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Construction of Zn^{2+}/Cd^{2+}-tolerant cyanobacteria with a modified metallothionein divergon: Further analysis of the function and regulation of *smt*

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SUMMARY

This paper reports the (*de novo*) construction of mutants of *Synechococcus* PCC 7942 lacking the repressor (SmtB) of the metallothionein gene, *smtA*. These *smtA*$^+$/*B*$^-$ cells are more tolerant to elevated [Zn^{2+}] and [Cd^{2+}] than cells containing an intact metallothionein divergon (*smt*). Previously selected (by step-wise adaptation) Cd^{2+}-tolerant mutants contain additional copies of *smtA* and possibly other undetected mutations. It is now confirmed that these cells also contain a deletion within 'all' copies of *smtB* and hence fail to revert to wild type following subculture in medium which has not been supplemented with Cd^{2+} or Zn^{2+}. Northern analysis showed enhanced accumulation of *smtA* transcripts, even in the absence of added metal ions in these mutants. An increase in the accumulation of Zn^{2+} is reported in cells containing an intact metallothionein divergon compared to cells deficient in both *smtA* and *smtB*. This supports the assumption that SmtA binds Zn^{2+} within cyanobacterial cells. We also describe the use of the above mentioned mutants to identify additional factors involved in the regulation of transcription from the *smtA* operator-promoter.

INTRODUCTION

Olafson and co-workers [13] reported the amino acid sequence of a metal-associated protein isolated from cyanobacteria. This protein is called a class II metallothionein (MT) [14,18]. In *Synechococcus* PCC 7942, and PCC 6301, *smtA* (encoding the class II MT) is located adjacent to a divergently-transcribed gene, *smtB* [7]. From inception it was considered probable that the product of *smtA* might sequester, and hence contribute towards the detoxification of, certain metal ions in these microorganisms [15]. An examination of Zn^{2+} accumulation in cells deficient in class II MT is reported herein.

In *Synechococcus* PCC 7942 containing reporter gene (*lacZ*) fusions, the *smtA* operator-promoter conferred metal-dependent expression of *lacZ* [7]. Of all metal ions tested at maximum permissive concentrations, greatest expression was observed in response to Zn^{2+} [7]. Highly elevated, metal-independent, expression of *lacZ* was detected in cells deficient in both chromosomal *smtB* and *smtA* [7]. Re-introduction of plasmid-borne *smtB* reduced *lacZ* expression, and restored metal-dependency. SmtB has been confirmed to be a metal-responsive repressor of transcription from the *smtA* operator-promoter [7,10,18]. However, the *smtB*-deficient mutants used for *lacZ* expression studies were also deficient in functional *smtA*, and therefore would have failed to discern any possible (indirect) contributions of SmtA to expression.

Mutants deficient in both *smtA* and *smtB* are sensitive to Zn^{2+} and (to a lesser degree) Cd^{2+} [19]. By contrast, *Synechococcus* PCC 6301 selected (by step-wise adaptation) for Cd^{2+} tolerance show increased *smtA* copy number [6]. However, these resistant cells could contain additional mutations which remain to be detected. Indeed, two cloned *smt*-containing fragments from these cells had undergone a rearrangement such that *smtB* was incomplete [5]. It is anticipated that if these cells are devoid of all functional copies of *smtB* they should show elevated (derepressed) expression of *smtA*. To investigate this further, we report here a more detailed characterization of these Cd^{2+}-resistant mutants including an examination of *smtA* transcript abundance in both the presence and absence of added metal ions. New (more defined) *smtA*$^+$/*B*$^-$ mutants of *Synechococcus* PCC 7942 (which is considered to belong to the same species as *Synechococcus* PCC 6301 but is readily transformed [4,22]) have been created. Metal tolerance and regulation of gene expression from the *smtA* operator-promoter have been examined in these new mutants.

MATERIALS AND METHODS

Materials and general methods

Cyanobacterial strains used were: *Synechococcus* PCC 6301, referred to herein as WT; C3.2 [described in 5], a Cd^{2+}-tolerant mutant of *Synechococcus* PCC 6301, showing amplification and rearrangement of *smt* (involving a deletion within the *smtB*-coding region, Fig. 1); R2-PIM8 [21], a small

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Abbreviations: MT, metallothionein; OD, optical density.

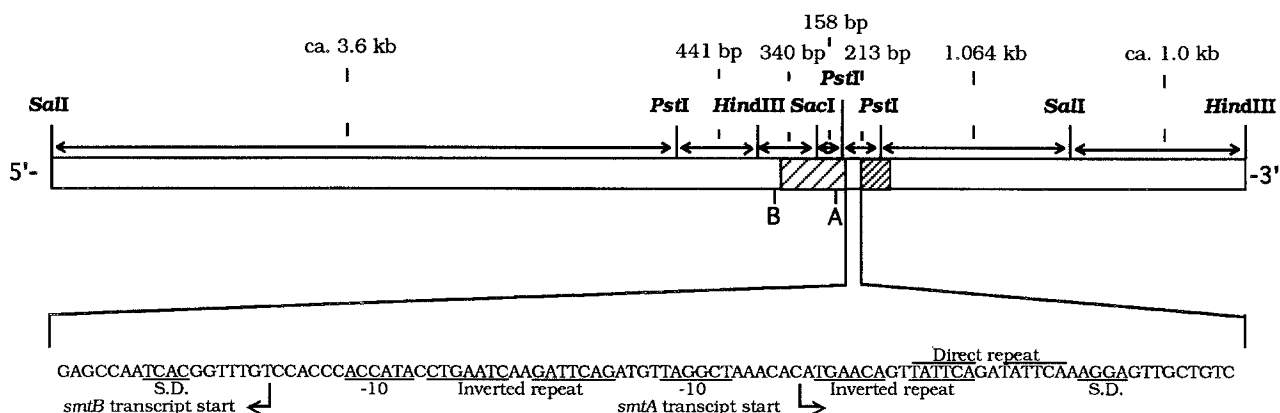


Fig. 1. Restriction map of the *smt* divergon. *smtA* and *smtB* are divergently transcribed and separated by a 100-bp operator-promoter region (sequence shown in full). Diagonal shading represents protein coding regions. Determined transcript start sites (bent arrows), –10 consensus sequences (underlined), Shine-Dalgarno sequences (S.D.) and inverted/direct repeats (under-/over-lined) are shown. A and B denote HIP1 sites involved in a deletion within Cd^{2+}-resistant mutant C3.2. A mutant lacking functional *smtA* and *smtB*, *smt*⁻, is deleted from the *Sac*I site to the 3′ distal *Pst*I site (which includes the operator-promoter region). A reporter gene construct, pB⁺, contains *smtA* 5′ sequences (including operator-promoter sequences and *smtB*) as far as the *Hin*dIII site. A truncated derivative of pB⁺, pB⁻, contains *smtA* 5′ sequences extending as far as the *Pst*I site within *smtB*.

plasmid-cured derivative of *Synechococcus* PCC 7942, referred to herein as R2; R2-PIM8(*smt*) [described in 19], a mutant of R2-PIM8 lacking functional *smtA* and *smtB* (in which the *smt* operator-promoter and 5′ regions of *smtA* and *smtB* are deleted, Fig. 1), referred to herein as *smt*⁻; *smtA*⁺/*B*⁺, *smt*⁻ with an intact *smt* divergon reintegrated into the chromosome (essentially equivalent to R2 [19]); and *smtA*⁺/*B*⁻, the novel mutants described herein. Cells were cultured as described previously [5,6,19].

Reporter gene constructs used were: pLACPB2 [16], carrying a promoterless *lacZ*, referred to herein as control; pLACPB2 (*smt*-5′) [described in 7], pLACPB2 containing *smtA* 5′ sequences (including the *smt* operator-promoter and *smtB*, Fig. 1), referred to herein as pB⁺; and pLACPB2 (*smtB*⁻) [described in 7], a deletion of pB⁺ (which contains the *smt* operator-promoter but has a deletion in *smtB*, Fig. 1), referred to herein as pB⁻.

DNA restriction/modification enzymes and agarose were supplied by Gibco-BRL Ltd, Paisley, UK. [α-^{32}P]dCTP (14.8 TBq $mmol^{-1}$) and nylon (Hybond N/N⁺) filters were obtained from Amersham International, Aylesbury, UK.

DNA isolation and Southern analyses were performed as described previously [6,19]. To examine *smtA* transcript abundance, total nucleic acids were isolated from cultures in mid-logarithmic phase, using standard techniques [2] and analysed as described previously [19]. DNA probes were prepared from an *smtA* polymerase chain reaction product [described in 15] and from restriction fragments of pJHNR49 (a *Sal*I/*Hin*dIII genomic *smt* fragment, from *Synechococcus* PCC 7942, in the vector pGEM4z [described in 7]), radiolabelled with [α-^{32}P]dCTP according to the procedure of Feinberg and Vogelstein [3].

Analysis of metal accumulation

Cells from mid/late logarithmic R2 and *smt*⁻ cultures were exposed to 0, 2.5 or 14 μM Zn^{2+} for up to 60 min under standard growth conditions. Zn^{2+}-exposed cells were harvested by centrifugation, washed in 50 mM Tris·HCl (pH 7.8), and solubilized overnight by incubation in 70% (v/v) HNO_3/water (atomic absorption spectrometer grade HNO_3, BDH). Metal content was determined by atomic absorption spectrometry (using a Perkin Elmer model HGA spectrophotometer according to manufacturer's protocols) and converted to amount of metal per 1×10^9 cells (assuming an optical density, OD, of 1 at 540 nm is equivalent to 3×10^8 cells ml^{-1}). ODs were estimated at the time of metal induction and immediately prior to centrifugation. No changes were detected during the incubation period.

Construction, and phenotypic analysis, of an smtB⁻ mutant

Zn^{2+}-sensitive *smt*⁻ cells were transformed to Zn^{2+}-tolerance with a 1423-bp *Sal*I/*Hin*dIII *smt* fragment (*smtA*⁺/*B*⁻) obtained from pAGNR12a (a *Sal*I/*Hin*dIII genomic *smt* fragment, from C3.2, in the vector pGEM4z [5]) using techniques described previously [19]. Transformants were selected on Allens agar (Bacto-Agar, Difco Laboratories, Detroit, MI, USA) plates [1] supplemented with 20 μM Zn^{2+}.

Reporter gene constructs were introduced into R2, *smt*⁻ and *smtA*⁺/*B*⁻ transformants essentially as described by van den Hondel and co-workers [20]. Recombinants were selected on Allens agar plates supplemented with chloramphenicol (7.5 μg ml^{-1}) and carbenicillin (50 μg ml^{-1}). β-galactosidase assays were performed using *o*-nitrophenol-β-D-galactopyranoside (ONPG) as the substrate [9]. OD readings at 414 nm and 595 nm were normalized against water and Allens media, respectively. Cultures were used with an OD at 595 nm of c. 0.08. Cells were lysed with chloroform/SDS. β-galactosidase activity was calculated using a modified equation: Activity (nmoles *o*-nitrophenol min^{-1} mg $protein^{-1}$) = $300(OD_{414(t)} - OD_{414(t=0)})/1.83\ (t \times v \times OD_{595})$, where t = time of reaction (min), v = volume of culture used in the assay (ml). Growth of R2 and *smtA*⁺/*B*⁻ transformants, in metal-sup-

plemented Allens media, was examined as previously described [19].

RESULTS

Accumulation of Zn^{2+} in an smt^- mutant

Cyanobacterial MT has been purified in association with certain metal ions and is assumed to sequester these ions *in vivo* [11,12,17]. To further test this assumption metal accumulation characteristics of smt^- mutants, deficient in both functional *smtA* and *smtB*, were examined. Cells (R2) containing the wild-type *smt* divergon accumulated more Zn^{2+} than smt^- mutants in each of three replicate experiments (Fig. 2). This was observed in cultures exposed (for 30 or 60 min) to a concentration of Zn^{2+} (2.5 μM) that allowed growth of both cell types and also at a concentration lethal to smt^- (14 μM).

Genotypic analysis of a Cd^{2+}-selected mutant after subsequent culture in Cd^{2+}-free media

Cells selected by step-wise adaptation for Cd^{2+} resistance (line C3.2) have previously been shown to contain increased *smtA* copy number [6]. In addition, two different *smt* fragments isolated from these cells contained a deletion within *smtB* [5]. If cell line C3.2 is deficient in all functional copies of *smtB*, elevated transcription of *smtA* would be anticipated even in the absence of metal supplements.

No fragments corresponding to an intact *smt* divergon (c. 5.8 kb for *Sal*I and c. 1.8 kb for *Sal*I/*Hin*dIII) were detected in DNA isolated from C3.2 following subculture in non-metal supplemented media (Fig. 3). However, fragments corresponding to *smt* with a deletion within *smtB* (c. 5.4 kb and 11 kb for *Sal*I and c. 1.4 kb for *Sal*I/*Hin*dIII) were observed. Furthermore, no hybridization was detected using part of the *smtB* deletion as a probe (data not shown). Figure 4 shows that total nucleic acid isolated from both C3.2 and 'wild-type' (WT) cells contained (relatively abundant) *smtA* transcripts (c. 300 bases) following a brief (2 h) exposure to Cd^{2+} (1.4 μM). In WT cells transcripts declined to undetected levels after return to Cd^{2+}-free media for a single subculture. However, *smtA* transcripts had previously remained abundant in C3.2 following three and five subcultures in the absence of Cd^{2+} (Fig. 4). Transcripts were most abundant in C3.2 exposed to Cd^{2+}, revealing an element of metallo-induction even in these cells devoid of SmtB.

Construction of an $smtB^-$ mutant and analysis of its phenotype

In addition to deletion within *smtB*, C3.2 contains additional copies of *smtA* and potentially other mutations selected during step-wise adaptation. To examine whether deletion within *smtB* alone can confer increased expression from the *smtA* operator-promoter, and hence increased metal tolerance, an $smtA^+/B^-$ genotype has been created. smt^- cells were transformed to Zn^{2+} tolerance via integration of a DNA fragment containing the modified *smt* divergon derived from C3.2. The structure of the *smt* region of two transformants was confirmed by Southern analysis (Fig. 5). A fragment containing the *smt* operator-promoter, absent from smt^- [19], hybridized to restricted DNA from both transformants (consistent with their recombinant status). Fragment sizes were

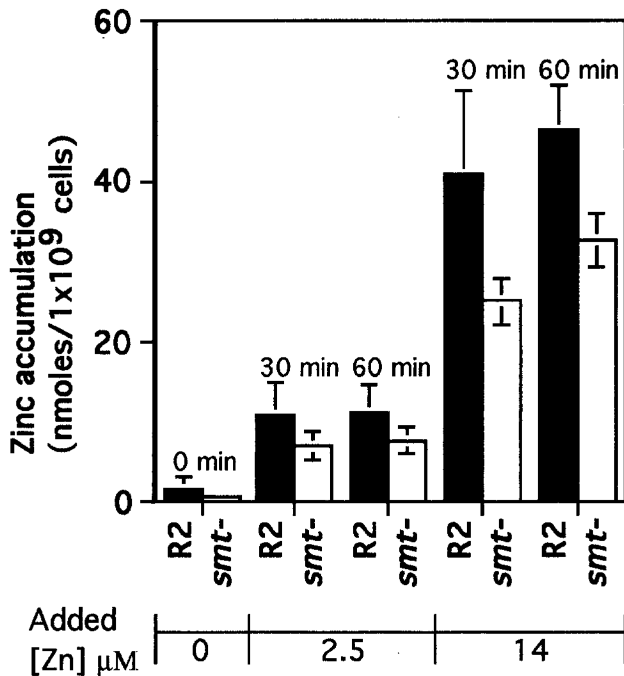


Fig. 2. Zn^{2+} accumulated by R2 and smt^- exposed to $ZnCl_2$. Cells exposed to 0 μM Zn^{2+}, 2.5 μM Zn^{2+} for 30 and 60 min or 14 μM Zn^{2+} for 30 and 60 min, were washed in 50 mM Tris·HCl (pH 7.8) and the Zn^{2+} content of 1×10^9 cells calculated. Data points represent the mean values from three replicate experiments, with standard deviation.

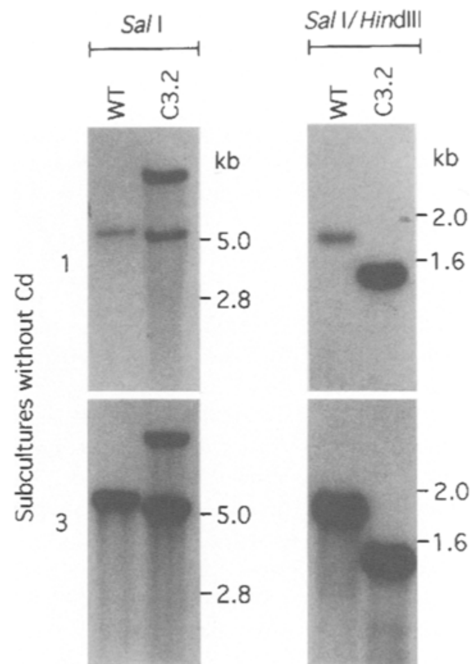


Fig. 3. Southern analyses of DNA from WT and Cd^{2+}-selected mutant, C3.2. *Sal*I and *Hin*dIII/*Sal*I digested DNA, isolated from WT and C3.2 following one and three subcultures in the absence of added Cd^{2+}, was resolved on 0.7% (w/v) agarose gels, transferred to nylon filters and probed with *smtA*.

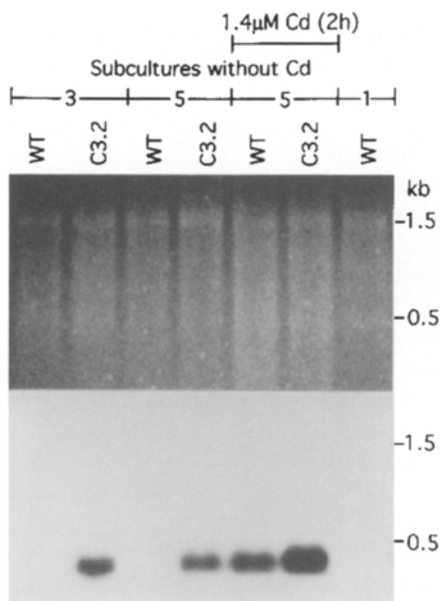


Fig. 4. Northern analysis of nucleic acid from WT and C3.2. Total nucleic acid was isolated from WT and C3.2 following; three and five subcultures in the absence of added Cd^{2+}; exposure to 1.4 μM Cd^{2+} for 2 h subsequent to five subcultures in the absence of added Cd^{2+}; and one subculture in the absence of added Cd^{2+} subsequent to the latter treatment (WT only). Nucleic acids were resolved on a 1.5% agarose-formaldehyde gel (upper panel) and the subsequent northern blot (lower panel) was probed with *smtA*.

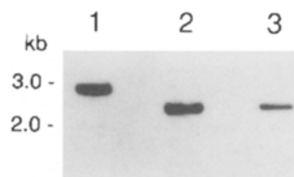


Fig. 5. Southern analysis of DNA from R2 and *smtA*$^+$/*B*$^-$ transformants. *Hin*dIII digested DNA from R2 (lane 1) and two *smtA*$^+$/*B*$^-$ transformants (lanes 2 and 3) was resolved on a 0.8% (w/v) agarose gel, transferred to a nylon filter and probed with a 213-bp *Pst*I fragment of *smt* (containing the *smt* operator-promoter, Fig. 1).

smaller than those for R2 confirming integration of the mutant divergon (with deleted *smtB*). These cells are hereafter referred to as *smtA*$^+$/*B*$^-$.

Figure 6 (column 3) shows that in *smtA*$^+$/*B*$^-$ expression of a reporter gene (*lacZ*) driven by the *smtA* operator-promoter (in pB$^-$) is elevated in the absence of added metal ions. However, no further increase in expression of the reporter gene was detected in response to added Zn^{2+}. Introduction of plasmid-borne *smtB* (pB$^+$) reduces this expression and restores metal-dependency (column 2), as anticipated. Several other features are also apparent from these data (Fig. 6), including the observations that; expression from pB$^-$ is greater in *smt*$^-$ cells than in *smtA*$^+$/*B*$^-$ (column 9 compared to column 3); in the absence of added metal ions expression from pB$^-$ is elevated in R2 despite the presence of chromosomal *smtB* (column 6).

Minimum concentrations of Zn^{2+} and Cd^{2+} which give

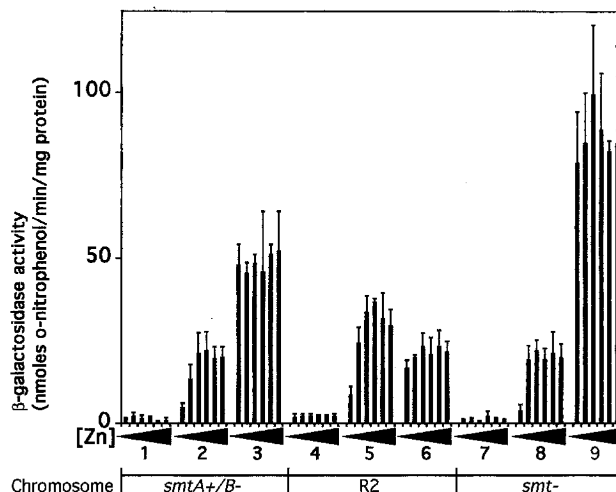
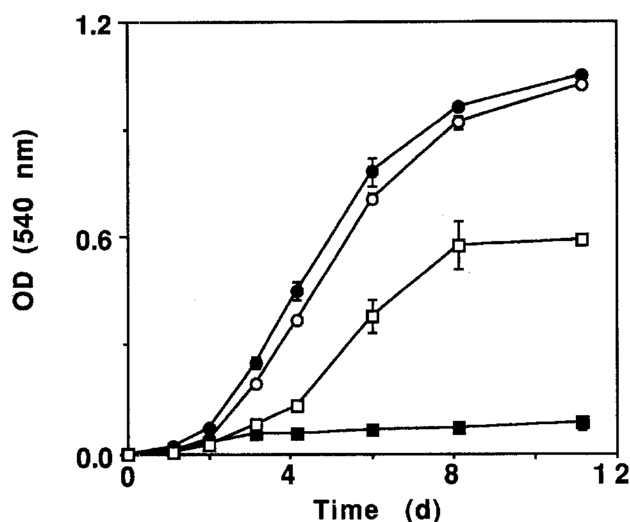


Fig. 6. β-galactosidase activity in *smtA*$^+$/*B*$^-$, R2 and *smt*$^-$. β-galactosidase activity measured in *smtA*$^+$/*B*$^-$ (columns 1, 2 and 3), R2 (columns 4, 5 and 6) and *smt*$^-$ (columns 7, 8 and 9) containing *lacZ* in the control (columns 1, 4 and 7), pB$^+$ (columns 2, 5 and 8) or pB$^-$ (columns 3, 6 and 9) plasmids. Cells were exposed to a range of concentrations of Zn^{2+} (0, 2.5 μM, 11 μM, 12 μM, 14 μM, or 16 μM) for 2 h prior to assay. Data points represent the mean values from three replicate experiments, with standard deviation.

some inhibition of growth of *smtA*$^+$/*B*$^-$ were estimated (data not shown). Growth of *smtA*$^+$/*B*$^+$ and *smtA*$^+$/*B*$^-$ was subsequently examined as a function of time in response to selected metal concentrations (Fig. 7). *smtA*$^+$/*B*$^+$ has a genotype equivalent to R2 (detected by Southern analyses) and identical growth characteristics to R2 [19], however *smtA*$^+$/*B*$^+$ has been constructed by similar procedures to *smtA*$^+$/*B*$^-$ and is therefore the preferred control. Most importantly, growth of *smtA*$^+$/*B*$^-$ was greater than *smtA*$^+$/*B*$^+$ at high metal concentrations (16 μM Zn^{2+}, 3 μM Cd^{2+}) (Fig. 7). It is noted that in past experiments [19] growth of R2 (and *smtA*$^+$/*B*$^+$) has been observed in 3 μM Cd^{2+} (although only following a prolonged lag) and R2 (consistently) shows growth at higher concentrations of Cd^{2+} than *smt*$^-$ mutants. Figure 7 also shows that in non-metal supplemented media there is a slight, but reproducible, reduction in apparent growth of *smtA*$^+$/*B*$^-$, relative to *smtA*$^+$/*B*$^+$. Cell number counts rather than OD measurements are required to confirm, or otherwise, this apparent subtle difference in growth rates, although previous experiments (data not shown) have shown a linear relationship between these two parameters in R2 cultures.

DISCUSSION

Increased accumulation of Zn^{2+} in R2 compared to *smt*$^-$ mutants supports the assertion that SmtA binds these metal ions within cyanobacteria (Fig. 2). Previous studies had already shown that expression of a recombinant SmtA fusion protein conferred enhanced accumulation of these ions in *Escherichia coli* [17]. SmtA clearly has a role in metal tolerance, although the mechanism of *smt*-mediated metal detoxification has not been defined [19]. By analogy to eukaryotic MTs, SmtA may serve as an intracellular 'sink' for metal ions.



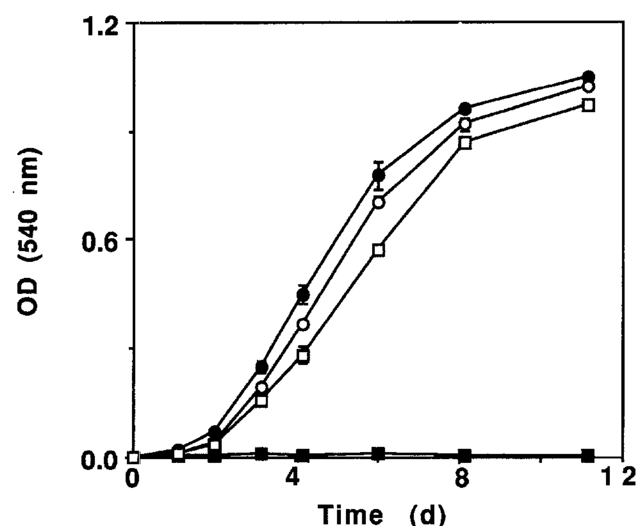


Fig. 7. Growth of *smtA*$^+$/*B*$^-$ and *smtA*$^+$/*B*$^+$ in medium supplemented with $ZnCl_2$ or $CdCl_2$. Growth of *smtA*$^+$/*B*$^-$ (open symbols) and *smtA*$^+$/*B*$^+$ (closed symbols) was examined in Allens medium supplemented with $ZnCl_2$ or $CdCl_2$ as a function of time. Cultures were inoculated at a cell density of 1×10^6 cells ml^{-1} with added: Upper panel, 0 (circles) or 16 (squares) μM Zn^{2+}; lower panel, 0 (circles) or 3 (squares) μM Cd^{2+}. Growth was estimated by measuring the OD at 540 nm. Data points represent the mean values from three separate cultures, with standard deviation. In addition, equivalent graphs were obtained in two further experiments (data not shown).

However, it has been considered that additional influx may negate such internal sequestration in an organism bathed in metal-containing media and that SmtA may be part of a more dynamic mechanism of metal detoxification [18]. Nonetheless, CUP1 is thought to perform an analogous role in the sequestration/detoxification of copper in yeast [cited in 18], and the data presented in Fig. 2 argue against a role for SmtA in donating metal ions to an efflux system. It therefore remains likely that *smt*-mediated metal tolerance is merely a function of the capacity of SmtA to sequester these ions within cyanobacteria. While it appears that *smt* causes some additional accumulation of Zn^{2+} it is proposed that this is less than the total amount of metal sequestered, SmtA thereby effecting 'some' reduction in the unbound cytoplasmic metal ion concentration.

The observation that cloned *smt* regions from a Cd^{2+}-tolerant cell line were mutated within *smtB* led to the proposal that loss of this repressor would enhance expression of *smtA*. This could in turn contribute towards metal tolerance [5]. Overexpression of *smtA* transcripts, even in non-metal supplemented media, has now been confirmed in these cells (Fig. 4). Furthermore it has been possible to engineer *de novo* metal-tolerant cyanobacteria by re-creating an *smtA*$^+$/*B*$^-$ genotype (Figs 5–7). These studies confirm that the previously observed [5] HIP1-mediated deletion within *smtB* does confer a selective advantage to cells continuously challenged with elevated concentrations of metal ions. In addition, the present work confirms that previously observed reduced metal tolerance of *smt*$^-$ mutants [19] is a function of the loss of *smtA* and not the accompanying loss of *smtB*.

It is of interest that growth of *smtA*$^+$/*B*$^-$ appears (very slightly) compromised when cultured in non-metal supplemented media (Fig. 7). This may be the result of excessive sequestration of essential metal ions by SmtA. Nonetheless, there was no evidence of reversion when cell line C3.2 was subcultured for many generations in the absence of added metal ions (Fig. 3). Presumably, the absence of any residual copies of the deleted region of *smtB* precluded this.

In addition to elevated 'constitutive' (in the absence of added metal ions) expression of *smtA* in C3.2, these data (Fig. 4) also show an 'unexpected' further increase in *smtA* transcript abundance in these cells (deficient in the metal-responsive repressor SmtB) upon exposure to Cd^{2+}. By contrast, Fig. 6 (column 3) shows no corresponding metal-induced expression (from the *smtA* operator-promoter) in *smtA*$^+$/*B*$^-$ in the absence of *smtB* (using plasmid pB$^-$). This could reflect an element of post-transcriptional control of *smtA* transcript abundance, although no metal-dependent changes in *smtA* transcript stability were detected in previous studies using R2 [7]. Comparison of the *smtA* 5′ flanking regions retained in cell line C3.2 and in pB$^-$ reveals the latter to be truncated by 44 bp (this 44-bp region coincides with part of the *smtB*-coding region). It has previously been suggested that there may be a 'remote' *cis*-acting element contained within the *smtB*-coding region required for full expression of *smtA*, which had been lost from pB$^-$ (previously termed pLACPB2(*smtB*$^-$) [10]. The 44-bp region contains a 5-3-5 hyphenated direct repeat (CCGTC-TCT-CCGTC) which is a candidate binding site for the putative metal-responsive activator.

SmtB-dependent repression (in the absence of added metal ions) is most pronounced when *smtB* is associated with (and divergently transcribed from) the *smtA* operator-promoter (Fig. 6, compare columns 5 and 6). In non-metal supplemented media, expression from pB$^+$ is always lower than expression from pB$^-$ even in cells containing chromosomal *smtB*. This indicates that SmtB (in common with many other known bacterial regulators [8]) acts more efficiently when synthesized

adjacent to its target DNA-binding site. Figure 6 also reveals that expression from the *smtA* operator-promoter (in pB^-) is strikingly reduced in the recreated metal-tolerant mutants, $smtA^+/B^-$, compared to previously studied [7] *smtB*-deficient mutants, smt^- (compare columns 3 and 9). An obvious difference between these two cell types is the absence of *smtA* in the latter. An equivalent reduction in expression was previously observed in smt^- cells when a truncated version of pB^- was used [10]. This truncation involved the loss of a region of the *smt* operator-promoter required for formation of an identified DNA-protein complex (MAC3) [10]. MAC3 was suggested to be an activator [10]. The current observations would support a model in which the MAC3-activator is subject to feedback inhibition by SmtA. The influence of SmtA on MAC3 may be indirect but, most importantly, this inhibition is not overcome by elevated Zn^{2+} (Fig. 6). It is now apparent that maximum expression from the *smtA* operator-promoter is not attained even in response to maximum permissive, or to inhibitory, metal concentrations when this feedback inhibition is operational (in cells containing SmtA). This could reflect, as yet unidentified, roles for SmtA in other (than metal-detoxification) biochemical processes.

At least two types of mutation were known to have occurred in Cd^{2+}- tolerant cell line C3.2 [5,6]. Based upon one of these it has now been possible to engineer, *de novo*, Zn^{2+}/Cd^{2+}-resistant cyanobacteria via generation of cells deficient in the MT repressor, SmtB. These studies have also revealed the importance of other (than SmtB) regulatory factors in the control of *smtA* expression. Modification of these other regulatory pathways may facilitate the engineering of even higher levels of expression from the *smtA* operator-promoter, potentially conferring greater metal resistance (and possibly greater metal accumulation).

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