
pI258   ArsR    ..............................MSYKELSTILKILSDSSRLEILDLL.SCGELCACDLLEHFQFSQ
pI258   CadC    ....MKKKDTCEIFCYDEEKVNRIQGDLQTVDISGVSQILKAIADENRAKITYALCQDEELCVCDIANILGVTI
OF4     CadC    ....VNKKDTCEIFCYDEEKVNRIQGDLKTIDIVSVAQMLKAIADENRAKITYALCQDEESCVCDIANIIGITA
        CadX    ....MSYENTCDVICVHEDKVNNALSFLEDDKSKKLLNILEKICDEKKLKIILSLIKEDELCVCDISLILKMSV
        MerR    ............MKSPALAGSLATAEVPCTHPDTTARFFRALADPTRLKLLQFI.LRGERTSAECVEHAGISQ
        NolR    ............MNFRMEHTMQPLPPEKHEDAEIAAGFLSAMANPKRLLILDSL.VKEEMAVGALAHKVGLSQ
Consensus       ....................................L....D..RL.I...L....ELCVCD.......S.

        SmtB    SAVSHQLRSLRNLRLVSYRKQGRHVYYQLQDH..HIVALYQNALDHLQECR.....................
R773    ArsR    PKISRHLAMLRESGILLDRKQGKWVHYRLSPHIPSWAAQIIEQAWLSQQDDVQVIARKLASVNCSGSSKAVCI
pSX267  ArsR    PTLSHHMKSLVDNELVTTRKNGNKHMYQL.NH..EFLDYINQNLDIINTSDQGCACKNMKSGEC.........
pI258   ArsR    PTLSHHMKSLVDNELVTTRKDGNKHWYQL.NH..AILDDIIQNLNIINTSNQRCVCKNVKSGDC.........
pI258   CadC    ANASHHLRTLYKQGVVNFRKEGKLALYSLGDEHIRQIMMIALAHKKEVKVNV.....................
OF4     CadC    ANASHHLRTLHKQGIVRYRKEGKLAFYSLDDEHIRQIMMIVLEHKKEVNVNV.....................
        CadX    ASTSHHLRLLYKNEVLDFYKDGKMAYYFIKDDEIREFFSKNHEGF.............................
        MerR    PRVSVHLSCLVDCGYVSARRDGKKLRYSVGDP..RVADLVMLARCLAADNAAALDCCTRIPGEGEQR......
        NolR    SALSQHLSKLRAQNLVSTRRDAQTIYYSSSSD..AVLKILGALSDIYGDDTDAVEEKPLVRKSA.........
Consensus       ...SHHL..L.....V..RK.G....Y.L.....................................
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Fig. 2. Multiple sequence alignment with SmtB. Amino acid sequences included are SmtB from *Synechococcus* PCC 7942; ArsR, *trans*-acting repressor of the *ars* operons from *Escherichia coli* (plasmid R773) [16,50], from *Staphylococcus xylosus* (plasmid pSX267) [16] and from *Staphylococcus aureus* (plasmid pI258) [16,18]; CadC, of unknown function that is essential for high level Cd^{2+} resistance, from *Staphylococcus aureus* (plasmid pI258) [16,67]; CadC, proposed to have a role in sodium/proton antiport, from *Bacillus firmus* OF4, [16,28]; CadX, of the *cadB* resistance operon, from *Staphylococcus aureus* (plasmid pII147) (K. Dyke, personal communication) [described by 53]; MerR, proposed regulator of the *mer* operon, from *Streptomyces lividans* [33, described by 53]; and NolR, regulator of *nod* gene expression, from *Rhizobium meliloti* [33]. A consensus sequence with a plurality of 6 is shown. The putative helix-turn-helix DNA binding motif of SmtB is underlined.

Increased transcription of smtA in response to metal ions

A range of metal ions (including Cd^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Au^{+}, Ag^{+}, Co^{2+}, Ni^{2+} and Bi^{3+}) induce MT synthesis in animal cells [cited in 19]. *cis*-acting metal-responsive elements (MREs) in the promoter region of animal MT genes are known [reviewed in 13,42]. Furthermore, *trans*-acting metal-responsive transcription factors that bind MREs in a metal-dependent manner and induce MT transcription, have been characterized [26,43]. Transcription from yeast class II MT genes in response to Cu^{2+} (or Ag^{+}) [10,24] is mediated by ACE1 (or CUP2). In its metallated form ACE1 binds to *cis*-acting MREs in the promoter region of the MT gene activating transcription [reviewed in 59].

The abundance of *smtA* transcripts increases in response to elevated concentrations of a number of trace metal ions (including Cd^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Co^{2+} and Ni^{2+}) but not heat shock [16]. However, at maximum permissive concentrations, only Zn^{2+}, and to a lesser extent Cd^{2+} and copper ions, increased (substantially) expression of a reporter-gene (*lacZ*) driven by the *smtA* operator-promoter region [16]. Accumulation of *smtA* transcripts in response to Cd^{2+} (Fig. 3) was shown to be exclusively mediated by increased transcription with no detectable effect of the metal ions on transcript stability [16].

In *smt*$^{-}$ mutants highly elevated expression of *lacZ* (driven by the *smtA* operator-promoter) was detected, even in the absence of added metal ions [16]. Repression, and metal-dependent expression, of *lacZ* was restored (at least in part) in cells containing plasmid-borne and/or chromosomal *smtB*. SmtB is thus a *trans*-acting repressor of expression from the *smtA* operator-promoter.

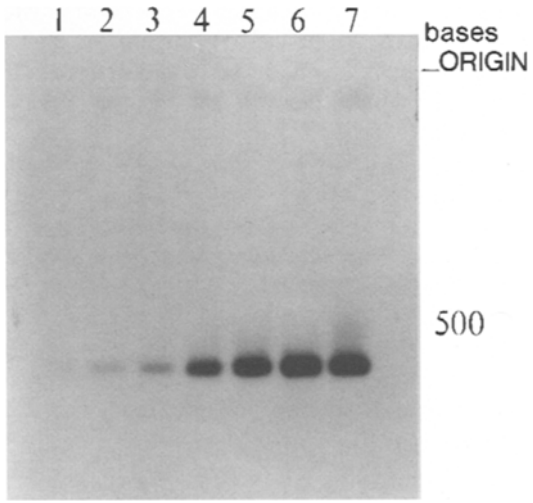

Fig. 3. Northern analysis of nucleic acid from *Synechococcus* PCC 6301 showing increase in *smtA* transcript abundance with length of exposure to $CdCl_2$. Total nucleic acid isolated from *Synechococcus* PCC 6301 cells incubated, under standard growth conditions, in the presence of 2.5 μM $CdCl_2$ for 0 min (lane1), 5 min (lane 2), 10 min (lane 3), 15 min (lane 4), 20 min (lane 5), 30 min (lane 6) or 60 min (lane 7), were resolved on a 1.5% agarose-formaldehyde gel and the subsequent northern blot was probed with *smtA* [15].

Protein interactions within the smt operator-promoter region

Three protein complexes with the *smt* operator-promoter (MAC1, MAC2 and MAC3, Fig. 4) have been identified by electrophoretic mobility shift assays [33]. MAC1 formed with a region of DNA immediately upstream of the ATG of *smtA* and was only observed in extracts from cells containing *smtB*

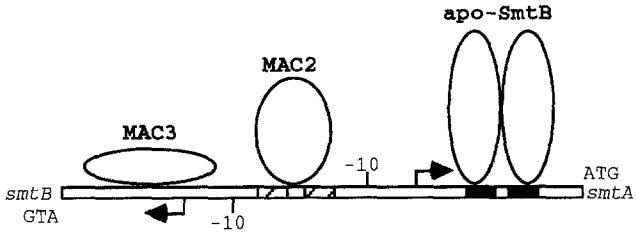

Fig. 4. Protein interactions with the *smt* operator-promoter region. Electrophoretic mobility shift assays have identified three protein-DNA complexes which form within the 100-bp *smt* operator-promoter region (represented in the diagram). One of the proteins, shown to correspond to apo-SmtB, binds to a region of DNA immediately upstream of *smtA* and dissociates in the presence of Zn^{2+}. The other two proteins which form the MAC2 and MAC3 complexes remain to be characterized. A 7-2-7 hyphenated inverted repeat (diagonal shading), an imperfect 6-2-6 hyphenated inverted repeat (solid) and transcription start sites (bent arrows) are marked.

(MAC2 and MAC3 were retained using extracts from *smt*$^-$ mutants). Based upon these observations, it was proposed that SmtB forms the protein component of MAC1 [33]. This proposal has most recently been confirmed following expression, affinity purification and sequencing, of SmtB in/from *E. coli* (unpublished observations, data not shown). The DNA-protein complex was less abundant using protein extracts from *Synechococcus* cells grown in the presence of elevated Zn^{2+}. Treatment with Zn^{2+} chelators facilitated reassociation in vitro indicating direct interaction of the protein component of MAC1 with metal. Metallation of SmtB has also been verified following production of SmtB in *E. coli* (unpublished observations, data not shown).

Helix-turn-helix DNA binding proteins generally bind to inverted repeats. A candidate SmtB-binding site is a degenerate 6-2-6 inverted repeat (TGAACA-GT-TATTCA) which also incorporates the left half of a 6-2-6 direct repeat (TATTCA-GA-TATTCA) (Fig. 5). A similar inverted repeat (TGAACA-GT-TGTTCA) is present within the operator-promoter region of the MT divergon, *mtn*, of *Synechococcus vulcanus* (Fig. 5). However, the locations of the repeats differ in *mtn* and *smt* in such a way as to suggest subtle variations in the mechanisms of transcriptional regulation.

The mode of action of SmtB appears similar to that of the regulator of the *ars* operon, ArsR (of the *E. coli* plasmid R773) [66], with SmtB acting as an inducible negative regulator of *smtA* transcription. This can be contrasted with the regulation of eukaryotic MT genes which are subject to metal-inducible positive regulation [26,43,59].

The regions of DNA involved in the formation of MAC2 and MAC3 have also been mapped by electrophoretic mobility shift assays using specific competitor DNA fragments [33] (Fig. 4). The MAC2 binding site contains a 7-2-7 hyphenated inverted repeat (CTGAATC-AA-GATTCAG) while MAC3 binds to a region most distal to *smtA*. Little is known about MAC2 and MAC3 although an activatory role has been suggested for MAC3 [33,63].

AMPLIFICATION AND REARRANGEMENT OF *smt* IN METAL-RESISTANT MUTANTS

Mammalian cell lines selected for Cd^{2+} resistance (which also show increased resistance to Zn^{2+}, Hg^{2+}, Cu^{2+} and Bi^{3+}) overproduce MT due, at least in part, to an increase in the number of copies of class I MT genes [5,7]. Mutliple tandem duplications of class II MT genes have also been observed in yeast cells selected for Cu^{2+} resistance [8,32].

Olafson and coworkers [40] noted a delay prior to growth of *Synechococcus* sp. in media supplemented with Cd^{2+}. The onset of growth coincided with accumulation of MT and extrachromosomal MT gene amplification was proposed. However, the *smt*-divergon has since been assigned to the chromosome [62], with the reservation that there may be a mega-plasmid in *Synechococcus* PCC 7942 [45]. Amplification [12] of *smtA* has subsequently been reported in *Synechococcus* PCC 6301 cells selected for Cd^{2+} resistance by stepwise adaptation.

Specific rearrangement within *smt* was also detected in some Cd^{2+}-tolerant *Synechococcus* PCC 6301 cell lines [11]. Further characterization revealed that a fragment of 352 bp was missing from within *smtB* (Fig. 6). It was considered that functional deletion of *smtB* may confer a selective advantage for continuously metal-challenged cells [11]. This has most recently been confirmed following reconstruction of an equivalent genotype in *Synechococcus* PCC 7942 [63].

An octameric palindrome (5′ GCGATCGC 3′) traversed the borders of the *smtB* deletion [11]. This palindrome, designed HIP1, is highly iterated within the region of the *smt* divergon (seven occurrences in 1326 nucleotides). Three HIP1 sites occur in similar locations in (and flanking) the *mtn* divergon of *Synechococcus vulcanus*, although it is noteworthy that sites in similar locations in the coding region of *mtnB* and *smtB* are in different reading frames (Fig. 6). HIP1 was estimated to be abundant in other cyanobacterial sequences within GenBank, occurring once every 664 bp in *Synechococcus* genera [11], and this has most recently been confirmed experimentally (unpublished observations, data not shown). HIP1 is proposed

smtA
5'-CCATACCTGAATCAAGATTCAGATTAGGCTAAACACA**TGAACA**GT**TATTCA**GATATTCAA**AGGA**GTTGCTGTC**ATG**-3'
-10 S.D. M

mtnA
5'-TTGACATA**TGAACA**GT**TGTTCA**AGTATACTGAACTAGAGGCGGAAAACAACCTCACTGATTCGTCATTTGG**AGGA**AAAGCC**ATG**-3
-35 -10 S.D. M

Fig. 5. Cyanobacterial MT promoter regions. The *smtA* and *mtnA* promoter regions are shown with −10 and −35 consensus sequences underlined (the *smtA* promoter has no region corresponding to an *E. coli* consensus −35 sequence) and Shine–Dalgarno sequences (S.D.) marked. A similar inverted repeat (bold/underlined) is present within the promoter region of both MT genes, although in *smt* it is imperfect and located 3′ of the extended −10 consensus sequence while in *mtn* it is located between the −10 and −35 consensus sequences.

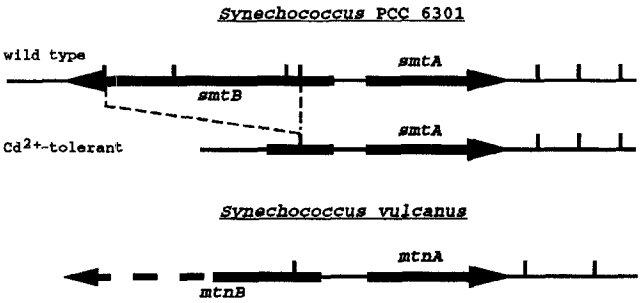

Fig. 6. Comparison of cyanobacterial metallothionein divergons and their HIP1 sites. Diagrammatic representation of the *smt* divergon (showing the MT gene, *smtA*, and the repressor gene, *smtB*) from wild type and Cd^{2+}-tolerant *Synechococcus* PCC 6301 and the *mtn* divergon (showing the MT gene, *mtnA*, and the partially sequenced ORF with similarity to *smtB*, *mtnB*) from *Synechococcus vulcanus*. The region of *smtB* which was lost in a Cd^{2+}-tolerant cell line (C3.2) is indicated. HIP1 sites within *smtB* and *mtnB* (solid line indicates the extent of known sequence) and at the 3′ ends of *smtA* and *mtnA* are shown (vertical lines).

to contribute to genome plasticity and hence adaptation to environmental change in cyanobacteria.

PROSPECTIVES AND PERSPECTIVES

Several lines of evidence suggest that the cyanobacterial MT divergon, *smt*, is involved in Zn^{2+} homeostasis. Mutants deficient in *smt* are hypersensitive to Zn^{2+} [62]. MT associates with, and accumulates in response to, Zn^{2+} in *Synechococcus* sp. [40]. Relative to equine MT recombinant SmtA has a high affinity for Zn^{2+} [51]. Expression from the *smtA* operator-promoter is maximally induced by Zn^{2+}, in comparison with other metal ions, at biologically significant (maximum permissive) concentrations [16].

A role for animal MT in Zn^{2+} homeostasis as it relates to the regulation of gene expression has been proposed [cited in 19]. Animal MT shows programmed expression during development and, at certain developmental states, is redistributed from the cytosol to the nucleus [cited in 1]. Zn^{2+} associated with animal MT is highly labile, a necessary attribute for an intracellular Zn^{2+} donor [cited in 19]. *In vitro* Zn^{2+} transfer between transcription factors and higher eukaryotic apo-MT has been demonstrated [68,69]. Zn^{2+} requiring transcription factors are not well characterized in prokaryotes. It has been proposed that prokaryotes may have avoided the 'hidden costs' of the precise Zn^{2+} homeostasis required for maintaining Zn^{2+} binding transcription factors [27]. However, intracellular Zn^{2+} buffering is likely to remain a requirement of these organisms, and *smt* may perform such a role in certain cyanobacteria. Viability of *smt*$^-$ mutants (in non-metal supplemented media) suggests no vital role for *smt*. Nonetheless, it remains a possibility that SmtA may perform functions in non-metal supplemented media (for example in the donation of metal ions to metallo-proteins), but other proteins could substitute for SmtA in its absence. It also remains to be established when/if *smt* is essential for survival in natural, generally more nutrient-limited, environments.

The mechanism by which *smt* confers increased tolerance to Zn^{2+} and Cd^{2+} is unknown. By analogy to eukaryotic MTs, SmtA may act as an intracellular 'sink' for metal ions. However, it has been considered that additional influx may negate such internal sequestration in cyanobacteria bathed in metal-containing media. SmtA may be part of a more dynamic mechanism of metal detoxification, possibly involving donation of metal ions to an efflux system, although the latter suggestion is not supported by observed metal accumulation characteristics of *smt*$^-$ mutants [63]. Clearly there is a need for rates of metal influx and efflux to be quantified in cells deficient in these genes.

There are potential applications for an environmentally-responsive cyanobacterial promoter in the controlled expression of 'transgenes' in these microorganisms. Metal-responsive expression from the *smtA* operator-promoter could be exploited for such work. A more detailed understanding of the mechanisms of *smt* metalloregulation, including the characterization of MAC3 and structure-function analyses of SmtB, is also needed. Gene expression driven by the *smt* operator-promoter may be applied to the detection of metals in aquatic environments. In addition, it has been considered that 'bioaccumulation' of trace metals could be engineered via modification of MT gene expression.

Amplification of, and rearrangement within, the MT divergon has been observed in cultured cyanobacteria selected for metal-tolerance via step-wise adaptation [11,12]. It will be of interest to examine whether similar mutations have occurred in cyanobacteria selected for metal resistance in metal-polluted 'natural' environments.

ACKNOWLEDGEMENTS

Work on the regulation of cyanobacterial metallothionein genes is supported by research grant GR/J37126 from the BBSRC. N.J.R. is a Royal Society University Research Fellow. We thank Dr J.W. Huckle for unpublished data shown in Fig. 3, Dr A.P. Morby for past collaborations/discussions and a draft version of Fig. 6, and P.D. Glands for unpublished observations relating to SmtB.

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