

Development of Bacterium-Based Heavy Metal Biosorbents: Enhanced Uptake of Cadmium and Mercury by *Escherichia coli* Expressing a Metal Binding Motif

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A gene coding for a de novo peptide sequence containing a metal binding motif was chemically synthesized and expressed in *Escherichia coli* as a fusion with the maltose binding protein. Bacterial cells expressing the metal binding peptide fusion demonstrated enhanced binding of Cd^{2+} and Hg^{2+} compared to bacterial cells lacking the metal binding peptide. The potential use of genetically engineered bacteria as biosorbents for the removal of heavy metals from wastewaters is discussed.

The discharge of heavy metals into the environment due to agricultural, industrial, and military operations and the effect of this pollution on the ecosystem and human health are growing concerns. Recent research in the area of heavy metal removal from wastewaters and sediments has focused on the development of materials with increased affinity, capacity, and selectivity for target metals (7, 8, 30). The use of microorganisms to sequester, precipitate, or alter the oxidation state of various heavy metals has been extensively studied (7, 8, 15, 24, 27). Expression of metallothioneins (1, 17, 20, 24, 25) or metalloproteins (4, 28) to increase the affinity and biosorptive capabilities of bacterial cells for heavy metals is a promising technology for the development of bacterium-based biosorbents. We are studying the expression of metal binding peptides with high affinity and selectivity for target metals in *Escherichia coli* and the potential use of such peptides as heavy metal biosorbents. We previously reported expression of the *Neurospora crassa* metallothionein gene in *E. coli* by fusion to the maltose binding protein of expression vector pMAL (New England Biolabs) (21). This recombinant *E. coli* organism was shown to remove heavy metals such as Cd^{2+} from simulated and actual wastewater samples (3, 20). The de novo design of metal binding motifs has been described and offers the potential for the design and expression of peptides with high affinity and selectivity for various metals (5, 9, 12, 14, 23). In addition to the metallothioneins, peptides which contain an abundance of cysteine residues or Cys-Gly motifs have been shown to have a high affinity for Cd^{2+} and Hg^{2+} (5, 22). In this study, heavy metal removal by bacterial cells expressing a repetitive metal binding motif, (Cys-Gly-Cys-Cys-Gly)₃, expressed as a fusion with the maltose binding protein is reported.

Synthesis of the metal binding gene. The metal binding gene encodes 21 amino acids with the motif Cys-Gly-Cys-Cys-Gly repeated three times and linked through a three-amino-acid sequence (Fig. 1A). Four overlapping oligonucleotides were synthesized (0.2 μM scale) on an Applied Biosystems 371 DNA synthesizer. Oligonucleotides were purified and subsequently phosphorylated, hybridized, and ligated by using standard molecular biology protocols as described previously (16).

The sequences for the four overlapping oligonucleotides used are as follows: SYNTOP1, 5' GATCCTGTGGTTGCTGTGGCAAAGGTCATTGTGGCTGT 3'; SYNTOP2, 5' TGCGGCAAAGGTCACCTGCGGTTGCTGTGGTA 3'; SYNBOT1, 5' TTTGCCGCAACAGCCACAATGACCTTTGCCACAGCAACCACAG 3'; and SYNBOT2, 5' AGCTTACCACAGCAACCGCAGTGACC 3'.

The constructed gene contains *Bam*HI and *Hind*III overhangs at the 5' and 3' ends, respectively, for directional cloning into expression vector pMAL-p digested with these restriction enzymes. Following ligation, bacterial cells were transformed and screened for colonies containing the gene insert by hybridization and restriction analysis. DNA sequencing of positive clones (performed by the University of Georgia DNA Sequencing Facility) confirmed the correct in-frame cloning of the gene into pMAL-p, yielding expression vector pMAL-p-syn. Figure 1C presents the DNA sequence of pMAL-p-syn showing the metal binding gene insert flanked by the *Bam*HI and *Hind*III sites of vector pMAL-p.

Expression and localization within the periplasm. The expression of small proteins in *E. coli* has often been problematic due to instability and degradation (18, 21). This is especially true in cases where the protein is repetitive in nature. These problems have been minimized by expressing these proteins as fusion proteins (18, 21, 25, 26). We previously expressed the *N. crassa* metallothionein gene within the cytoplasm and periplasm of *E. coli* by using the fusion protein of vector pMAL-c and pMAL-p (encoding the maltose binding protein), respectively (21).

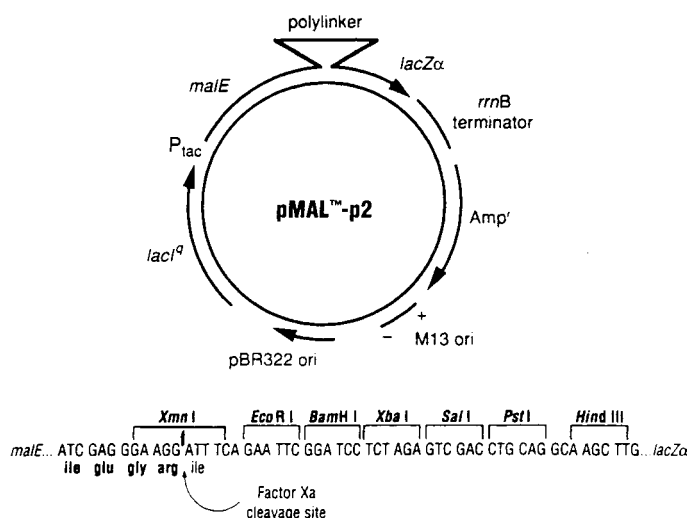
These and other studies have shown that bacterial cells expressing metallothionein within the cytosol are less efficient in accumulating heavy metals from solutions than cells expressing the metallothionein within the periplasm or outer membrane (4, 21, 26). In the present study only the pMAL-p vector was used to express the metal binding gene as a fusion with the maltose binding protein within the periplasm. To determine the level of expression and cellular localization of the metal binding peptide fusion, *E. coli* TB1 cells were transformed with either the pMAL-p-syn plasmid (containing the metal binding gene) or the parent pMAL-p plasmid as a control. Cells were grown to an optical density of 0.6 in Luria broth (LB) containing ampicillin and induced with 2 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 1 h as reported previously (21). Induced cells were harvested by centrifugation at 6,000 \times g and

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A.

5' TGT	GGT	TGC	TGT	GGC	AAA	GGT	CAT 3'
3' ACA	CCA	ACG	ACA	CCG	TTT	CCA	GTA 5'
CYS	GLY	CYS	CYS	GLY	LYS	GLY	HIS
5' TGT	GGC	TGT	TGC	GGC	AAA	GGT	CAC 3'
3' ACA	CCG	ACA	ACG	CCG	TTT	CCA	GTG 5'
CYS	GLY	CYS	CYS	GLY	LYS	GLY	HIS
5' TGC	GGT	TGC	TGT	GGT 3'			
3' ACG	CCA	ACG	ACA	CCA 5'			
CYS	GLY	CYS	CYS	GLY			

B.



C.

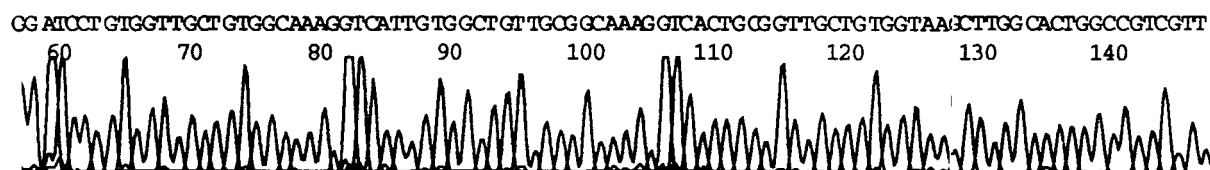


FIG. 1. Cloning of the metal binding gene into the pMAL-p vector. (A) Nucleotide and amino acid sequence of the metal binding gene. (B) Map of vector pMAL-p. (C) DNA sequence of metal binding gene cloned in pMAL-p. Ori, origin of replication.

separated into cytoplasmic and periplasmic fractions utilizing the cold osmotic shock method of Neu and Heppel (19) as described previously (21). The maltose binding protein containing the metal binding peptide fusion and the maltose binding protein lacking the metal binding peptide fusion (from control cells) were purified from the cytoplasmic and periplasmic fractions by using an amylose resin affinity column under conditions recommended by the manufacturer (New England Biolabs) (21). Protein content within each fraction was determined by using the Bradford method (2) with the Bio-Rad (Richmond, Calif.) protein assay kit. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (13) as described previously (21). More than 85% of the expressed protein was found to be within the periplasm. This is consistent

with results obtained from the expression of metallothionein and other proteins by using the pMAL-p vector (21). β -Galactosidase activity was used as a marker to ensure that no cytoplasmic proteins had leaked into the periplasm during fractionation (21).

Figure 2 shows an SDS-PAGE gel of the purified metal binding peptide fusion protein isolated from the periplasmic fraction of induced cells. The purified protein appears as a 44-kDa band which is similar in size to the purified protein isolated from cells lacking the metal binding peptide (expressing only the maltose binding protein). To confirm the presence of the metal binding peptide, the purified proteins were subjected to amino acid composition analysis (performed by Baylor College of Medicine Core Protein Sequencing Facility) (21). Since the maltose binding protein does not contain any

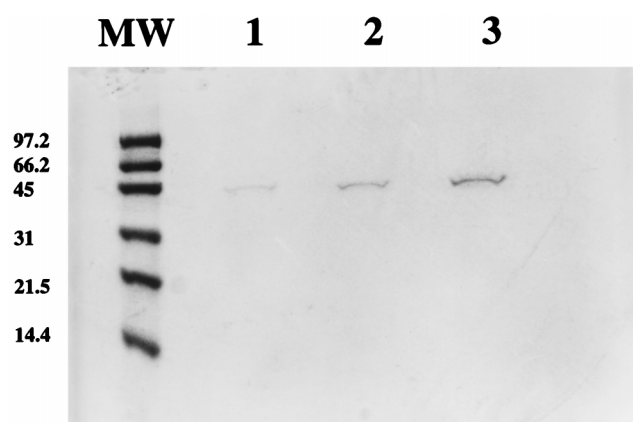


FIG. 2. SDS-PAGE of purified metal-binding peptide fusion. The gel was stained with 0.1% Coomassie blue. Lane MW contains molecular weight markers, in thousands; lanes 1 to 3 contain 0.5, 1, and 2 μ g of purified metal binding peptide, respectively.

cysteine residues (6), the expression of cysteine-containing peptides by using this fusion provides a convenient and direct method for determining the authenticity of the desired product. Amino acid analysis of the purified maltose binding protein containing the putative metal binding peptide revealed that the protein contained 1.8 mol% cysteine, reflecting the addition of nine cysteines, whereas the purified maltose binding protein lacking the metal binding peptide contained 0 mol% cysteine, as expected. This is comparable to the 1.5-mol% cysteine content for the expressed metallothionein previously reported, which contains seven cysteine residues (21).

Heavy metal removal. The ability of cells expressing the metal binding peptide fusion to remove heavy metals from solutions was tested and compared to that of control bacteria lacking the metal binding peptide (expressing only the maltose binding protein). Induced bacterial cells (1 liter) were harvested by centrifugation and resuspended in 10 ml of LB. Several aliquots of the resuspended cells were placed in 1.5-ml centrifuge tubes and centrifuged at high speed to pellet the cells for wet weight estimation. Cells (100 mg [wet weight]) were resuspended in 30 ml of LB and then 30 μ l of 5 mM stock solution of Cd^{2+} (CdCl_2), Hg^{2+} (HgCl_2), Pb^{2+} [$\text{Pb}(\text{NO}_3)_2$], or Cu^{2+} (Cu_2SO_4) was added, yielding a final concentration of 5 μ M. Samples were incubated for 1 h at 37°C. Following incubation, samples were centrifuged to pellet bacterial cells and the supernatant was analyzed for heavy metal content by Accura Analytical (Norcross, Ga.). As shown in Table 1, cells expressing the metal binding peptide fusion efficiently remove Cd^{2+} and Hg^{2+} (1.1 and 1.3 nmol removed/mg of cells [wet weight]), whereas control cells lacking the metal binding peptide remove less than 0.1 nmol/mg of cells (wet weight). Removal of Pb^{2+}

and Cu^{2+} was greater than in control cells, but the difference was not as great as for Cd^{2+} and Hg^{2+} (Table 1).

Although the level of heavy metal contamination is variable at different sites, the most promising use for a bacterium-based biosorbent may be the removal of very low levels of target metals in the presence of other metals. In this case, the bulk of the heavy metal is removed through a primary treatment, and a secondary, or polishing, application is added to further reduce the levels of target metals in the effluent (3, 17). The ability of cells expressing the metal binding peptide to remove low levels of heavy metal was tested by using part-per-billion levels of ^{109}Cd . A stock solution was prepared by addition of 0.5 μCi of radioisotopic ^{109}Cd (specific activity, 180 Ci/mol) to a 5-ml aliquot of unlabeled CdCl_2 , yielding a final specific activity of 5 $\mu\text{Ci/mol}$. Bacterial cells (20 mg [wet weight]) were resuspended in 5 ml of LB and incubated with 50 μl of the ^{109}Cd stock solution, yielding a final concentration of 0.2 μM (approximately 22 ppb). Samples were incubated for 1 h at 37°C and then centrifuged to pellet the bacterial cells. Radioactivity was measured in both the supernatant and pellets by using a Packard liquid scintillation counter as described previously (21). Figure 3 shows the total amount (in nanomoles) of ^{109}Cd which is cell bound or remains in the supernatant after incubation. Of the 1.0 nmol of ^{109}Cd added, cells expressing the metal binding peptide bound 0.92 nmol, whereas cells lacking the metal binding peptide bound only 0.13 nmol. The effect of coinubation with increasing concentrations of unlabeled heavy metals on the removal of ^{109}Cd by cells was tested. In these experiments 20 mg of cells was coinubated in 5 ml of LB with 0.2 μM ^{109}Cd and unlabeled Cd^{2+} , Hg^{2+} , Pb^{2+} , or Cu^{2+} at a concentration of 0.2, 1, or 5 μM . Figure 4 shows the effect of increasing concentrations of the unlabeled metals on the ability of cells to remove ^{109}Cd from solutions. Cd^{2+} and Hg^{2+} have the most inhibitory effect on ^{109}Cd removal, indicating that these metals have a high affinity for the peptide. Pb^{2+} is also inhibitory at higher concentrations, but Cu^{2+} does not have any inhibitory effect, even at the highest concentration tested. The ability of cells expressing the metal binding peptide fusion to effectively and selectively remove Cd^{2+} and Hg^{2+} from solutions with a low initial (part-per-billion) concentration (Fig. 3 and 4) along with the data presented in Table 1 demonstrates the potential for these cells to be used at various metal-contaminated sites.

Our approach of using genetically engineered organisms for heavy metal removal has focused on the expression of peptides with high cysteine content within the periplasmic space of bacterial cells and the use of such bacteria as an immobilized biomass. The metal binding peptide described in this study is similar in size and cysteine content to the previously expressed *N. crassa* metallothionein (21), and it was designed for high affinity to Cd^{2+} and Hg^{2+} through the incorporation of Cys-Gly and Cys-Cys-Gly motifs. The potential benefits of a de novo-constructed metal binding peptide versus naturally occur-

TABLE 1. Heavy metal removal by cells expressing the metal binding peptide and by control cells expressing only the maltose binding protein

Heavy metal	Total amt added (nmol)		Amt in supernatant after incubation		Calculated amt bound (nmol/mg of cells [wet wt])	
	Calculated	Measured	Cells expressing peptide	Control cells	Cells expressing peptide	Control cells
Cd^{2+}	150	149.6 \pm 1.8	46.0 \pm 8.4	145.2 \pm 3.6	1.10 \pm 0.12	0.09 \pm 0.03
Hg^{2+}	150	137.3 \pm 6.1	8.3 \pm 1.2	142.5 \pm 12.7	1.30 \pm 0.02	ND ^a
Pb^{2+}	150	136.3 \pm 8.9	40.6 \pm 11.5	121.6 \pm 6.2	0.95 \pm 0.11	0.14 \pm 0.06
Cu^{2+}	150	145.0 \pm 2.8	89.3 \pm 6.1	153.6 \pm 9.5	0.55 \pm 0.06	ND

^a ND, not determined.

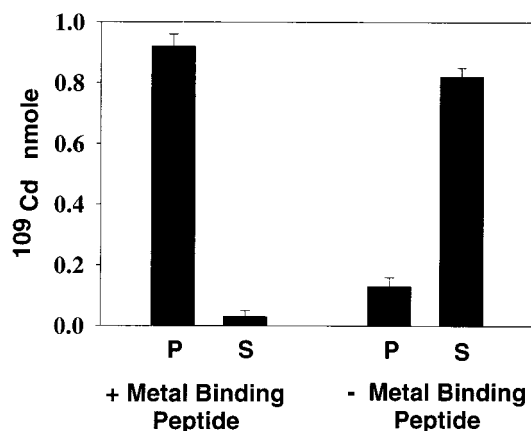


FIG. 3. ¹⁰⁹Cd binding by cells expressing or lacking the metal binding peptide. S, supernatant; P, pellet; + metal binding peptide, cells expressing the metal binding peptide; - metal binding peptide, control cells lacking the metal binding peptide.

ring metal binding proteins include the ability to incorporate metal binding sites for several metals within one protein as well as the potential for addition of amino acid sequences for increased protein stability (11). To further increase the biosorption of heavy metals by *E. coli*, the expression of tandem repeat copies of the metallothionein and this peptide is currently being studied. The construction of genetically engineered organisms for heavy metal bioaccumulation has recently been reported by other investigators. Chen and Wilson (4) reported the simultaneous expression of a metallothionein gene and a mercury transport system in *E. coli*. This study showed that expression of the metallothionein in the cytosol without coexpression of the transport system resulted in diminished bioaccumulation of Hg²⁺ by the cells. These results are consistent with our previous work and other studies indicating that maximal bioaccumulation of metal occurs with expression of metal binding proteins outside the cytosol (4, 21, 25, 29). In addition, expression outside the cytosol may allow for the use of nonviable cells for metal accumulation and for efficient desorption of the bound metal (3, 7, 20). Sousa et al. reported the expression of yeast and mammalian metallothioneins within the outer membrane of *E. coli* when LamB was used as an anchoring domain (29). The binding of Hg²⁺ and Cd²⁺ by cells expressing the metal binding peptide fusion described in the present

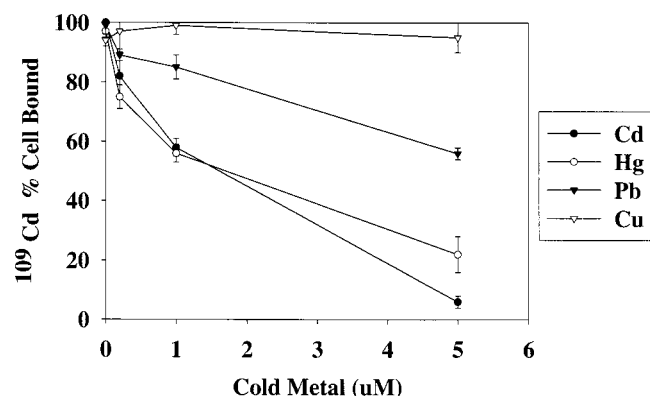


FIG. 4. Effect of coinubation with cold metal on the binding of ¹⁰⁹Cd by cells expressing the metal binding peptide. A total of 100% cell bound is equal to 1.0 nmol of ¹⁰⁹Cd, which is the total amount added.

study is comparable to those described by Chen and Wilson (4) and Sousa et al. (28, 29). The metal binding studies described by these investigators, however, were performed under different conditions and at heavy metal concentrations of 5 μM (4) and 20 to 30 μM (28, 29), whereas our studies were performed at concentrations of 0.2 to 5 μM.

The development of an immobilized biomass from bacterial cells has been discussed as an approach for the development of heavy metal removal systems (17) and involves the immobilization of nonviable cells or cell remnants containing the metal binding peptide within a suitable matrix. The ultimate utility of such a system will depend on binding capacity, affinity, selectivity, stability, and ease of production, among other factors. The current expression system in *E. coli* provides several benefits for the study of metal binding peptides, including periplasmic localization, enhanced stability of the expressed peptide, and lack of cysteine in the fusion, thus providing a convenient method for analysis of cysteine-containing peptides. However, alternate cost-effective promoters (e.g., heat induced), increased levels of metallothionein expression (10), and potential for expression of metal binding peptides in other organisms need to be studied for further development. The expression and incorporation of amino acid sequences which are known to stabilize proteins against heat, pH, and salinity may further increase the utility of such a system (11).

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