Journal of Industrial Microbiology (1996) 17, 41–46
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Expression of mouse metallothionein in the cyanobacterium *Synechococcus* PCC7942

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A cDNA encoding mouse metallothionein was cloned into the shuttle vector pUC303, creating a translational fusion with the bacterial chloramphenicol acetyltransferase gene. The resulting fusion protein has been expressed in the cyanobacterium *Synechococcus* PCC7942. Cyanobacterial transformants expressed mouse metallothionein-specific mRNA species as detected by RNA slot blots. In addition, the transformants expressed a unique cadmium ion-binding protein corresponding to the predicted size of the mouse metallothionein fusion protein. Expression of this fusion protein conferred a two- to five-fold increase in cadmium ion tolerance and accumulation on *Synechococcus* PCC7942.

Keywords: metallothionein; cyanobacteria; metals; bioremediation

Introduction

The application of heavy metal ion-resistant microbes in the removal of heavy metal ions from polluted effluents has become a focus for environmental bioremediation. A number of microbial bioremediation strategies have been examined. For example, bioreactors based on viable immobilized bacteria have been constructed [4,5]. Microbial and algal biomass has been employed in metal ion removal technologies [2,18,19,30,34,36]. The application of microbial mats for such purposes has recently been reviewed [3].

Microbially-based metal ion remediation systems must be cost-effective in order to compete with traditional metal ion removal technologies [30]. The photosynthetic cyanobacteria provide advantageous hosts for the development of such systems since they utilize solar energy and carbon efficiently from the air. Kuritz and Wolk [16] have reported the genetic engineering of cyanobacteria for enhanced degradation of organic pollutants and proposed their use in the low-cost, low-maintenance remediation of surface waters. Similarly, development of cyanobacterial strains having increased metal ion tolerance would offer a cost-effective alternative to microbes currently used in metal ion removal technologies. Moreover, cyanobacteria are compatible with existing microbial bioremediation technologies such as microbial mats, of which cyanobacteria are natural components [3].

The potential of using microbes genetically engineered to express a metal ion-binding protein, metallothionein, as filters for the removal of heavy metals from contaminated waters has been recognized [10,12,26,27]. Metallothionein (MT) is a low-molecular weight, cysteine-rich protein which binds a variety of heavy metal ions with stability constants on the order of 10^{11} to 10^{19} M^{-1} [9]. While MT is ubiquitous among eukaryotes, to date only two prokaryotes, *Pseudomonas putida* and the cyanobacterium *Synechococcus* sp [35] have been found to express MT-like proteins. One molecule of mammalian MT binds seven ions of cadmium, zinc, mercury, cobalt, lead, or nickel, and binds 12 ions of copper, silver, or gold [9]. On the other hand, the prokaryotic MT of *Synechococcus* sp complexes only about half as much metal ion per mole as its mammalian counterparts [22] and demonstrates a high affinity for Zn^{2+} as compared with equine MT [29]. Considerable effort has been made towards expression of recombinant MT from a variety of species in a number of hosts [10,13,23,27,33]. In general, expression of recombinant MT confers increased heavy metal ion tolerance and/or accumulation on the host.

The present work addresses the potential of engineering cyanobacteria for increased bioaccumulation of heavy metal ions via expression of a recombinant mammalian MT. As a first step, *Synechococcus* PCC7942 was transformed with a mouse MT (*mMT*) cDNA fused to a portion of the bacterial chloramphenicol acetyltransferase (*cat*) gene. The results demonstrate that expression of the recombinant CAT-mMT fusion protein confers increased tolerance to heavy metal ions as well as increased heavy metal ion accumulation to a cyanobacterial host.

Materials and methods

Host strains and culture conditions

Escherichia coli JM109 was used for initial plasmid characterization. Transformants were grown at 37°C with 25 μg of streptomycin ml^{-1} in DYT medium. One liter of DYT medium contains 10 g yeast extract, 10 g tryptone, and 5 g NaCl (pH 7.4). Bacterial growth in liquid cultures was monitored at $A_{595\ nm}$.

Synechococcus PCC7942 was grown at 30°C with a light intensity of 2500 lux in shake flasks containing liquid BG-11 medium or on BG-11 solidified with 1.5% agar which

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Received 10 July 1995; accepted 13 June 1996

was further supplemented with 1 mM sodium thiosulfate. BG-11 medium was prepared as described [25] and supplemented with 2 mM HEPES (pH 7.0). Transformation of cyanobacteria and selection of transformants was carried out as previously described [15]. Cyanobacterial growth in liquid cultures was monitored at $A_{730\ nm}$.

Materials

Restriction endonucleases were obtained from Pharmacia (Piscataway, NJ, USA). T4 DNA ligase, Klenow DNA polymerase, *Taq* DNA polymerase, and double-stranded DNA Cycle Sequencing Systems were supplied by BRL (Gaithersburg, MD, USA) and used according to the manufacturer's recommendations. Radioactive cadmium ($^{109}CdCl_2$) was supplied by Amersham (Cleveland, OH, USA). Wizard Minipreps and PCR Preps DNA purification systems were obtained from Promega (Madison, WI, USA). DNA oligonucleotides were synthesized by National Biosciences (Plymouth, MN, USA). Genius System digoxigenin labeling kits were from Boehringer Mannheim (Indianapolis, IN, USA). *mMT* cDNA was the gift of Richard Palmiter (University of Washington) and *Synechococcus* PCC7942 R2-SPc was provided by WE Borrias (University of Utrecht).

Plasmid construction

DNA manipulations were performed as described by Sambrook *et al* [28]. Mouse metallothionein expression plasmids were constructed by modification of the shuttle vector pUC303 [14]. An *mMT* fragment containing *Nco*I and *Sal*I restriction enzyme sites at the 5′ and 3′ ends, respectively, was prepared by polymerase chain reaction (PCR) amplification of *mMT* cDNA using the following primers: N-terminal primer 5′GGCGCCATGGACCCCAACTGCTC3′, C-terminal primer 5′CGGTCGACGCTGTTCGTCACATCA3′ (underlined sequences represent restriction endonuclease sites for *Nco*I and *Sal*I, respectively).

Mouse metallothionein expression plasmid pJLE16 was constructed by subcloning the *Nco*I-*Sal*I *mMT* fragment into pUC303, previously digested with *Nco*I and *Sal*I, creating a translational fusion with the first 172 amino acids of CAT (Figure 1). Plasmid pJLE21 was constructed to express a truncated version of the CAT-mMT fusion protein by deletion of a 339-bp *Dra*I fragment from the *cat* region of pJLE16. Plasmid pJLE24 was constructed by subcloning a part of the *cat* gene, a portion of the *Synechococcus* PCC7942 *smt* locus including *smtB*, the 100-bp operator/promoter region, and the first codon of *smtA* as an *Eco*RI fragment from plasmid pJLE22 into the unique *Eco*RI site of plasmid pJLE16 (Figure 1). Plasmid pJLE22 is identical to plasmid pJLE22-R [7], except that the *smt* locus with respect to the *cat* gene is in the opposite orientation. Expression of the *cat-mMT* gene fusion in pJLE24 is under the control of the *smtA* promoter. In pJLE16 and pJLE21 expression is under the control of the *cat* promoter. Plasmid construction was verified by restriction digestion and DNA sequencing.

Plasmid recovery

Bacterial and cyanobacterial plasmid DNA were prepared using the Wizard Minipreps System according to the Wizard protocol. Cell extracts of cyanobacterial cultures were prepared as described [15]. After removal of cell debris, Promega Magic DNA Binding Resin was added to the supernatant fluid and the DNA was recovered. RNase, 20 $\mu g\ ml^{-1}$, was added to the final eluent.

Plasmid DNA recovered from transformed cyanobacteria was used in the transformation of *E. coli* JM109. Restriction endonuclease digests were subsequently performed on plasmid DNA recovered from the *E. coli* transformants.

RNA isolation and RNA slot blots

Total RNA from 300-ml cultures of mid-log phase cyanobacteria was isolated by the method of Reith *et al* [24]. Five-microgram aliquots of RNA were denatured with 10 mM NaOH, 1 mM EDTA and applied to a nylon membrane using a Bio-Dot SF microfiltration apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Following a 2 h prehybridization at 43°C, blots were hybridized for 16 h at 43°C to either a *mMT* DNA probe or to a *smtB* DNA probe. Probes were labeled with digoxigenin (DIG) using the Genius System. The *mMT* DNA probe was obtained by PCR amplification of *mMT* cDNA. The *smtB* DNA probe was obtained by restriction endonuclease digestion of plasmid pJLE22-R [7]. Hybridization and chemiluminescent detection of DIG-labeled probes was carried out according to the manufacturer's instructions.

Protein blotting and detection

Cyanobacterial cell extracts were fractionated by denaturing electrophoresis on 15% polyacrylamide-SDS gels and proteins were electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). The CAT and CAT-mMT proteins were detected by probing the membranes with rabbit polyclonal antibodies raised against CAT (5 Prime to 3 Prime, Boulder, CO, USA). Following application of anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody, blots were developed using SigmaFast BCIP/NBT buffered substrate tablets (Sigma, St Louis, MO, USA) according to the manufacturer's directions.

The expression of CAT-mMT fusion proteins was also detected using a variation of a previously described radioactive cadmium ion protocol [1]. Cyanobacterial cell extracts were fractionated as above and blotted onto Immobilon-P membranes. Following incubation in 10 mM Tris-Cl (pH 7.6) for 1 h at room temperature, blots were incubated in 10 mM Tris-Cl (pH 7.6), 0.1 mM $ZnCl_2$, and 0.1 M KCl containing 1 $\mu Ci\ ml^{-1}$ $^{109}CdCl_2$, for 10 min at room temperature. Blots were then washed twice, 10 min per wash, in 10 mM Tris-Cl (pH 7.6), 0.1 M KCl at room temperature. Cadmium ion-binding proteins were detected by autoradiography or phosphorimaging (Molecular Dynamics, San Diego, CA, USA).

Chloramphenicol resistance assay

BG-11 (25 ml) supplemented with 7.5 $\mu g\ ml^{-1}$ chloramphenicol was inoculated with 500 μl of the appropriate stationary phase cyanobacterial culture and incubated under standard conditions. Growth was monitored by $A_{730\ nm}$ measurements.

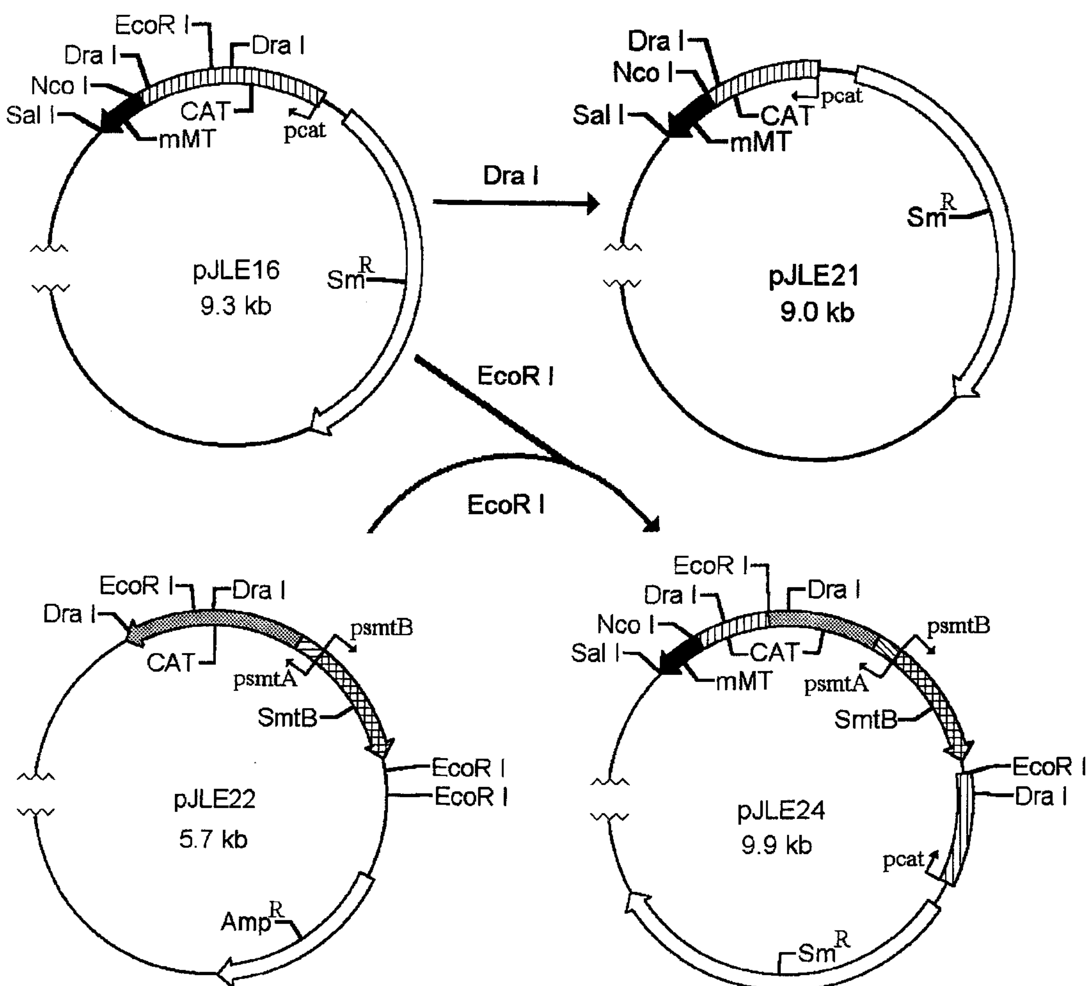

Figure 1 Construction of *mMT* expression vectors. Mouse metallothionein expression plasmid pJLE16 was constructed by subcloning a *Nco*I-*Sal*I *mMT* fragment into pUC303, previously digested with *Nco*I and *Sal*I, creating a translational fusion with the first 172 amino acids of CAT. Plasmid pJLE21 was constructed by deletion of a 339-bp *Dra*I fragment from the *cat* region of pJLE16. Plasmid pJLE24 was constructed by subcloning a portion of the *Synechococcus* PCC7942 *smt* locus as an *Eco*RI fragment from plasmid pJLE22 into the unique *Eco*RI site of plasmid pJLE16. Plasmid pJLE22 was identical to plasmid pJLE22-R [7], except that the *smt* locus with respect to the *cat* gene was in the opposite orientation. A given insert is the same size in each plasmid map for clarity. Each inserted segment has unique shading to indicate origin and destination.

Cadmium ion tolerance

BG-11 (1 ml) containing appropriate antibiotic and $CdCl_2$ (1.5 μM to 3 μM) was inoculated with *Synechococcus* PCC7942 or *Synechococcus* PCC7942 (pJLE16). Cultures were incubated in 15-ml polypropylene tubes at 29°C, agitated at 200 rpm, with 2500 lux illumination. Growth was monitored by $A_{730\,nm}$ measurements. Experiments were performed in triplicate.

$^{109}Cd^{2+}$ accumulation

Uptake of $^{109}Cd^{2+}$ by *E. coli* was determined according to Hou *et al* [10] and $^{109}Cd^{2+}$ uptake by cyanobacteria was determined according to a similar protocol. A 1-ml aliquot of culture was mixed with 5 μl of $^{109}CdCl_2$ (122 μCi ml^{-1}) and incubated at 29°C for 16 h with 2500 lux illumination. After measuring the $A_{730\,nm}$, the cells were transferred to microcentrifuge tubes and harvested by centrifugation for 5 min at room temperature. The pellet was washed twice with saline followed by solubilization in Laemmli [17] sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol) at 100°C for 10 min. The radioactivity of the resulting cell lysate was determined in a liquid scintillation counter.

Results

RNA slot blots

Transcription of the *cat-mMT* gene fusion in cyanobacteria was verified by RNA slot blots (Figure 2). Total RNA from wild type cells as well as from *Synechococcus* PCC7942 (pJLE16) and *Synechococcus* PCC7942 (pUC303) was immobilized on nylon membranes and the membranes were screened for *cat-mMT* mRNA by hybridization with a digoxigenin-labeled *mMT* DNA probe (Figure 2, row A). Transcription of the *mMT* occurred only in *Synechococcus* PCC7942 (pJLE16). RNA from wild type cells and RNA from *Synechococcus* PCC7942 (pUC303) did not hybridize to the *mMT* probe. The *mMT* probe was subsequently stripped and the blots were rehybridized with a probe consisting of the cyanobacterial *smtB* gene to verify that all slots contained RNA (Figure 2, row B).

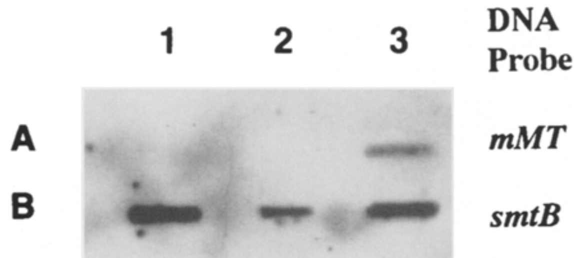

Figure 2 RNA slot blot analysis of *CAT-mMT* transcription in *Synechococcus* PCC7942. Row A: 5 μg of total RNA from *Synechococcus* PCC7942 (lane 1), *Synechococcus* PCC7942 (pUC303) (lane 2), or *Synechococcus* PCC7942 (pJLE16) (lane 3) was blotted and probed with digoxigenin-labeled *mMT*. Row B: The blot shown in row A was subsequently stripped and reprobed with a digoxigenin-labeled probe consisting of the cyanobacterial *smtB* gene as a loading control.

Detection of CAT-mMT protein in cyanobacterial cell extracts

To measure expression of the CAT-mMT fusion protein in transformed cyanobacterial cells, cell extracts were prepared from *Synechococcus* PCC7942, *Synechococcus* PCC7942 (pUC303), and *Synechococcus* PCC7942 (pJLE16). Following separation of cellular proteins by SDS-PAGE [17] and blotting, fusion proteins were detected by immunostaining with a polyclonal antibody to the CAT amino terminus and by the binding of $^{109}Cd^{2+}$.

Immunostaining with CAT polyclonal antibodies revealed a band corresponding to the CAT protein (25.7 kDa) in extracts from *Synechococcus* PCC7942 (pUC303) (Figure 3, lane 3) and to a protein of similar molecular weight, presumably the CAT-mMT fusion (26.4 kDa), in *Synechococcus* PCC7942 (pJLE16) (Figure 3, lane 5). Wild type cell extracts did not contain a corresponding CAT protein (Figure 3, lane 1). The additional, higher molecular weight band in the *Synechococcus* PCC7942 (pUC303) and *Synechococcus* PCC7942 (pJLE16) samples was presumed to be the result of nonspecific interaction with the polyclonal antibody.

To confirm that the immunopositive protein in the *Synechococcus* PCC7942 (pJLE16) extracts was indeed the CAT-mMT fusion protein, as opposed to full-length CAT, protein blots were probed with $^{109}Cd^{2+}$. Autoradiographs verified the presence of $^{109}Cd^{2+}$-binding proteins in the extract from *Synechococcus* PCC7942 (pJLE16) at a position which corresponded to the immunopositive band from the same extract (Figure 3, compare lanes 5 and 6). Similar cadmium ion-binding proteins were not detected in extracts from *Synechococcus* PCC7942 or *Synechococcus* PCC7942 (pUC303) (Figure 3, lanes 2 and 4). The apparent absence of a band corresponding to the prokaryotic MT is possibly the result of its strong affinity for Zn^{2+} [29] which is a component of the incubation buffer (see Materials and Methods).

Synechococcus PCC7942 (pUC303), which expresses a functional CAT protein, was chloramphenicol-resistant in media containing chloramphenicol (7.5 $\mu g\ ml^{-1}$). As expected, *Synechococcus* PCC7942 and *Synechococcus* PCC7942 (pJLE16) were chloramphenicol-sensitive (Figure 3), since they do not express an entire CAT protein.

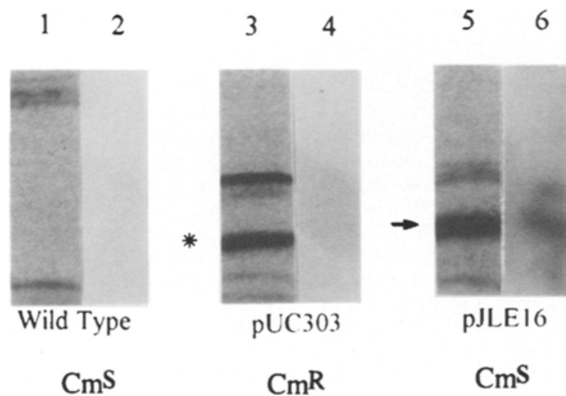

Figure 3 Detection of CAT and CAT-mMT proteins. Protein extracts (100 μg) from *Synechococcus* PCC7942 (lanes 1–2), *Synechococcus* PCC7942 (pUC303) (lanes 3–4), and *Synechococcus* PCC7942 (pJLE16) (lanes 5–6) were separated by denaturing electrophoresis on a 15% polyacrylamide-SDS gel and transferred to Immobilon-P membranes. Membranes were probed with polyclonal antibodies to the CAT amino terminus (lanes 1,3,5) or with $^{109}Cd^{2+}$ (lanes 2,4,6). For presentation purposes, corresponding lanes from the respective Western blot and autoradiograph have been juxtaposed. The asterisk indicates the CAT protein. The arrow indicates the CAT-mMT fusion protein. Cm^R and Cm^S indicate chloramphenicol resistance and sensitivity, respectively.

Cadmium ion tolerance

Increased heavy metal ion tolerance by transformed cyanobacteria expressing recombinant mMT was also examined. *Synechococcus* PCC7942 (pJLE16) was capable of sustained growth upon direct inoculation into media containing up to 3 μM $CdCl_2$. Strain PCC7942 did not survive in metal ion concentrations exceeding the maximum permissive concentration of 1.5 μM, a level set by SmtA [11] (data not shown).

$^{109}Cd^{2+}$ accumulation

The extent of heavy metal ion accumulation by cyanobacteria expressing recombinant mMT was measured during growth in the presence of $^{109}Cd^{2+}$. *Synechococcus* PCC7942 (pUC303) and *Synechococcus* PCC7942 (pJLE16) accumulated 1.6 and 2.5 times more $^{109}Cd^{2+}$ than wild type cells, respectively. *Synechococcus* PCC7942 (pJLE24), in which CAT-mMT expression was under the control of the native cyanobacterial *smtA* promoter, accumulated 4.8-fold more $^{109}Cd^{2+}$ than *Synechococcus* PCC7942 (data not shown).

To determine whether the CAT portion of the fusion protein decreased metal ion accumulation, a plasmid (pJLE21) containing a 339-bp deletion within the CAT portion of the fusion protein was constructed (Figure 1). Accumulation of $^{109}Cd^{2+}$ by *E. coli* JM109 transformed with either pJLE16 or pJLE21 was essentially identical (55 000 CPM/$A_{595\ nm}$).

Discussion

Evidence presented here indicates that a low level of *cat-mMT* mRNA was produced from plasmid pJLE16. As determined by RNA slot blot analysis, the level of *cat-mMT* mRNA appeared to be less than that produced from the *smtB* gene which, by analogy to the divergently transcribed *smtA*, occurs at a low copy number (probably one) on the cyanobacterial chromosome [8]. The expression levels demonstrated here may simply reflect the relative activity

of the CAT promoter in cyanobacteria. For example, expression of CAT under the control of its own promoter conferred resistance to chloramphenicol (7.5 μg ml^{-1}) in *Synechococcus* PCC7942, while expression of CAT under the control of the strong chloroplast *ps2B* promoter confers resistance up to 40 μg ml^{-1} of chloramphenicol in *Synechococcus* PCC6301 R2 [6] (considered to belong to the same species as *Synechococcus* PCC7942 [8]). The present work demonstrates that *Synechococcus* PCC7942 (pJLE24), expressing an identical CAT-mMT fusion protein under the control of the native cyanobacterial *smtA* promoter, accumulated 2-fold more Cd than *Synechococcus* PCC7942 (pJLE16).

Western blots probed with antibodies to CAT demonstrated that translation of the *cat-mMT* mRNA occurred, resulting in a level of CAT-mMT fusion protein similar to the level of CAT protein achieved with the same promoter. Because the CAT-mMT fusion protein is similar in size to native CAT, it is difficult to distinguish the two based solely upon molecular weight. To confirm that *Synechococcus* PCC7942 (pJLE16) expressed the CAT-mMT fusion protein, blots identical to the immunostained blots were probed for MT by incubation with $^{109}Cd^{2+}$. The results verified that *Synechococcus* PCC7942 (pJLE16) extracts contained a Cd-binding protein corresponding to the immunopositive protein from the same extracts, whereas control extracts did not (Figure 3). Furthermore, *Synechococcus* PCC7942 (pUC303) cells were chloramphenicol-resistant, indicating the expression of a functional CAT protein. *Synechococcus* PCC7942 (pJLE16) expressed a truncated CAT protein, as a result of the *cat-mMT* gene fusion, and were chloramphenicol-sensitve.

Expression of the CAT-mMT fusion protein in *Synechococcus* PCC7942 (pJLE16) conferred an approximate 2-fold increase in cadmium ion tolerance and cadmium ion accumulation as compared to wild type organisms. *Synechococcus* sp adapts to step-wise increases in heavy metal ion concentration via upregulation of prokaryotic MT expression [11,21]. The results presented here, however, indicate increased metal ion tolerance associated with *Synechococcus* PCC7942 (pJLE16) upon direct exposure to elevated cadmium ion concentrations in a single step. Wild type cells, on the other hand, did not tolerate direct exposure to cadmium ions in excess of the maximum permissive concentration. This suggests that the increased tolerance associated with *Synechococcus* PCC7942 (pJLE16) was not the result of upregulated prokaryotic MT expression, but rather was due to expression of the recombinant mMT.

While the levels of recombinant mMT expression achieved in this study appear to be low, the biological results (ie a 2 to 5-fold increase in cadmium ion accumulation) validate optimization of this approach. Recent studies by Takeshima *et al* [32] and Soltes-Rak *et al* [31], for example, clearly demonstrate the importance of factors such as the nucleotide sequence, position of ribosome-binding sequences, and codon usage on the efficiency of foreign protein expression in cyanobacteria. In addition, further analysis of mMT expression under the control of the native cyanobacterial metallothionein promoter, *smtA*, in *Synechococcus* PCC7942 and under the control of the strong phycocyanin promoter [20], *cpc*, in *Synechococcus* PCC7002 is in progress.

Acknowledgements

This work is a result of research sponsored in part by the Tennessee Valley Authority, contract TV-85653V, by the National Oceanic and Atmospheric Administration, US Department of Commerce under Grant #NA016RG0155, Project E/O-16, and the Mississippi-Alabama Sea Grant Consortium. We gratefully acknowledge the gifts of *Synechococcus* PCC7942 (R2-SPc) from WE Borrias and *mMT* cDNA from R Palmiter. The authors also thank Amy Adams for technical assistance.

References

1 Aoki Y, C Tohyama and KT Suzuki. 1991. A Western blotting procedure for detection of metallothionein. J Biochem Biophys Meth 23: 207–216.

2 Barkley NP. 1991. Extraction of mercury from groundwater using immobilized algae. J Air Waste Manage Assoc 41: 1387–1393.

3 Bender J, RF Lee and P Phillips. 1995. Uptake and transformation of metals and metalloids by microbial mats and their use in bioremediation. J Ind Microbiol 14: 113–118.

4 Collard JM, P Corbisier, L Diels, Q Dong, C Jeanthon, M Mergeay, S Taghavi, D van der Lelie, A Wilmotte and S Wuertz. 1994. Plasmids for heavy metal resistance in *Alcaligenes eutrophus* CH34: mechanisms and applications. FEMS Microbiol Rev 14: 405–414.

5 Diels L, Q Dong, D van der Lelie, W Baeyens and M Mergeay. 1995. The *czc* operon of *Alcaligenes eutrophus* CH34: from resistance mechanism to the removal of heavy metals. J Ind Microbiol 14: 142–153.

6 Dzelzkalns VA, GC Owens and L Bogorad. 1984. Chloroplast promoter driven expression of the chloramphenicol acetyl transferase gene in a cyanobacterium. Nucl Acids Res 12: 8917–8925.

7 Erbe JL, KB Taylor and LM Hall. 1995. Metalloregulation of the cyanobacterial *smt* locus: identification of SmtB binding sites and direct interaction with metals. Nucl Acids Res 23: 2472–2478.

8 Gupta A, BA Whitton, AP Morby, JW Huckle and NJ Robinson. 1992. Amplification and rearrangement of a prokaryotic metallothionein locus *smt* in *Synechococcus* PCC6301 selected for tolerance to cadmium. Proc R Soc London B 248: 273–281.

9 Hamer DH. 1986. Metallothionein. Annu Rev Biochem 55: 913–951.

10 Hou YM, R Kim and SH Kim. 1988. Expression of the mouse metallothionein-I gene in *Escherichia coli*: increased tolerance to heavy metals. Biochim Biophys Acta 951: 230–234.

11 Huckle JW, AP Morby, JS Turner and NJ Robinson. 1993. Isolation of a prokaryotic metallothionein locus and analysis of transcriptional control by trace metal ions. Mol Microbiol 7: 177–187.

12 Jacobs FA, FM Romeyer, M Beauchemin and R Brousseau. 1989. Human metallothionein-II is synthesized as a stable membrane-localized fusion protein in *Escherichia coli*. Gene 83: 95–103.

13 Kille P, P Stevens, A Cryer and J Kay. 1990. The expression of a synthetic rainbow trout metallothionein gene in *E. coli*. Biochim Biophys Acta 1048: 178–186.

14 Kuhlemeier CJ, AAM Thomas, A van der Ende, RW van Leen, WE Borrias, CAMJJ van den Hondel and GA van Arkel. 1983. A host-vector system for gene cloning in the cyanobacterium *Anacystis nidulans* R2. Plasmid 10: 156–163.

15 Kuhlemeier CJ and GA van Arkel. 1987. Host-vector systems for gene cloning in cyanobacteria. In: Methods in Enzymology, Vol 153 (Wu R and L Grossman, eds), pp 199–215, Academic Press, San Diego, CA.

16 Kuritz T and CP Wolk. 1995. Use of filamentous cyanobacteria for biodegradation of organic pollutants. Appl Environ Microbiol 61: 234–238.

17 Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

18 Maquieira A, HAM Elmahadi and R Puchades. 1994. Immobilized cyanobacteria for on-line trace metal enrichment by flow injection atomic absorption spectrometry. Anal Chem 66: 3632–3638.

19 Matis KA and AI Zouboulis. 1994. Flotation of cadmium-loaded biomass. Biotech Bioeng 44: 354–360.
20 Murphy RC and SEJ Stevens. 1992. Cloning and expression of the *cryIVD* gene of *Bacillus thuringiensis* subsp *israelensis* in the cyanobacterium *Agmenellum quadruplicatum* PR-6 and its resulting larvicidal activity. Appl Environ Microbiol 58: 1650–1655.
21 Olafson RW, S Loya and RG Sim. 1980. Physiological parameters of prokaryotic metallothionein induction. Biochem Biophys Res Com 95: 1495–1503.
22 Olafson RW, WD McCubbin and CM Kay. 1988. Primary- and secondary-structural analysis of a unique prokaryotic metallothionein from a *Synechococcus* sp cyanobacterium. Biochem J 251: 691–699.
23 Pan AH, F Tie, ZW Duau, MZ Yang, ZX Wang, LY Li, ZL Chen and BG Ru. 1994. Alpha-domain of human metallothionein I-A can bind to metals in transgenic tobacco plants. Mol Gen Genet 242: 666–674.
24 Reith ME, DE Laudenbach and NA Straus. 1986. Isolation and nucleotide sequence analysis of the ferredoxin I gene from the cyanobacterium *Anacystis nidulans* R2. J Bacteriol 168: 1319–1324.
25 Rippka R, J Deruelles, JB Waterbury, M Herdman and RY Stanier. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111: 1–61.
26 Romeyer FM, FA Jacobs and R Brousseau. 1990. Expression of a *Neurospora crassa* metallothionein and its variants in *Escherichia coli*. Appl Environ Microbiol 56: 2748–2754.
27 Romeyer FM, FA Jacobs, L Masson, Z Hanna and R Brousseau. 1988. Bioaccumulation of heavy metals in *Escherichia coli* expressing an inducible synthetic human metallothionein gene. J Biotechnol 8: 207–220.
28 Sambrook J, EF Fritsch and T Maniatis. 1989. Molecular Cloning. A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
29 Shi J, WP Lindsay, JW Huckle, AP Morby and NJ Robinson. 1992. Cyanobacterial metallothionein gene expressed in *Escherichia coli*: metal-binding properties of the expressed protein. FEBS Lett 303: 159–163.
30 Simmons P, JM Tobin and I Singleton. 1995. Considerations on the use of commercially available yeast biomass for the treatment of metal-containing effluents. J Ind Microbiol 14: 240–246.
31 Soltes-Rak E, DJ Kushner, DD Williams and JR Coleman. 1995. Factors regulating *cryIVB* expression in the cyanobacterium *Synechococcus* PCC7942. Mol Gen Genet 246: 301–308.
32 Takeshima Y, N Takatsugu, M Sugiura and H Hagiwara. 1994. High-level expression of human superoxide dismutase in the cyanobacterium *Anacystis nidulans* 6301. Proc Natl Acad Sci USA 91: 9685–9689.
33 Thiele DJ, MJ Walling and DH Hamer. 1986. Mammalian metallothionein is functional in yeast. Science 231: 854–856.
34 Tobin JM, C White and GM Gadd. 1994. Metal accumulation by fungi: applications in environmental biotechnology. J Ind Microbiol 13: 126–130.
35 Turner J and NJ Robinson. 1995. Cyanobacterial metallothioneins: biochemistry and molecular genetics. J Ind Microbiol 14: 119–125.
36 Volesky B and I Prasetyo. 1994. Cadmium removal in a biosorption column. Biotech Bioeng 43: 1010–1015.