Construction of Zn\textsuperscript{2+}/Cd\textsuperscript{2+} Hypersensitive Cyanobacterial Mutants Lacking a Functional Metallothionein Locus*

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Eukaryotic metallothioneins (MTs) have been extensively studied, but the precise functions of most of these molecules are not yet fully understood. Prokaryotes are often more tractable for the analysis of gene function and we report here the generation of mutants of *Synechococcus* PCC 7942 (strain R2-PIM8) deficient in the MT locus, smt. Viability of these mutants, designated R2-PIM8(smt), reveals that prokaryotic MT performs no "vital" role (such as donation of metals to metallo-proteins) in *Synechococcus*. R2-PIM8(smt) has reduced (~5-fold) tolerance to elevated Zn\textsuperscript{2+}, with detectable hypersensitivity to Cd\textsuperscript{2+}. Restoration of Zn\textsuperscript{2+} tolerance was used as a selectable marker to isolate recombinants derived from R2-PIM8(smt) after reintroduction of a linear DNA fragment containing an uninterrupted smt locus. These smt-complemented cells also exhibited restored Cd\textsuperscript{2+} tolerance. Hypersensitivity to Cu\textsuperscript{2+} was not detected in R2-PIM8(smt) indicating independence of Cu\textsuperscript{2+} resistance from smt mediated metal (Zn\textsuperscript{2+}/Cd\textsuperscript{2+}) tolerance.

A role proposed for MTs\textsuperscript{†} in all of the organisms in which they have been detected is the sequestration of excess amounts of certain metal ions. The specific metals sequestered by MTs vary for the structurally distinct proteins/polypeptides occurring in different organisms (reviewed in Kagi (1991)). Several lines of evidence also suggest other functions for some MTs, in particular those in higher eukaryotes where the inducing, and co-ordinated, metal ions include Zn\textsuperscript{2+}.

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

Lower eukaryotic (fungal) MTs bind copper in vivo and their synthesis is regulated by copper. The high frequency of homologous recombination in *Saccharomyces cerevisiae* has been exploited to construct strains deficient in the MT gene, *CUPl*, facilitating detailed analysis of MT function in this organism (Thiele et al., 1987). *CUPl* performs no essential role(s) required for cell growth, differentiation, or normal Cu\textsuperscript{2+} metabolism. *CUPl* mutants grow with normal doubling times in standard low Cu\textsuperscript{2+} media and are capable of mating, diplophase growth, sporulation, germination, accumulation of copper, and accumulation/activation of a copper requiring enzyme, copper-dependent superoxide dismutase (Thiele et al., 1987). However, MT-deficient *S. cerevisiae* are hypersensitive to elevated concentrations of Cu\textsuperscript{2+}.

A number of studies have indicated that some prokaryotes contain MT-like proteins (cited in Silver and Misra (1988)) but only a *Synechococcal* MT sequence has been reported (Olafson et al., 1988). This MT increased in abundance following exposure of cyanobacterial cells to elevated concentrations of either Cd\textsuperscript{2+} or Zn\textsuperscript{2+}, but not Cu\textsuperscript{2+}, and the purified protein was associated with either Cd\textsuperscript{2+} or Zn\textsuperscript{2+} (dependent upon the metal administered to the cells) with copper as a minor component (Olafson et al., 1980). Polymerase chain reaction fragments corresponding to the prokaryotic MT gene have been generated from *Synechococcus* PCC 6301 genomic DNA (Robinson et al., 1990), and we have recently cloned and structurally characterized the MT locus, designated smt, from *Synechococcus* PCC 7942 (Huckle et al., 1993). The locus includes the MT gene, smt\textsubscript{A}, and a divergent gene, smt\textsubscript{B}, which encodes a trans-acting repressor of transcription from the smt\textsubscript{A} operator-promoter (Huckle et al., 1993). The abundance of smt\textsubscript{A} transcripts increase in response to elevated concentrations of a range of trace metal ions (Huckle et al., 1993). Gene-fusion experiments indicate that the smt\textsubscript{A} operator-promoter is most responsive to maximum permissive concentrations of Zn\textsuperscript{2+} compared to other metal ions (Huckle et al., 1993). Following expression of SmtA as a recombinant fusion protein in *Escherichia coli*, the pH of half dissociation of different metal ions indicated a particularly high affinity for Zn\textsuperscript{2+} in comparison to mammalian MT (Shi et al., 1992). Amplification (Gupta et al., 1992) and specific rearrangement (Gupta et al., 1993) of the smt locus has been detected in *Synechococcus* PCC 6301 cells selected for growth in elevated concentrations of Cd\textsuperscript{2+}.

We report the generation of smt-deficient mutants via insertional inactivation/partial gene deletion and phenotypic analysis of these cells.

**EXPERIMENTAL PROCEDURES**

*Materials and Methods*—R2-PIM8, a small plasmid cured derivative of *Synechococcus* PCC 7942 (van der Plas et al., 1990), was...
cultured under constant light (100 μmol of photon m⁻² s⁻¹ photosynthetically active radiation) at 32 °C in Allen medium (Allen, 1968) with 0.012 g liter⁻¹ citric acid, the omission of Na₂SO₄·H₂O and supplemented with 30 μg of D-treonine ml⁻¹, 5 μg of streptomycin ml⁻¹, and 7.5 μg of chloramphenicol ml⁻¹ as appropriate. Solid media were prepared in the medium described by Agar (Bacto-Agar, Difco Laboratories). Modified Allen medium contains 0.77 μM Zn²⁺ and 0.32 μM Cu²⁺. E. coli DH5α was used as a host in all recombinant plasmid constructions, transformed cells were grown on LB medium (Sambrook et al., 1989) supplemented with 100 μg ml⁻¹ carbenicillin, or 34 μg ml⁻¹ chloramphenicol as necessary. Restriction enzymes were supplied by Northumbria Biologicals Ltd., Cramlington, United Kingdom (UK)or Boehringer Mannheim Ltd., East Sussex, UK. Taq polymerase was supplied by Stratagene, Cambridge, UK, or Promega Ltd., Southampton, UK. [α-³²P]dCTP (14.5 TBq mmol⁻¹) and nylon (Hybond N) filters were obtained from Amersham International, Aylesbury, UK. Phosphoramidite derivatives of all nucleotide bases were obtained from Applied Biosystems, Warrington, UK. Other chemicals and antibiotics were purchased from Sigma, Dorset, UK.

DNA probes were prepared from an smtA polymerase chain reaction product (described by Robinson et al., 1990) and from restriction fragments of pJHNR49 (Sambrook et al., 1989) as described in the supplier’s protocol (Model 370A DNA sequencing system; User’s Manual Version 1.3A, October 1988, pp. 3.22-3.25).

**Interruption of the smt Locus**—The scheme for inactivation of the smt locus is given in Fig. 2. R2-PIM8 was transformed with linearized plasmid pRECSU which consists of smt flanking sequences interrupted by E. coli plasmid pSU19 (a derivative of pSU2719 described by Martinez et al. (1988) containing the chloramphenicol acetyl transferase gene (cat). The smt flanking sequences in pRECSU, obtained from pJHNR49, include a 340-bp HindIII/SacI 5’ fragment and a 1073-bp PstI/SacI 3’ fragment, cloned into the Smal/SacI and PstI/SalI sites, respectively, of the pSU19 polylinker. XbaI was subsequently used to linearize pRECSU prior to transformation.

Plasmid DNA isolated by alkaline lysis was used to transform R2-PIM8 to Cm⁻ essentially as described by van den Hondel et al. (1980). Transformants were selected on Allen’s agar plates containing chloramphenicol. Individual Cm⁻ transformants were serially streak-purified at least three times to ensure segregation of homozygous mutants. A 1775-bp SalI/HindIII smt fragment from pJHNR49 (Fig. 2A) was used to complement pRECSU transformants, and transformants were selected on Allen’s agar plates supplemented with 20 μM Zn²⁺.

**DNA Isolation and Southern Analysis**—Genomic DNA was isolated from liquid cultures in late logarithmic to early stationary phase using plasmid isolation protocol described previously for the isolation of nucleic acids from plant cell cultures (Robinson et al., 1988), but excluding CsCl gradients. Genomic Southern blotting was performed using 10 or 20 μg of DNA for each restriction digest, followed by standard agarose gel electrophoresis, and transfer to nylon filters. Filters were washed to a final stringency of 0.5 x SSC, 1 g liter⁻¹ SDS at 65 °C (Sambrook et al., 1989). 

**Results**

**Evidence of Chromosomal Localization of the smt Locus**—Synechococcus PCC 7942 has two plasmids of ~8.0 and 48.5 kb (Lau and Dolittle, 1979; Laudenbach et al., 1983). Southern analysis of R2-PIM8 DNA probed with smtA identified the gene on unique SalI, HindIII, and BamHI fragments (Fig. 1A), confirming its presence in the small plasmid-cured strain. No hybridization was detected following probing of pPLANB2 (an E. coli plasmid carrying the 8.0-kb plasmid of Synechococcus PCC 7942 (Laudenbach et al., 1983) with smtA (data not shown)).

pPLANB2 (plasmids 1 to 7), carry seven BamHI restriction fragments of the 48.5-kb plasmid (Laudenbach et al., 1983). There was only faint hybridization to pPLANB2 (data not shown). Hybridization to PstI digested pPLANB2 is weak relative to an equivalent number of copies of pJHNR49 and a 213-bp PstI smtA fragment was only detected in pJHNR49 (Fig. 1B). Probing with the weakly hybridizing ~2.0-kb fragment from pPLANB2 identified the homologous sequence in R2-PIM8, in addition to weak cross-hybridization to smtA (data not shown).

**Interruption of the smt Locus**—The plasmid pRECSU (Fig. 2), containing smt flanking sequences interrupted by E. coli plasmid pSU19, was generated. The smt flanking sequences in pRECSU are separated by 371-bp in the smt locus which includes the smtA and smtB transcription/translation start sites and promoter sequences, which were therefore absent from pRECSU. The orientation of fragments in pRECSU was confirmed by sequencing using both forward and reverse M13 primers. R2-PIM8 was transformed to Cm⁻ with linearized pRECSU. Stable Cm⁻ transformants were selected on Allen’s agar plates containing 7.5 μg ml⁻¹ chloramphenicol. After several rounds of streaking to isolate homoygous for an interrupted smtA locus, a number of transformants were grown in liquid culture and then plated to obtain single colonies.

**Fig. 1. Southern analysis of smtA—confirming chromosomal localization.** A, total nucleic acids from R2-PIM8 (a small plasmid-cured strain of Synechococcus PCC 7042) was digested with SalI (lane 1), HindIII (lane 2), and BamHI (lane 3). B, equivalent copy numbers of pJHNR49 (lane 2) and pPLANB2 (lanes 1 and 3) digested with PstI. The digested DNA was electrophoresed on 0.8% agarose gels, transferred to nylon filters, and probed with smtA.
The structure and homozygosity of pRECSU transformants, hereafter referred to as R2-PIM8(smt), were confirmed by Southern analyses (Fig. 3). A ~5.8-kb SalI smt fragment in R2-PIM8 DNA was not detected in R2-PIM8(smt) DNA probed with part of the diagnostic-deletion region. Upon prolonged exposure a faint band was visible at ~7.8 kb in R2-PIM8(smt) DNA, due to weak cross-hybridization to pSU19. SalI was used to linearize pRECSU prior to transformation of R2-PIM8. B, the upper diagram represents the smt locus of R2-PIM8. Transformation with linearized pRECSU should result in homologous recombination with cat directed into the smt locus (lower diagram).

The orientation of the cloned smt sequences resulted in the interruption by the vector DNA (including cat) of the Synechococcus PCC 7942 smt flanking sequences. The smt flanking sequences in pRECSU are separated by 371 bp in the smt locus which includes the smtA and smtB transcription/translation start sites and promoter sequences, which are therefore absent from pRECSU. XbaI was used to linearize pRECSU from R2-PIM8 DNA; lane 2, PstI-digested DNA probed with pSU19.

A ~0.9-kb PstI fragment containing smtB in R2-PIM8 DNA and a ~3.1-kb fragment in R2-PIM8(smt) DNA was detected upon probing with retained smtB sequences (Fig. 3B). The latter corresponds to the anticipated size of the smt locus containing pSU19 with concomitant deletion of a 371-bp region. Probing with plasmid pSU19 identified ~3.1-kb PstI, ~2.7-kb HindIII, and ~7.8-kb SalI fragments in R2-PIM8(smt) DNA as expected, confirming the site of integration of pRECSU (Fig. 3C). An anomalous additional band was thought to result from homology between the probe and other sequences within the genome.

Plasmid Recovery—A ~7.8-kb plasmid (pJSTNR4.1) was generated from a SalI fragment of R2-PIM8(smt) DNA by plasmid recovery in E. coli. The restriction pattern (Fig. 4) is that expected for digestion of DNA containing the interrupted smt locus. The smt-derived regions of pJSTNR4.1 were sequenced using the M13 forward and reverse primer sites within pSU19 and were identical to the known genomic sequences, further confirming the correct site of integration of pRECSU.

Analysis of smtA Transcript Abundance—smtA transcripts were only detected in RNA isolated from Cd2+ exposed E. coli, was digested with SalI (lane 1), PstI (lane 2), HindIII (lane 3), or PstI/HindIII (lane 4) and electrophoresed on a 1% agarose gel.

Phenotypic Analysis of R2-PIM8(smt)—The proportion of cultures of R2-PIM8 and R2-PIM8(smt) growing in Allens medium supplemented with a range of concentrations of CdCl2, ZnCl2, and CuCl2 were monitored, and minimum inhibitory/maximum permissive concentrations of these metal ions were determined for both strains. Growth of R2-PIM8 and R2-PIM8(smt) was subsequently examined as a function of time in response to the selected metal concentrations (Fig. 6, A, B, and C). R2-PIM8 survived in ~5-fold higher concentrations of Zn2+ than R2-PIM8(smt). A higher tolerance to
Cd\(^{2+}\) was observed for R2-PIM8 in comparison to R2-PIM8\(^{(smt)}\), however this was only detected after a prolonged lag of \(>148\) h (Fig. 6B). There was no marked difference in the minimum inhibitory concentration of Cu\(^{2+}\), and similar growth rates were observed for both R2-PIM8 and R2-PIM8\(^{(smt)}\) at selected Cu\(^{2+}\) concentrations (Fig. 6C).

Complementation of R2-PIM8\(^{(smt)}\)—A 1775-bp SalI/HindIII smt fragment (Fig. 2A), was used to transform Zn\(^{2+}\)-hypersensitive R2-PIM8\(^{(smt)}\) to Zn\(^{2+}\) and Cm\(^{+}\) by homologous recombination. After several rounds of streaking on Zn\(^{2+}\)-supplemented plates (20 \(\mu\)M) to segregate mutants homozygous for an uninterrupted functional smt locus, a number of colonies were grown in liquid culture and plated to obtain single colonies. Fig. 7 depicts the tolerance of R2-PIM8 (A), R2-PIM8\(^{(smt)}\) (B), and smt-complemented cells (C) on Allen's agar plates supplemented with chloramphenicol (7.5 \(\mu\)g ml\(^{-1}\)) or Zn\(^{2+}\) (20 \(\mu\)M). The structure and homozygosity of the smt-complemented cells was confirmed by Southern analysis, using the 213-bp PstI diagnostic-deletion region as a probe (Fig. 8). The restriction pattern was as observed for R2-PIM8 (Fig. 7A), confirming reintegration of a functional smt locus. Fragments hybridizing to pSU19 in R2-PIM8\(^{(smt)}\) DNA (Fig. 3C) were not detected in smt-complemented cells (data not shown).

Growth of smt-complemented cells in Allen's medium supplemented with increasing levels of CdCl\(_2\), ZnCl\(_2\), and CuCl\(_2\) was identical to that observed for R2-PIM8 (data not shown).

**DISCUSSION**

Previous circumstantial evidence suggesting a role for prokaryotic MT in Zn\(^{2+}\) homeostasis includes; an observed high Zn\(^{2+}\)-affinity (relative to equine MT) of a recombinant GST-SmtA fusion protein following expression in E. coli (Shi et al., 1991, 1991b); MT induction by, and association with, Zn\(^{2+}\) in *Synechococcus* sp. (Olafson et al., 1980); an observed maximal induction of expression from the smt operator-promoter in response to Zn\(^{2+}\) (Huckle et al., 1993). Here we describe the production of R2-PIM8\(^{(smt)}\) mutants which lack a functional smt locus and show reduced (~5-fold) tolerance to Zn\(^{2+}\).

smt-mediated restoration of Zn\(^{2+}\) tolerance can be used as a selectable marker for transformation of R2-PIM8\(^{(smt)}\). Zn\(^{2+}\)-hypersensitive R2-PIM8\(^{(smt)}\) was transformed to normal Zn\(^{2+}\) tolerance with a linear DNA fragment containing the smt locus. All of the resulting Zn\(^{2+}\) colonies exhibited restored Cm\(^{+}\), growth curves indicated Zn\(^{2+}\) and Cd\(^{2+}\) tolerance characteristics identical to R2-PIM8, and Southern analyses confirmed reintegration of the functional smt locus with concomitant loss of pSU19. Thus, the Zn\(^{2+}\)- and Cd\(^{2+}\)-hypersensitive phenotype of R2-PIM8\(^{(smt)}\) was solely due to loss of the smt locus.

In vitro Zn\(^{2+}\) transfer between transcription factors and higher eukaryotic apo-MT has implicated the latter in Zn\(^{2+}\) homeostasis as it relates to the regulation of gene expression (Zeng et al., 1991a, 1991b). Zn\(^{2+}\)-requiring transcription factors are not well characterized in prokaryotes, and 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 (Luisi, 1992). However, intracellular "Zn\(^{2+}\) buffering" remains a requirement of these organisms, and reduced tolerance of R2-PIM8\(^{(smt)}\) to elevated Zn\(^{2+}\) reveals such a function for the SmtA protein. Viability of R2-PIM8\(^{(smt)}\) confirms no essential role for...
et posed. However, plasmid-encoded functions have not previously been identified in cyanobacteria (Ciferri et al., 1989) and the smt locus is now confirmed to be chromosomal. Gupta et al. (1992, 1993) observed amplification and highly iterated palindrome-mediated rearrangement of the smt locus in Synechococcus PCC 6301 selected for Cd\(^{2+}\) resistance. However, no rearrangement or amplification of smt was detected (by Southern analysis) in cultures growing at 4.0 \(\mu\)M Cd\(^{2+}\) and 4.5 \(\mu\)M Cd\(^{2+}\) in this study (data not shown). The cause of the observed lag in response to Cd\(^{2+}\) remains to be elucidated.

There was no marked difference in Cu\(^{2+}\) tolerance of R2-PIM8 and R2-PIM8(smt). Olafson (1986), observed a protracted growth lag in cells exposed to elevated Cu\(^{2+}\) but no coincident MT synthesis. Energy-dependent copper efflux has been proposed as an alternative mechanism of Cu\(^{2+}\) resistance in Synechococcus sp. (Olafson, 1986) and in another cyanobacterium, Nostoc calcicola (Verma and Singh, 1991).

In conclusion, these data show that the prokaryotic MT locus, smt, is involved in Zn\(^{2+}\) and Cd\(^{2+}\) detoxification, but performs no vital role required for growth in non-metal-supplemented media. The mechanism of smt-mediated metal detoxification is unknown. By analogy to eukaryotic MTs, SmtA may serve as an intracellular “sink” for excess metal. The possibility that SmtA may be part of a more dynamic mechanism of metal homeostasis (e.g. involving enhanced detoxification) is unknown. By analogy to eukaryotic MTs, SmtA may serve as an intracellular “sink” for excess metal.

SmtA in the donation, or removal, of Zn\(^{2+}\) to, or from, apo-proteins.

Reduced tolerance of R2-PIM8(smt) to Cd\(^{2+}\) was detected upon prolonged (>148 h) exposure. Olafson et al. (1980) noted a marked growth lag of ~6 days in Synechococcus sp. exposed to Cd\(^{2+}\). The onset of growth coincided with accumulation of MT, and extrachromosomal MT gene amplification was proposed. However, plasmid-encoded functions have not previously been identified in cyanobacteria (Ciferri et al., 1989) and the smt locus is now confirmed to be chromosomal. Gupta et al. (1992, 1993) observed amplification and highly iterated palindrome-mediated rearrangement of the smt locus in Synechococcus PCC 6301 selected for Cd\(^{2+}\) resistance. However, no rearrangement or amplification of smt was detected (by Southern analysis) in cultures growing at 4.0 \(\mu\)M Cd\(^{2+}\) and 4.5 \(\mu\)M Cd\(^{2+}\) in this study (data not shown). The cause of the observed lag in response to Cd\(^{2+}\) remains to be elucidated.

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FIG. 7. Growth of R2-PIM8, R2-PIM8(smt), and smt-complemented cells on Allen's agar plates. Colonies of A, R2-PIM8; B, R2-PIM8(smt); C, smt-complemented cells were streaked onto Allen's agar plates (control) supplemented with chloramphenicol (7.5 \(\mu\)g ml\(^{-1}\)) or Zn\(^{2+}\) (20 \(\mu\)M).

FIG. 8. Southern analysis of genomic DNA from smt-complemented cells. Genomic DNA was digested with BamHI (lane 1), SalI (lane 2), and HindIII (lane 3). The Southern blot was probed with part of the 371-bp diagnostic-deletion region.