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Construction and Characterization of *Escherichia coli* Genetically Engineered for Bioremediation of Hg^{2+}-Contaminated Environments

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***Escherichia coli* strains were genetically engineered to express an Hg^{2+} transport system and metallothionein. Overexpression of a glutathione *S*-transferase fusion protein of *Saccharomyces cerevisiae* or pea metallothionein significantly increased the bioaccumulation of Hg^{2+} transported by MerT and MerP and protected the cells from the accumulated Hg^{2+}. The recombinant strains have excellent properties for bioremediation of Hg^{2+}-contaminated environments.**

Mercury is one of the most toxic heavy metals in the environment. Adsorption methods for removing Hg^{2+} with either ion-exchange resins or biosorbents have been shown to be sensitive to ambient conditions, e.g., pH, ionic strength, and the presence of other inorganic and organic components (4, 13). They also fail to remove and recover metal ions when they are complexed or adsorbed on soil. Finally, adsorption methods lack specificity in metal binding, which may cause difficulty in the recovery and recycling of the desired metal(s).

Intracellular-bioaccumulation processes with microorganisms optimized by genetic engineering could overcome the deficiencies of common metal cleanup processes and may be an alternative for removal and recovery of heavy metals such as Hg^{2+} from contaminated water or soil. In this study, genetically engineered *Escherichia coli* strains were constructed to simultaneously express an Hg^{2+} transport system and overexpress a metallothionein (MT) as a carboxyl-terminal fusion to glutathione *S*-transferase (GST-MT), and the strains' ability to accumulate Hg^{2+} was investigated.

Overexpression of MT. MTs are a class of low-molecular-weight metal-binding proteins rich in cysteine residues (8). They are capable of binding a variety of heavy metals, including Hg^{2+} and Cd^{2+}. A cloned and overexpressed MT would allow a microorganism to hyperaccumulate the metal transported by a metal transport system. Though a number of MTs have been expressed in *E. coli*, their stability has been a problem (2). In this study, a GST fusion system (16) was used for overexpressing and stabilizing MT. Figure 1 shows the construction of plasmid pGYMT, which codes for *Saccharomyces cerevisiae* MT (YMT) fused to GST (GST-YMT). The YMT gene coding sequence was isolated as a 0.2-kb *Nde*I-*Bam*HI fragment from plasmid pET3a-MT_0 (15). The fragment was ligated into the *Hin*dIII and *Bam*HI sites of pBluescript-SK(+) (Stratagene) by using an *Nde*I-*Hin*dIII synthetic linker containing a *Bam*HI site. The YMT cassette from pBlueYMT was removed by *Bam*HI digestion and ligated into the *Bam*HI site of pGEX-2T (16) to yield pGYMT and pGRYMT, in which the MT gene is in the reverse orientation. Plasmid pGHMT (Table 1) was constructed by cloning a human hepatic MT-II (HMT-II) gene into pGEX-2T. pGHMT encodes HMT-II fused to GST (GST-HMT).

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To express GST-MT, cultures were grown and induced as described previously (17). Lysates of induced cells were electrophoresed on a sodium dodecyl sulfate–14% polyacrylamide gel and visualized by staining with Coomassie brilliant blue R-250 (Sigma). The GST-YMT fusion protein migrated at the expected molecular mass (33 kDa for a GST-YMT in-frame fusion) (Fig. 2, lane 4). A protein similar to GST (26.5 kDa) was detected in lysates of cells transformed with plasmid pGRYMT (Fig. 2, lane 5). This was expected due to an in-frame stop codon at the 5′ end of the reversed YMT sequence. While GST-YMT is soluble in *E. coli* cells, the GST-HMT fusion protein (Fig. 2, lane 1) formed inclusion bodies after induction. Therefore, GST-HMT was not tested for Hg^{2+} bioaccumulation. GST-YMT, GST-HMT, and GST-pea (*Pisum sativum* L.) MT (GST-PMT) (17) was expressed at approximately 25% of the total protein in *E. coli*, as determined by laser densitometry of stained gels.

Construction of strains expressing MerT-MerP and GST-MT. The products of the *merT* and *merP* genes catalyze Hg^{2+} transport across the cell membrane (10). To express the Hg^{2+} transport system and GST-MT simultaneously, the ColE1-compatible vector pCL1921 (9) was used to clone the *merT* and *merP* genes from pDU1358. Plasmid pCLTP (Table 1), containing *merT* and *merP*, was constructed by digesting plasmid pDH1 (11) with *Hin*dIII and *Eco*RI. The 1.8-kb fragment containing *merT* and *merP* was inserted between the *Hin*dIII and *Eco*RI restriction sites of pCL1921, producing pCLTP. *E. coli* strains harboring pCLTP or pCL1921 were transformed separately with plasmid pGEX-2T, pGYMT, pGRYMT, pGHMT, or pGPMT3 (17) by electroporation (6). Transformed cells were selected on Luria-Bertani agar (LB) plates containing ampicillin and spectinomycin.

Hg^{2+} resistance. To test Hg^{2+} resistance, *E. coli* cells harboring various plasmids were grown in LB containing the appropriate antibiotics. When the optical density at 600 nm (OD_{600}) reached 0.5 to 0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) was added to 1.0 mM and Hg^{2+} was added to various final concentrations. Cells were then incubated at 37°C and the OD_{600} was read at various times. The expression of the *merT* and *merP* genes in pCLTP caused *E. coli* cells to be hypersensitive to Hg^{2+}, leading to cell lysis (data not shown). As shown in Fig. 3, the growth of induced JM109(pCLTP/pGRYMT) cells, expressing the Hg^{2+} transport system but not GST-YMT, was inhibited by 5 μM Hg^{2+},

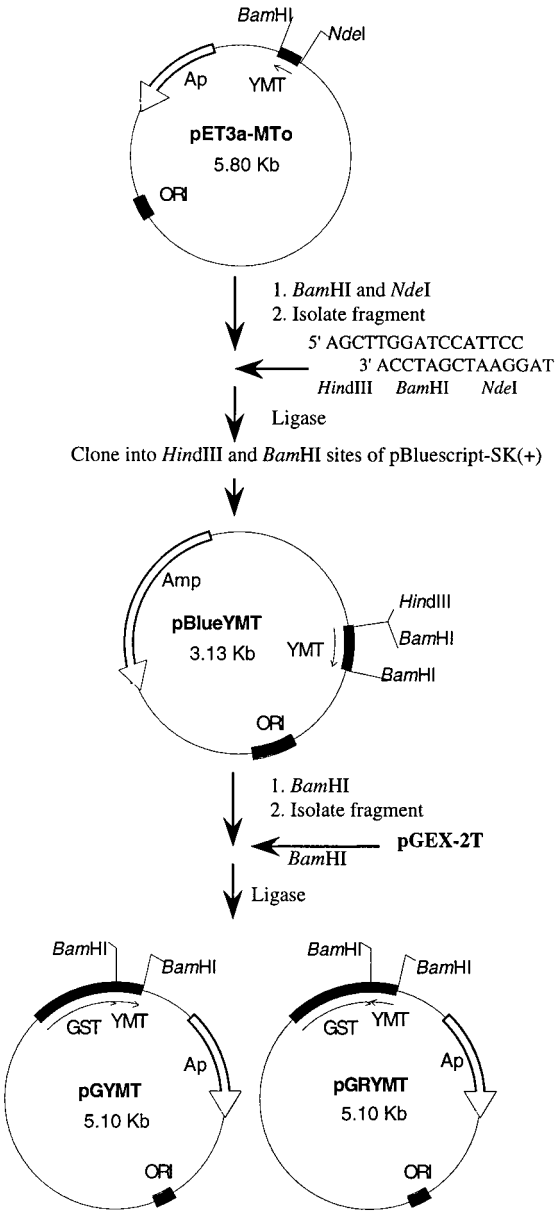

FIG. 1. Construction of the GST-YMT-expressing plasmid pGYMT.

while the growth of JM109(pCLTP/pGYMT) cells, expressing both the Hg^{2+} transport system and GST-YMT, was not. pET3a-MT_0 and pGPMT3, containing the YMT and GST-PMT genes, respectively, also caused *E. coli* cells to be resistant to Hg^{2+} after induction, while pGHMT, encoding the production of GST-HMT as inclusion bodies, did not. The resistance to Hg^{2+} of the recombinant strains is significant since it would allow the cells to be retained inside a reactor when the strains are used for bioremediation of Hg^{2+}-contaminated environments and would make it possible to eventually recover the accumulated Hg^{2+}.

Hg^{2+} bioaccumulation. To measure Hg^{2+} bioaccumulation, induced cells were harvested by centrifugation, washed, and resuspended at a final OD_{600} of 1.0 in either LB (pH 7.0) containing 30 μg of chloramphenicol per ml, MOPS [3-(*N*-morpholino)propanesulfonic acid] minimum medium (pH 7.3)

TABLE 1. Plasmids and strains

Strain or plasmid	Description	Reference or source
Strains		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)	18
XL1-blue	*supE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac* F′[*proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tet^r)]	3
Plasmids		
pCL1921	Cloning vector; pSC101 origin; Spc^r; *lac* promoter	9
pDH1	Ap^r; Contains the entire broad-spectrum *mer* operon of pDU1358 except *merD*	11
pCLTP	Spc^r; pCL1921 with the 1.8-kb *Hin*dIII-*Eco*RI fragment, containing the *merT* and *merP* genes, of pDH1	This work
pGEX-2T	GST gene fusion vector; ColE1 origin; Ap^r; *lacI*^{q}; *tac* promoter	16
pET3a-MT_0	Ap^r; Contains the YMT gene	15
pGYMT	Ap^r; pGEX-2T containing the GST-YMT gene	This work
pGRYMT	Ap^r; pGEX-2T containing the YMT gene in the reverse orientation	This work
pING1-HMT	Ap^r; Contains the HMT-II gene	14
pGHMT	Ap^r; pGEX-2T containing the GST-HMT gene	This work
pGEX-3X	GST gene fusion vector with a multiple-cloning site different from that of pGEX-2T	16
pGPMT3	Ap^r; pGEX-3X containing the GST-PMT gene	17

(12), or phosphate buffer (pH 7.0), all containing 5 μM Hg^{2+}; cells were then incubated at 37°C for an additional hour, harvested by centrifugation, and washed twice with LB containing chloramphenicol. The Hg^{2+} content of the harvested cells was determined by cold-vapor atomic absorption spectroscopy (7). To assay accumulation with time, cells were harvested at various times by filtration with 0.45-μm-pore-diameter nitrocellulose filters (Millipore), and each entire filter with cells was subjected to Hg^{2+} analysis. A 5 μM Hg^{2+} concentration was

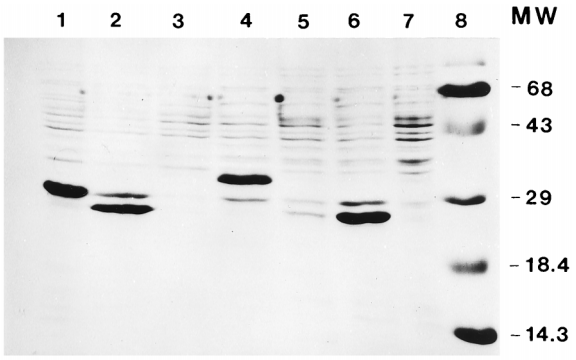

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *E. coli* cell extracts. All cells were induced with IPTG. Lanes: 1 and 2, XL1-blue cells containing pGHMT and pGEX-2T, respectively; 3, XL1-blue cells; 4 through 6, JM109 cells containing pGYMT, pGRYMT, and pGEX-2T, respectively; 7, JM109 cells; 8, protein molecular weight markers. MW, molecular weight (shown in thousands).

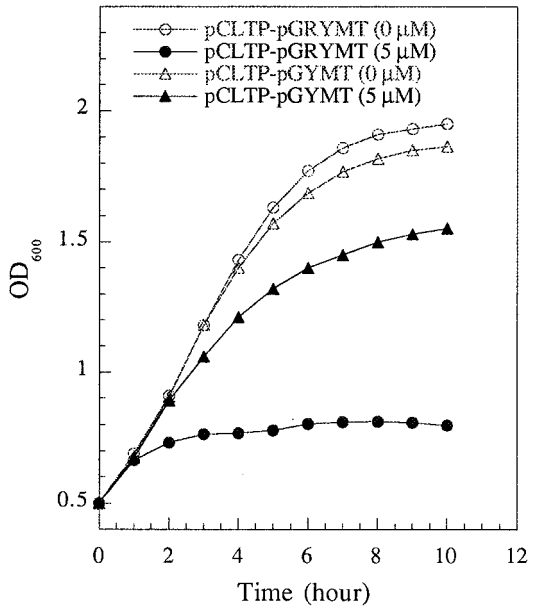

FIG. 3. Effect of Hg^{2+} on the growth of JM109 cells containing the indicated plasmids. Cells were grown in LB containing ampicillin and spectinomycin. When the OD_{600} reached 0.5, IPTG was added to 1 mM and Hg^{2+} was added to the indicated concentrations. OD_{600} values were read at the indicated times.

used for the bioaccumulation assay since it is in the concentration range of Hg^{2+}-contaminated groundwater monitored in industrial sites of the United States (1).

As shown in Fig. 4, *E. coli* cells without an Hg^{2+} transport system yielded no significant accumulation of Hg^{2+} (bar A). In the presence of an Hg^{2+} transport system, intracellular accumulation of Hg^{2+} was significantly increased (bar B). Cells expressing an Hg^{2+} transport system and either GST-YMT (bar C) or GST-PMT (bar D) accumulated approximately fivefold-more Hg^{2+} than cells expressing *merT* and *merP* but not MT (bar B). These results indicate that both an Hg^{2+} transport system and MT were required for *E. coli* cells to accumulate the highest level of Hg^{2+}.

We further tested Hg^{2+} bioaccumulation by strains expressing either GST-YMT or GST-PMT and expressing MerT-MerP

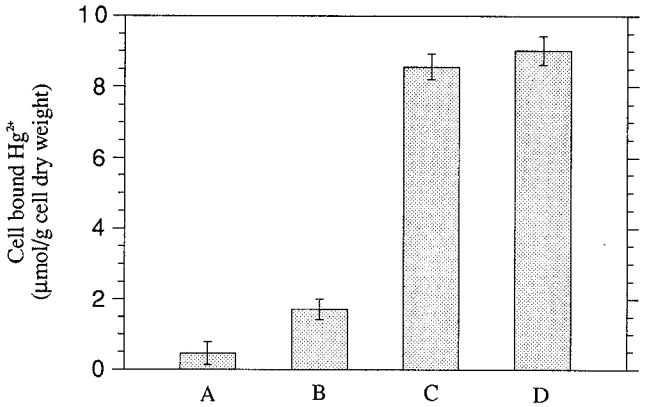

FIG. 4. Bioaccumulation, by induced JM109 cells, of Hg^{2+} from LB containing 5 μM Hg^{2+} after 1 h of exposure. JM109 cells contained pGYMT and pCL1921 (bar A), pGEX-2T and pCLTP (bar B), pGYMT and pCLTP (bar C), or pGPMT3 and pCLTP (bar D). Means ± standard deviations (n = 3) are shown.

under various conditions. It was found that bioaccumulation from phosphate buffer containing an initial concentration of 5 μM Hg^{2+} was fast, as over 90% of the maximum accumulation was reached within the first 10 min of incubation, and at the maximum accumulation, over 80% of the total added Hg^{2+} was removed (data not shown). Bioaccumulation was not enhanced in MOPS minimum medium, which contains various nutrients, or by the addition of a carbon source such as glucose (data not shown). These features are significant for engineering the bioaccumulation process for Hg^{2+} removal and recovery. In another paper (5), we documented the bioaccumulation of Hg^{2+} at various initial concentrations and the resistance of the recombinant strains to ambient conditions, such as pH, ionic strength, and the presence of metal chelators, as well as their selectivity against other metals, such as sodium, magnesium, and cadmium. Metal chelators and complexing agents, such as EDTA and cyanides, have been used in a broad range of industrial processes and have been found to interfere with metal cleanup processes. Our studies indicate that neither EDTA, citrate, nor cyanide (data not shown) affected Hg^{2+} bioaccumulation. Hg^{2+} bioaccumulation was also found to be unaffected by Na^+, Mg^{2+}, and Cd^{2+}. Besides high affinity and selectivity, the Hg^{2+}-bioaccumulating strains exhibited resistance to extremes of pH and ionic strength. As common Hg^{2+} cleanup processes, e.g., ion exchange, are sensitive to the presence of copollutants and ambient conditions, these features of the Hg^{2+}-bioaccumulating strains are significant. The strains may be used to remove Hg^{2+} selectively from contaminated waters which are resistant to common treatments. The high specificity of the Hg^{2+} bioaccumulation process may make it feasible to recover and recycle the accumulated Hg^{2+}. Further studies are under way to improve the specificity and affinity of the Hg^{2+} bioaccumulation process and to design and test bioreactors for the Hg^{2+}-bioaccumulating strains.

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