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Cyanobacterial metallothionein gene expressed in *Escherichia coli*

Metal-binding properties of the expressed protein

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The recently isolated Synechococcus gene *smtA* encodes the only characterised prokaryotic protein designated to be a metallothionein (MT). To examine the metal-binding properties of its product the *smtA* gene was expressed in *Escherichia coli* as a carboxyterminal extension of glutathione-*S*-transferase. The pH of half dissociation of Zn, Cd and Cu ions from the expressed protein was determined to be 4.10, 3.50, 2.35, respectively, indicating a high affinity for these ions (in particular for Zn in comparison to mammalian MT). *E. coli* expressing this gene showed enhanced (ca. 3-fold) accumulation of Zn.

smtA; SmtA protein; Prokaryotic metallothionein; Cyanobacteria; Synechococcus; *Anacystis nidulans*; Metal-accumulation; Metal-tolerance

1. INTRODUCTION

Although metallothionein (MT)-'like' metal-ligands have been reported in several different prokaryotes (cited in [1]) only one such protein has been isolated (from a cyanobacteria by Olafson et al. [2]) and sequenced. There is no significant similarity between the amino acid sequence of this Synechococcus protein and any other sequenced MT. The amount of this protein in Synechococcus cells was found to increase following supplementation of the growth medium with either Zn or Cd, but not Cu [3]. The native protein was isolated associated predominantly with either Zn or Cd corresponding with the metal administered to the cells. In addition, a small amount of Cu was detected following induction with either Cd or Zn. CD spectral analysis of the native Zn-protein identified some characteristics resembling mammalian Zn-MT [2]. However, the conformation of the metal-binding site(s) has (have) not been determined and the metal affinities of this protein have not previously been reported. Whether or not this prokaryotic protein has high affinities for metals, equivalent to those of eukaryotic MTs, needs to be established.

We have recently isolated PCR fragments corresponding to the gene encoding this cyanobacterial protein [4]. Genomic clones have subsequently been isolated and the gene designated *smtA*. Several different eukaryotic MT genes have been expressed in *E. coli* and their proteins shown to form conformations which allow association with metal ions [5–10]. In some cases eukaryotic MTs have been demonstrated to bind metal ions when expressed as fusions with another protein [7,9]. In addition, *E. coli* cells expressing murine MT-1 over-accumulated Cd and showed elevated resistance to Hg, Ag, Cu, Cd and Zn [10]. In contrast *E. coli* expressing rainbow trout MT did not show any significant increase in resistance to trace metals but did show increased accumulation of Cd [8]. Expression of *Neurospora crassa* MT, and variants of this protein, in *E. coli* led to increased accumulation exclusively of Cd and Cu from a mixture of 16 different metal ions [9]. In this letter we describe the expression of *smtA* in *E. coli* in order to facilitate analysis of the metal-binding characteristics of the expressed protein. In addition, *E. coli* cells expressing this gene have been examined for phenotypic effects in terms of modified metal-tolerance or -accumulation.

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2. MATERIALS AND METHODS

2.1. *In vitro amplification, cloning and sequence analysis of smtA coding region*

The following two oligonucleotide primers were synthesized using an Applied Biosystems 381A DNA synthesizer; 5'GGCGGATCCC-CATGACCTCAACAACCTTGGTC 3'; 5'GGCGAATTCACTA-CAGTTGCAGCCGGTGTGGCC 3'. These primers facilitated amplification by the polymerase chain reaction (PCR) of the *smtA* protein coding region and incorporated *Bam*HI and *Eco*RI restriction endonuclease recognition sites at opposite ends. These sites allowed cloning (in frame) into the glutathione-*S*-transferase (GST) fusion protein expression vector, pGEX3X (Pharmacia).

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PCR was performed using 200 ng of plasmid pJHNR4.9 (a genomic clone in the vector pGEM4z containing a 1.8 kb *Hin*dIII-*Sal*I fragment of *Synechococcus* PCC7942 DNA which includes the *smtA* gene) as template and standard conditions as described previously [4]. Purified PCR products were digested with *Bam*HI and *Eco*RI and then ligated into the equivalent sites of pGEX3X.

The sequence and orientation of the cloned *smtA* fragment in pGEX3X (pGPMT1) was verified by sequencing in both directions using the PCR oligonucleotide primers. The determined sequence of the *smtA* coding region and part of GST is given in Fig. 1. DNA sequencing was performed by modified dideoxy termination (Dye Deoxy Termination: Applied Biosystems) and reaction products were analysed using an Applied Biosystems 370A DNA sequencer.

2.2. *Expression, purification and characterisation of the recombinant SmtA protein*

The recombinant protein was expressed in *E. coli* and purified using glutathione Sepharose 4B (Pharmacia) as described previously [11]. Protein content was determined using a Coomassie blue based reagent (Bio-Rad) and bovine serum albumin as a standard. Metal concentration of samples was determined by atomic absorption spectrophotometry. Proteins were resolved on 15% SDS-PAGE gels [12] and visualised following Coomassie brilliant blue staining.

The protein sequence between GST and SmtA includes a specific protease recognition sequence for blood coagulation factor Xa [13] which facilitated cleavage of SmtA protein from GST. SmtA protein (purified from cells grown in LB medium supplemented with 0.5 mM Zn) was cleaved from GST while the fusion protein was associated with glutathione Sepharose 4B [14]. Column eluant containing factor Xa and SmtA protein was subsequently fractionated on Sephadex G-50. Fractions (2.5 ml) were collected and analysed for protein and metal. The amino acid sequence of the protein in the Zn-peak fraction was determined as described previously [11].

2.3. *Analysis of metal-binding properties of the expressed protein*

Aliquots of GST-SmtA fusion protein (purified from cells supplemented with 2 mM Cu; 0.5 mM Zn; 0.5 mM Cd, or 20 μM Hg) were incubated in different pH buffers and the proportion of bound metal ions determined as previously described [11]. Preparations of Zn-GST-SmtA fusion protein were also incubated with a two fold molar excess (with respect to Zn) of Cu. Unbound metal was removed by gel filtration (PD-10, Pharmacia) and the pH of half-dissociation of in vitro associated metal ions determined. The experiment was also repeated with a commercial preparation of equine metallothionein (Sigma).

2.4. *Analysis of metal-resistance and -accumulation*

Overnight cultures containing pGEX3X, or pGPMT1 were diluted 90% (v/v) in fresh LB medium supplemented with 50 $\mu g \cdot ml^{-1}$ ampicillin, then grown for 1 h at 37°C. Cultures were diluted again into fresh LB media containing 50 $\mu g \cdot ml^{-1}$ ampicillin such that the final OD_{600} of all cultures was 0.025. After a further incubation of 1 h, production of GST, or GST-SmtA, was induced by the addition of IPTG to a final concentration of 1 mM and metal was added to inhibitory concentrations (1 mM $ZnSO_4$; 2 mM $CuSO_4$; 0.6 mM $CdSO_4$). Cells were incubated at 37°C and growth-monitored by OD_{600} after 1 h and at hourly intervals thereafter.

To determine the amount of metal accumulated by *E. coli* cells, standardised aliquots (equivalent absorption at 600 nm) were removed from cultures grown as described above. The cells were harvested by centrifugation, washed twice in LB medium and solubilised overnight by incubation in 70% (v/v) nitric acid/water. Metal content was determined by atomic absorption spectrophotometry and converted to amount of metal per 8×10^8 cells (assuming an optical density of 1 is equivalent to 8×10^8 cells ml^{-1}). Additionally, metal accumulation was analysed in a similar manner for cells grown in the presence of lower concentrations of each metal (0.5 mM $ZnSO_4$, 0.5 mM $CuSO_4$, 0.3 mM $CdSO_4$.

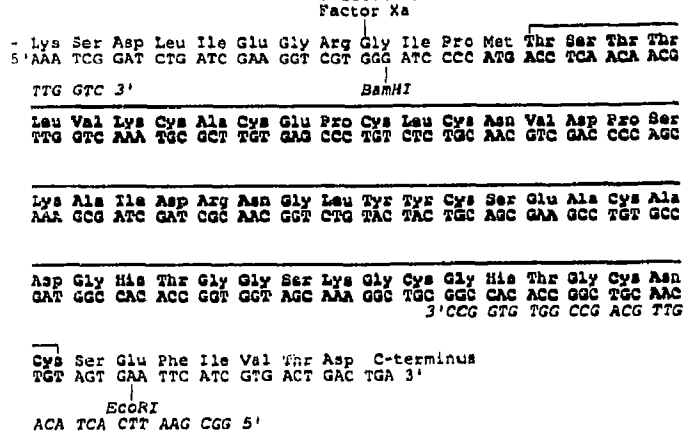

Fig. 1. The determined nucleotide sequences of part of the GST coding sequence from the expression vector pGEX3X and of the *smtA* coding region (shown in bold) generated from a genomic clone by PCR using oligonucleotide primers shown in italics. The PCR product was ligated using *Eco*RI and *Bam*HI recognition sites to produce an in-frame translational fusion which may be cleaved with factor Xa at the indicated site. The SmtA protein sequence is in bold type and is denoted with a line.

3. RESULTS AND DISCUSSION

Sequence analysis of pGPMT1 confirmed the orientation and frame of the *smtA* coding region fused to GST (Fig. 1). The predicted amino acid sequence of the product of *smtA* is in agreement with the reported sequence of the protein purified from *Synechococcus* sp. [2], with the exception of Ser^{32}, a discrepancy which we have previously observed [4]. Most recently we have also established that the *smtA* gene from *Synechococcus* PCC7942 contains an additional two codons adding a further ^{N}His-Gly^{C} to the C-terminus of the SmtA protein in this organism. These two amino acids are absent from the protein expressed here.

A protein corresponding to the predicted size (M_r 35,500) of the GST-SmtA fusion protein was detected in GSH-affinity purified lysates of induced JM101 cells containing plasmid pGPMT1 following growth in media supplemented with 0.5 mM Zn (Fig. 2A). A protein corresponding to the predicted size (M_r 26,500) of

Table I

Metal content of GST and GST-SmtA, purified from lysates of cells grown in media supplemented with Cd, Cu, Hg or Zn.

	Metal ion/polypeptide			
Protein	Cd	Cu	Hg	Zn
GST	0.87 ± 0.14	0.35 ± 0.32	0.80 ± 0.18	0.89 ± 0.25
GST-SmtA	5.89 ± 1.35	1.72 ± 1.03	6.78 ± 0.25	4.34 ± 0.27

The data shows the estimated number of moles of metal associated with each mol of protein. Mean values are given for extracts from three replicate cultures with standard deviations.

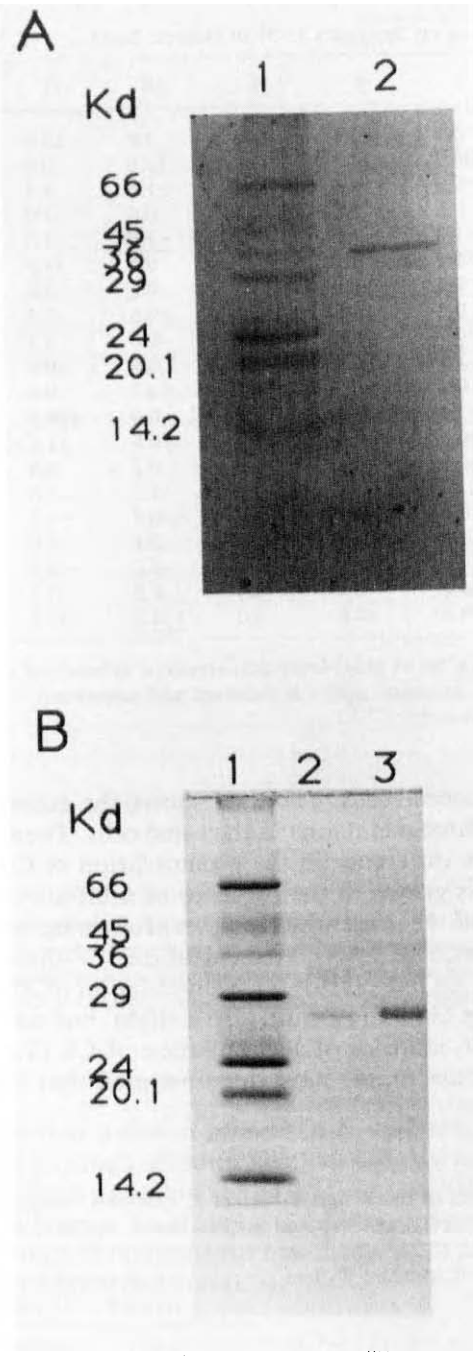

Fig. 2. Analysis of *E. coli* cell extracts by SDS-PAGE. Gels were stained with Coomassie blue. Origin of protein samples: molecular weight markers (both panels, lane 1); glutathione-Sepharose 4B purified lysates from IPTG-induced cells containing pGEX3X (B, lane 3) or pGPMT1 (both panels, lane 2). Protein was purified from cells grown in media supplemented with Cu (B, lane 2) or Zn (A, lane 2).

GST alone was detected in equivalent isolates from cells containing the pGEX3X vector alone (Fig. 2B). The bound metal content of GST-SmtA and GST alone was determined for protein purified from *E. coli* grown in media supplemented with Cu, Cd, Hg or Zn (Table I).

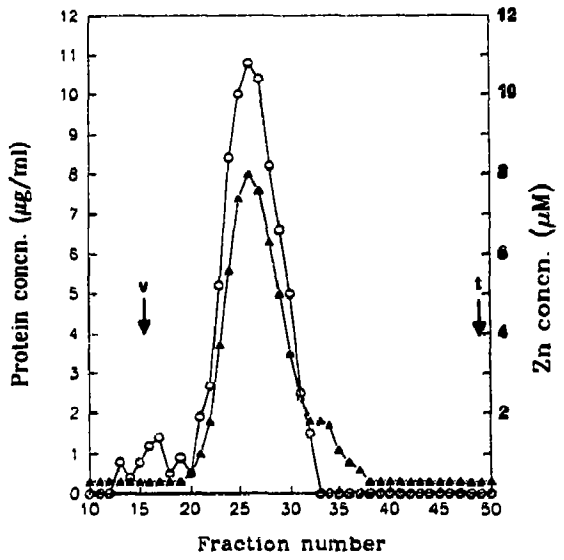

Fig. 3. Gel filtration chromatography on Sephadex G-50 of purified protein eluted from glutathione Sepharose 4B following incubation with factor Xa. Protein (○) was determined by dye binding assay and Zn (▲) was quantified by atomic absorption spectrophotometry. The void (v) and total (t) volumes of the column were determined by calibration with Blue dextran and Zn (II) respectively.

Relatively more metal was found to be associated with the fusion protein than with GST alone. However, variability in reactivities of different proteins (bovine serum albumin; GST; SmtA) in the protein assay may cause inaccuracies in the estimated stoichiometries. Furthermore, it is apparent that isolates from cells grown in media supplemented with Cu were impure (Fig. 2B). This could partly account for the lower estimates for the amount of Cu associated with the protein relative to the other three metal ions.

Data in Table I indicate that Cd, Cu, Hg and Zn all bind to the SmtA portion of the GST-SmtA fusion protein. To establish that metal ions associate with this part of the fusion protein, the SmtA domain was released from the fusion protein (purified from *E. coli* grown in Zn supplemented media) while associated with glutathione-Sepharose, using factor Xa. This releases SmtA plus an extension of three amino acids at the amino terminus (^{N}Gly-Ile-Pro^{c}) and an extension of seven amino acids at the carboxy terminus (^{N}Ser-Glu-Phe-Ile-Val-Thr-Asp^{C}). These additional amino acids arise from the GST coding sequence into which the *smtA* coding sequence is inserted in plasmid pGPMT1 (refer to Fig. 1). Eluate from glutathione-Sepharose was fractionated on Sephadex G-50 and fractions analysed for Zn and for protein (Fig. 3). A large coincident peak for Zn and protein was detected (fraction 26). Amino acid sequence analysis of an aliquot of this Zn-peak fraction gave the first 12 amino acids of SmtA, plus the anticipated N-terminal extension (Table II). A less abundant second amino acid was also detected at each cycle of Edman degradation. This sequence corresponded to SmtA se-

Table II

Amino acid sequence analysis of Zn peak fraction after gel filtration on Sephadex G-50 of cleaved SmtA

Cycle	1	2	3	4	5	6	7	8	9	10	11	12
Ala	15.8	0.0	−0.8	−3.8	−0.7	−0.4	0.1	3.9	*83.9*	0.0	15.6	0.0
Arg	102.7	0.0	0.0	0.0	−2.3	0.0	36.9	14.8	9.5	13.6	0.0	0.0
Asn	−0.2	0.3	2.9	−0.4	0.2	0.4	−0.7	0.4	−0.2	−1.0	1.5	−1.1
Asp	53.4	0.0	0.0	−0.3	−1.3	−0.4	−0.6	0.0	3.0	0.0	1.0	0.0
Cys	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	*−1.0*	−1.0	*−1.0*	−1.0	**−1.0**
Glu	2.4	−1.5	−0.6	33.4	0.0	0.0	−1.5	−0.5	−0.5	0.5	*54.5*	0.0
Gln	0.5	0.2	−0.1	−1.1	0.7	−0.8	−0.5	1.1	1.1	0.0	2.6	0.0
Gly	**478.3**	0.0	0.0	−1.4	0.0	0.0	0.0	23.1	−1.1	−0.5	0.7	10.7
His	−0.4	0.7	1.7	0.1	2.4	4.0	−0.9	0.0	8.3	0.0	5.3	0.6
Ile	−0.3	**528.6**	0.0	0.0	0.0	0.0	−1.5	24.8	14.8	0.0	0.0	0.0
Leu	−1.2	46.7	0.0	−1.4	*67.4*	0.0	−0.3	−6.3	**169.7**	92.2	0.0	0.0
Lys	21.2	0.0	0.0	−1.0	−1.9	−0.3	*51.4*	0.0	16.1	0.0	**159.5**	110.2
Met	4.9	−0.3	0.0	**433.6**	0.0	0.0	0.0	0.0	0.4	10.8	11.5	0.0
Phe	0.4	−0.1	−0.3	−0.1	0.4	0.0	−0.8	−0.1	0.2	0.2	0.6	0.2
Pro	0.3	7.6	**391.6**	0.0	0.0	0.0	−11.3	−3.8	2.5	11.3	−5.8	*34.9*
Ser	1.9	*23.1*	0.0	−2.1	0.0	**48.6**	0.0	4.2	0.0	0.4	−1.3	2.1
Thr	*76.2*	−0.2	*25.7*	*25.6*	**116.0**	0.0	**51.5**	**110.2**	22.7	2.1	0.0	0.0
Trp	0.7	−0.5	−0.4	−0.1	−0.1	0.1	0.3	0.1	0.2	−0.2	−0.2	0.2
Tyr	3.3	−0.1	−0.1	−0.3	0.2	0.3	−0.2	0.5	−0.4	−1.0	−0.7	0.2
Val	1.3	−0.8	−2.0	−1.3	5.5	*84.0*	0.0	14.8	0.0	**174.2**	105.9	0.0

The yield (pmol) of each amino acid is shown at each cycle with the anticipated sequence of SmtA plus N-terminal extension in bold and underlined. A secondary sequence corresponding to the anticipated sequence initiated at amino acid 5 is italicised and underlined.

quence initiated four amino acids beyond the factor Xa cleavage site. These data confirm that metal ions associate with the SmtA portion of the recombinant protein.

Comparison of the pH at which 50% of metal is dissociated is a criterion which has been used to distinguish MT from non-MT metal-binding proteins. From the graphs shown in Fig. 4 (panels A) such values can be estimated for the GST-SmtA fusion protein to be 4.10, 3.50 and 2.35 for Zn, Cd and Cu respectively. These values were obtained for fusion protein isolated from cells grown in media supplemented with the respective metal ions. A similar value was also obtained for Cu associated with GST-SmtA by exchange binding in vitro. Bound Hg could not be displaced under the conditions used in these experiments indicative of high affinity for this metal. From Fig. 4 the pH of half-dissociation of Zn from equine MT is estimated to be 4.50, which is in agreement with previously reported values [15,16]. This implies that SmtA may have a higher affinity for Zn than does equine MT and could have an important role in Zn metabolism/detoxification in Synechococcus cells. Metal displacement curves for Cu and Cd indicate that SmtA has a lower affinity for these metals than does equine MT.

Expression of the GST-SmtA fusion protein under the conditions reported here did not confer any detectable increase in Cd, Cu or Zn tolerance in *E. coli* containing plasmid pGPMT1 compared to cells containing the pGEX3X vector alone (data not shown). This does not preclude the possibility that metal-regulated expression of *smtA* confers enhanced tolerance to certain metals in Synechococcus cells. Table III shows the accumulation of these three metal ions in the same cells. There was no significant difference in the accumulation of Cd, Cu or Zn in cells grown in the presence of inhibitory concentrations of these metals. However, following growth in the presence of lower concentrations of these metals there was an increase in the accumulation of Zn in cells expressing GST-SmtA and also a slight, but statistically significant, increase in accumulation of Cu (Table III).

In conclusion, we have demonstrated that following

Table III

Accumulation of metal ions by intact *E. coli* cells containing either pGPMT1, or pGEX3X expressing GST-SmtA, or GST, respectively

	Expressed protein	Accumulated metal (nmol/8 × 10^8 cells)	
		[high]	[low]
Cd	GST	24.406 ± 3.825	5.789 ± 0.858
	GST-SmtA	23.531 ± 5.168	8.717 ± 2.410
Zn	GST	11.178 ± 0.885	1.998 ± 0.205
	GST-SmtA	12.172 ± 0.834	6.049 ± 1.038
Cu	GST	16.778 ± 7.371	6.193 ± 0.595
	GST-SmtA	21.762 ± 8.96	8.869 ± 1.164

Cells were grown in media supplemented with either inhibitory concentrations ([high]) of 0.6 mM $CdSO_4$, 1 mM $ZnSO_4$ or 2 mM $CuSO_4$ or lower concentrations ([low]) of 0.3 mM $CdSO_4$, 0.5 mM $ZnSO_4$ or 0.5 mM $CuSO_4$. Values are the mean of nine replicate determinations with standard deviation.

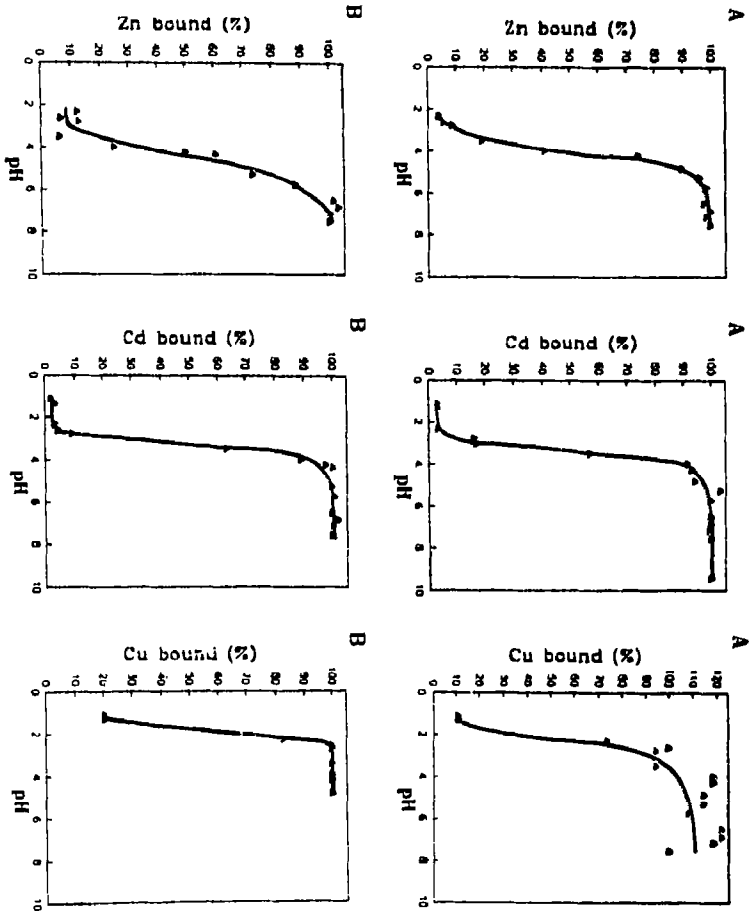

Fig. 4. Hydrogen ion competition of metal binding to GST-SmtA fusion protein (panels A). The data is expressed as a proportion of metal bound at the highest pH. Protein was purified from *E. coli* grown in media supplemented with either Zn, Cd or Cu. Aliquots of purified (via glutathione Sepharose 4B) protein were incubated for 1 h at the indicated pH. Free and bound Zn, Cd and Cu were resolved by gel filtration on columns of Sephadex G-25 (PD-10, Pharmacia) equilibrated with equivalent pH buffer. The analysis was also repeated with a commercial preparation of equine MT (panels B).

expression as a fusion protein in *E. coli*, the *smtA* gene product has high affinities for Cd, Cu, Hg and Zn supporting its designation as the first characterised prokaryotic MT gene. Continuing studies are elucidating the significance of this gene for metal-tolerance and -metabolism in cyanobacterial cells.

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