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Bacterium-Based Heavy Metal Biosorbents: Enhanced Uptake of Cadmium by *E. coli* Expressing a Metallothionein Fused to β -Galactosidase

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ABSTRACT

*We investigated the potential utility of a recombinant *E. coli* that expresses the human metallothionein II gene as a fusion protein with β -galactosidase as a heavy metal biosorbent. *E. coli* cells expressing the metallothionein fusion demonstrated enhanced binding of Cd^{2+} compared to cells that lack the metallothionein. It was shown that the metallothionein fusion was capable of efficiently removing Cd^{2+} from solutions. Approximately 40% of the Cd^{2+} accumulated by the recombinant cells free in suspension was associated with the outer cell membrane, and 60% of that was present in the cytoplasm.*

INTRODUCTION

The discharge of heavy metals into the environment caused by agricultural (4), industrial (1,26), and military operations (7) and the effect of this pollution on human health and the ecosystem (16,19) are growing concerns. The 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 (17,18,23,32). The use of microorganisms to sequester, precipitate, or alter the oxidation state of various heavy metals has been studied extensively (8).

Chemical oxidation-reduction, precipitation, adsorption, solidification, electrolytic recovery, and ion exchange are some of the physicochemical wastewater treatment processes that are used for metal removal. However, the application of such processes is sometimes restricted because of technical or

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economic constraints. Biosorption has distinct advantages over conventional methods. The process does not produce chemical sludge, and it can be highly selective, efficient, easy to operate, and cost effective for the treatment of large volumes of wastewaters containing low metal concentrations. For example, Purnanik et al. (23) reported that biosorbent beads prepared by immobilizing the *Citrobacter* biomass in polysulfone matrix exhibited high metal-loading capacities. The column containing 500 g biosorbent beads could effectively treat greater than 1000 L industrial wastewater that contained lead (65 mg/mL). The ultimate utility of such a system will depend on binding capacity, affinity, selectivity, and stability.

Metallothioneins are low molecular weight, cysteine-rich proteins that have been found in a broad range of eukaryotic species (9,14) and in a few prokaryotes (10,21). These proteins bind a variety of heavy metals, such as Cd^{2+} , Zn^{2+} , Cu^{2+} , Co^{2+} , Pb^{2+} , Ni^{2+} , and Fe^{2+} , with extremely high affinity through coordination bond to their cysteine residues (6,20). The expression of metallothioneins (2,22) or metalloproteins (5) 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 human metallothionein II (hMT-II) in *E. coli* and the potential use of such proteins as heavy metal biosorbents. We previously reported (33) the expression of hMT-II gene in *E. coli* by fusion to the β -galactosidase of expression vector pUEX2. This recombinant metallothionein was shown to bind heavy metals such as Cd^{2+} , Cu^{2+} , and Zn^{2+} . In this study, we report the heavy metal removal by *E. coli* cells expressing hMT-II expressed as a fusion with the β -galactosidase.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

We used *E. coli* JM109 (34) in this study. The vectors constructed were derivatives of pUEX2 (3) (Amersham Biosciences, Buckinghamshire, UK), which

is a derivative of pEX2 (29) and contains a thermolabile repressor, cI857, the λ P_R promoter, and a *cro-lacZ* gene.

Gene Expression and Heavy Metal Accumulation

E. coli JM109 (100 mL) harboring the expression vector pZMT001, which expresses hMT-II as a fusion protein with β -galactosidase (33), was cultured in LB medium, pH 7.0, at 28°C. At the mid-logarithmic phase, the cell culture was shifted to 42°C for 1 h. We then added 0.5 mM heavy metal ions such as Cd^{2+} , Cu^{2+} , or Zn^{2+} and further incubated the culture at 37°C for four days. The biomass was separated by centrifugation at 3000× *g* for 10 min, and the supernatants were analyzed for residual metal concentration on an atomic absorption spectroscopy (Shimadzu AA-646®; Shimadzu, Tokyo, Japan). We evaluated the metal removal by measuring the change in metal concentration in this supernatant. Indeed, *E. coli* cells were harvested by centrifugation at 3000× *g*, and their pellet was incubated for 24 h at 100°C for dry weight estimation.

Cellular Distribution of Cd^{2+}

E. coli JM109 harboring pZMT001 induced by heat shift was incubated in 100 mL LB medium, including 0.5 mM Cd^{2+} at 37°C for 1–4 days, and was harvested by centrifugation at 3000× *g* for 10 min. The supernatant was analyzed for residual Cd^{2+} content by atomic absorption spectroscopy. The cell pellet was washed twice with 100 mL 0.9% NaCl and washed twice with 100 mL 1 mM EDTA as a final concentration by centrifugation at 3000× *g* for 10 min for the determination of Cd^{2+} from the cell surface. Adsorbed Cd^{2+} on the cell surface was determined from this supernatant. Final cell pellets were dissolved in 100 mL 0.9% NaCl and subjected to atomic absorption spectroscopy to determine the intracellular Cd^{2+} concentrations after removing Cd^{2+} bound on the surfaces of cells. The value of the Cd^{2+} content of each sample was obtained using atomic absorption spectroscopy. This value is an average of five replicate determinations. The calibration and standard curve of the CdCl_2 solution were prepared in distilled water.

Electron Microscopy

E. coli JM109 harboring pZMT001, which expresses hMT-II as a fusion protein with β -galactosidase induced by heat shift, was incubated in LB medium including 0.5 mM Cd^{2+} at 37°C for two days. The cells were harvested by centrifugation at 3000× *g* for 10 min, washed with distilled water to remove unbound metal, and then fixed for transmission electron microscopy with a solution of 0.1% ruthenium red (Sigma, St. Louis, MO, USA) and 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, stained with 0.25% uranyl acetate in 0.1 M sodium acetate buffer, pH 6.3, dehydrated with ethanol, and embedded in Quetol 651® (Nishin EM, Tokyo, Japan). Finally, the sections were stained with lead acetate and uranyl acetate and examined with an electron microscope (model H4800MU; Hitachi, Tokyo, Japan) at 100 kV. The energy-dispersive X-ray analysis was performed with an electron microscope equipped with an EMAX-2000® microanalysis system (Horiba Ltd., Tokyo, Japan).

RESULTS

Gene Expression

E. coli cells carrying plasmid pUEX2 or pZMT001 were incubated at 28°C. At the mid-logarithmic phase of growth, we shifted the temperature to 42°C to inactivate the cI857 repressor and then to 37°C for 16 h. A band of a 122-kDa protein was observed during the SDS-PAGE analysis of the cell extract from *E. coli* JM109 (pZMT001). This protein is the same size as predicted from the sum of the 116-kDa β -galactosidase and the 6-kDa hMT-II. The induced cells carrying the plasmid vector pUEX2 only accumulated a 116-kDa protein, the size of which corresponds to that of β -galactosidase. Densitometric analysis revealed that the hMT-II fused with β -galactosidase accounted for greater than 24% of the total protein in the cell extract. This value indicates that the recombinant protein is synthesized at a rate of about 2 g/L culture broth (33).

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Table 1. Heavy Metal Removal by Cells Expressing hMT-II Fused to β -Galactosidase and by Control Cells Expressing Only the β -Galactosidase Protein

Heavy metal	Total amount added (mg)		Amount in supernatant after incubation (mg)		Calculated amount of uptake [$\mu\text{g}/\text{mg}$ cells (dry weight)]	
	Cells expressing		Cells expressing		Cells expressing	
	hMT-II	Control cells	hMT-II	Control cells	hMT-II	Control cells
Cd^{2+}	5.59	5.53	4.48	5.19	10.5	3.11
Cu^{2+}	3.24	3.09	3.11	3.14	1.22	N.D. ^a
Zn^{2+}	3.79	3.45	3.45	3.25	3.37	2.07

These values represented an average of three separate experiments.
^aNot determined.

Heavy Metal Removal

The ability of cells expressing the hMT-II fused to the β -galactosidase to remove heavy metals from medium was tested and compared to that of control *E. coli* JM109 lacking the hMT-II (expressing only the β -galactosidase). The recombinant *E. coli* cells (100 mL) were incubated in the presence of 0.5 mM each metal ions at 37°C for four days after the heat shift for the induction of the hMT-II gene. Table 1 shows that cells expressing the hMT-II fusion efficiently removed Cd^{2+} (10.5 $\mu\text{g}/\text{mg}$ dry cells), whereas control cells that lacked the hMT-II removed 3.11 $\mu\text{g}/\text{mg}$ dry cells. The removal of Cu^{2+} and Zn^{2+} was greater than in control cells, but the difference was not as great as for Cd^{2+} . The results indicate that metal absorbing ability of *E. coli* (pZMT001)

was intensified when compared with that of control cells, and *E. coli* (pZMT001) exhibited a preferential order of metal sorption as $\text{Cd}^{2+} > \text{Zn}^{2+} > \text{Cu}^{2+}$.

Cellular Distribution of Cd^{2+}

Figure 1 shows the amount of Cd^{2+} that bound to the cell surface, accumulated in cytoplasm, and remained in the supernatant after the incubation. The final uptake capacity was reached within three days. Of the 6.16 mg (0.5 mM) Cd^{2+} added to 100 mL of the broth, the cells expressing hMT-II bound and uptook 1.63 mg Cd^{2+} after two days of incubation. At this time, cells corresponding to 110 mg dry weight were found in the broth. The amount of Cd^{2+} in the medium was reduced to 74% from the initial amount. The rate of up-

take by *E. coli* (pZMT001) was calculated with 310 ng/mg dry cell/h. Bacteria sorb heavy metals at the cell surface through physicochemical interactions of lipopolysaccharide (18). Indeed, *E. coli* JM109 cells potentially had the ability to bind Cd^{2+} on the outer cell membrane, resulting in 0.57, 0.70, 0.80, 0.88 mg for 1–4 days of incubation, respectively. On the other hand, The Cd^{2+} of 0.93 mg existed in cytoplasm of *E. coli* JM109 (pZMT001) for two days of incubation. We did not detect any precipitation of heavy metals in the media under these conditions. Since the hMT-II fusion protein represents approximately 0.2 g in the cells from 100 mL culture broth, we could calculate the ratio of Cd^{2+} taken up by the cells to the amount of fusion protein produced. This was estimated at 6 molecules of Cd^{2+} per molecule of hMT-II fusion, which corresponds approximately to the full loading of the protein.

E. coli JM109 (pZMT001) cells obtained from the medium containing 0.5 mM Cd^{2+} were analyzed by energy-dispersive X-ray analysis. Figure 2A shows a transmission electron microscopy micrograph of a thin section of the *E. coli* JM109 (pZMT001) cell treated with uranyl acetate. The large black structure, which may be fusion protein, was present in the cytoplasm. Figure 2, B and C, shows Cd^{2+} existed in cytoplasm and cell membrane, respectively. The cytoplasm and membrane produced a specific Cd^{2+} absorption peak of 2.5 kV. The peak energy equivalent to Cd^{2+} in the cytoplasm and cell membrane from 10 thin sections of cells was measured. The average peak energy equivalent to Cd^{2+} in the cyto-

Figure 1. In vivo bioaccumulation of Cd^{2+} during four days of incubation by *E. coli* JM109 (pZMT001) expressing fusion hMT-II proteins. Open bar, supernatant; hatched bar, outer cell membrane; and solid bar, cytoplasm. The bar represents the mean value of three independent experiments.

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plasm (77.4) was also larger than that in the cell membrane (46.1). Clearly, more Cd^{2+} was accumulated in the cytoplasm during the two days of incubation after the induction.

DISCUSSION

Other studies have shown that bacterial cells expressing metallothionein within the cytosol were less efficient in accumulating heavy metals from solutions than cells expressing the metallothionein within the periplasm or outer membrane (22,24,30). Chen and Wilson (5) reported the simultaneous expression of a metallothionein gene and a mercury transport system in *E. coli*. This study showed that the expression of the metallothionein in the cytosol without co-expression of the transport system resulted in a diminished bioaccumulation of Hg^{2+} by the

cells. These results are consistent with other studies that indicate that maximal bioaccumulation of metal occurs with the expression of metal-binding proteins outside the cytosol (15,28,31).

The ability of cells expressing the hMT-II fusion within the cytoplasm to remove Cd^{2+} effectively and selectively from the medium, along with the data presented in Table 1, demonstrates the potential for these cells to be used at various metal-contaminated sites. Incidentally, Romeyer et al. (25) reported that the *E. coli* cells expressing metallothionein fused to *araB'* accumulate a greater absolute amount of Cd^{2+} , up to 4.48 $\mu\text{g}/\text{mg}$ dry weight *E. coli*. Moreover, cells that display hMT ∂ -domain fused to LamB accumulate Cd^{2+} at 5.04 $\mu\text{g}/\text{mg}$ dry weight (15). Thus, the *E. coli* (pZMT001) has a high capacity for bioaccumulation in comparison with the two *E. coli* strains previously described.

Romeyer et al. (24) also reported

that *E. coli* expressing a *Neurospora crassa* metallothionein protein was able to accumulate much more Cd^{2+} than other metals, and Kotrba et al. (15) reported that *E. coli* displaying hMT α -domain fused to LamB led to a 17-fold increase of Cd^{2+} bioaccumulation from media compared with control cells expressing LamB alone. The bioaccumulation of Cu^{2+} and Zn^{2+} was found to be only slightly increased. *E. coli* JM109 (pZMT001) also accumulated Cd^{2+} more than Cu^{2+} and Zn^{2+} . These results explain that the metal affinity of fusion protein is $\text{Cd}^{2+} > \text{Zn}^{2+}$, Cu^{2+} in culture medium. The regulation of Zn^{2+} levels is achieved by paired uptake and efflux systems (27). The level of Zn^{2+} in *E. coli* cells is maintained constantly by these systems. Cd^{2+} can also be taken up via the Zn^{2+} transport system. Cd^{2+} caused by high toxicity may inhibit its efflux system and then be accumulated in the cell.

This recombinant *E. coli* JM109 (pZMT001) used as a biosorbent may be able to treat industrial wastewater containing a high concentration (50–60 mg/L) of Cd^{2+} . To further increase the biosorption of heavy metals by *E. coli*, the expression of tandem repeat copies of the metallothionein is currently being studied (12). However, alternative cost-effective promoters (e.g., heat-induced), increased levels of metallothionein expression (11), and the 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 that are known to stabilize proteins against heat, pH, and salinity may further increase the utility of such a system (13). To improve resistance mechanisms through a high accumulation of the metal in the cell, it may prove necessary to produce higher levels of metallothionein, in conjunction with the design of new metallothioneins using synthetic DNA.

Current efforts in our laboratory are also focused on immobilization strategies that would afford mechanical and chemical stability to the bacteria, without compromising the binding of target metals. In scale-up studies, columns containing biosorbent beads will be able to effectively treat industrial wastewater containing Cd^{2+} .

Figure 2. Energy-dispersive X-ray analysis of Cd^{2+} accumulated by *E. coli* JM109 (pZMT001) in LB amended with 0.5 mM Cd^{2+} . (A) Transmission electron microscopy of a thin section of *E. coli* JM109 (pZMT001). (B) Energy-dispersive X-ray spectra of cytoplasm and (C) cell membrane were obtained from thin sections that are shown in the circle and the arrow. Energy levels are indicated on the x-axis. The emission line for Cd is at 2.5 kV. The highest peak indicates Cu from the copper electron microscopy grids. All spectra were taken by counting times of 100 s.

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