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Genetic engineering of bacteria and their potential for Hg^{2+} bioremediation

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Abstract

Ion exchange or biosorptive processes for metal removal generally lack specificity in metal binding and are sensitive to ambient conditions, e.g. pH, ionic strength and the presence of metal chelators. In this study, cells of a genetically engineered *Escherichia coli* strain, JM109, which expresses metallothionein and a Hg^{2+} transport system after induction were evaluated for their selectivity for Hg^{2+} accumulation in the presence of sodium, magnesium, or cadmium ions and their sensitivity to pH or the presence of metal chelators during Hg^{2+} bioaccumulation. The genetically engineered *E. coli* cells in suspension accumulated Hg^{2+} effectively at low concentrations (0–20 µM) over a broad range of pH (3 to 11). The presence of 400 mM sodium chloride, 200 mM magnesium chloride, or 100 µM cadmium ions did not have a significant effect on the bioaccumulation of 5 µM Hg^{2+}, indicating that this process is not sensitive to high ionic strength and is highly selective against sodium, magnesium, or cadmium ions. Metal chelators usually interfere with ion exchange or biosorptive processes. However, two common metal chelators, EDTA and citrate, had no significant effect on Hg^{2+} bioaccumulation by the genetically engineered strain. These results suggest that this *E. coli* strain could be used for selective removal of Hg^{2+} from waste water or from contaminated solutions which are resistant to common treatments. A second potential application would be to remove Hg^{2+} from Hg^{2+}-contaminated soil, sediment, or particulates by washing them with a Hg^{2+} chelator and regenerating the chelator by passing the solution through a reactor containing the strain.

Abbreviations: GST – glutathione S-transferase; MT – metallothionein; GST-MT – GST fusion protein of MT; GST-PMT – GST fusion protein of pea MT

Introduction

Mercury has been recognized as one of the most toxic heavy metals and has featured in the most serious outbreaks of metal poisoning among the general population, e.g. Minamata disease (Kurland et al. 1960). In the aqueous environment, Hg^{2+}in sediment is subject to methylation by microorganisms and abiotic processes (Spry & Wiener 1991), forming more toxic methylmercury (CH_3Hg^+). Bioaccumulation of methyl mercury through food chains causes a potential risk to consumers of fish or shellfish.

To clean up sediment or soil polluted by Hg^{2+}, extraction processes using Hg^{2+} complexing ligands have been studied in recent years (Wasay et al. 1995; Sandstede et al. 1993). Common treatments to remove Hg^{2+} from contaminated water are mostly based on sorption to materials such as ion exchange resins (Osteen & Bibler 1991; Ritter & Bibler 1992). To develop and implement new processes to meet newer regulations, many attempts have been made to employ microbial products and activities to remove and recover heavy metals such as Hg^{2+}. Microbial processes being studied for metal remediation include biotransformation [through microbial oxidation and reduction of metals (Gounot 1994; Barkay et al. 1992)], bioprecipitation [by precipitating metal ions at the cell surface through microbial mechanisms such as cation efflux to change pH (Diels et al. 1995) or using a cell-bound phosphatase to release phosphate at the cell surface (Macaskie et al. 1994)], and biosorption [using natural (Volesky & Holan 1995) or recombinant (Pazirandeh et al. 1995) microbial biomass to adsorb metal ions]. Among them, biosorption has been one of the most actively studied processes. However, ion exchange technology as well as biosorption generally fail to remove and recover metal ions such as Hg^{2+} when the metal ions are strongly adsorbed on soil or sediment or in complex forms. The adsorptive treatments and bioprecipitation process are also sensitive to ambient conditions, e.g. pH and the presence of other inorganic and organic components (Bedell & Darnall 1990; Chang & Hong 1994). In particular, they lack specificity in metal binding, which may cause difficulties in the recovery and recycling of the desired metal(s).

Though genetic engineering has rarely been applied to metal bioremediation, it has the potential to improve or redesign microorganisms so that biological metal-sequestering systems have higher intrinsic capability and specificity and are more resistant to ambient conditions. To construct strains that are capable of specifically accumulating Hg^{2+} from dilute solutions, we have genetically engineered *E. coli* cells to simultaneously express a Hg^{2+} transport system (the products of the *merP* and *merT* genes) and overexpress metallothionein (MT) (a class of metal binding protein rich in cysteine residues) as a carboxyl terminal fusion to glutathione S-transferase (GST-MT) (Chen & Wilson, unpubl.). The Hg^{2+} accumulation system was designed so that overexpressed MT would serve as a Hg^{2+} accumulator; a Hg^{2+} transport system would make the cells specifically accumulate Hg^{2+}; and the intracellular accumulation process would allow the bioaccumulation system to be less sensitive to ambient conditions than adsorptive ones. To test these hypotheses, we studied Hg^{2+} accumulation by one of the genetically engineered *E. coli* strains and evaluated its specificity for Hg^{2+} accumulation, the effect of pH and the presence of chelators on Hg^{2+} accumulation by the strain. In another paper, we documented the construction of the genetically engineered strains and the effect of MT or GST-MT and MerT-MerP expression on Hg^{2+} bioaccumulation (Chen & Wilson, unpubl.). There are two reasons why *E. coli* has been chosen for these initial studies to test our hypotheses. The first is because *E. coli* greatly facilitates genetic engineering experiments and the second is that in comparison with yeast or fungi, *E. coli* has more surface area per unit of cell mass which potentially should give higher rates of Hg^{2+} removal from solution.

Materials and methods

Plasmids and bacterial strains

Two compatible plasmids, pSUTP (Chen & Wilson, unpubl.) and pGPMT3 (Tommey et al. 1991), were used in this study for expression of a Hg^{2+} transport system and the GST fusion protein of pea MT (GST-PMT), respectively. pSUTP was constructed by inserting a *merT* and *merP* coding sequence from pDH1 (Mukhopadhyary et al. 1991) between the HindIII and EcoRI restriction sites of pSU39 (Bartolome et al. 1991). pSU39 is a vector with the p15a replicon, kanamycin resistance, inducible lacZα and pUC19 mutiple cloning sites (MCS). A BamHI/EcoRI fragment containing the pea MT gene was cloned into pGEX3X (Pharmacia), resulting in pGPMT3. pGEX3X is a glutathione S-transferase gene fusion vector with the CoE1 replicon, ampicillin resistance, and a tac promoter followed by a MCS for gene fusion. The host strain used in this study was *E. coli* JM109, a derivative of *E. coli* K12 (Yanisch-Perron et al. 1985). Cells were cultured in Luria broth (LB) or on LB agar plates. Ampicillin, kanamycin, and chloramphenicol were used at concentrations of 50, 25, and 30 µg/ml, respectively. All chemicals used in this study are at least of analytical grade.

Hg^{2+} bioaccumulation

E. coli cells harboring either pSUTP and pGPMT3 or pSUTP and pGEX3X were grown overnight in LB containing kanamycin and ampicillin, then diluted to an OD_{600} of 0.10 with fresh LB containing kanamycin and ampicillin and incubated at 37 °C with vigorous shaking. When the OD_{600} reached 0.5 to 0.7, isopropyl-β-D-thiogalactoside (IPTG) was added to 1.0 mM. After four-hours of induction, the cells were harvested by centrifugation at 4 °C, washed and resuspended in 10 mM phosphate buffer (pH 7.0) containing different concentrations of Hg^{2+} (1000 ppm mercury reference standard solution, Fisher Chemical, USA) to an OD_{600} = 1.0. The cells were shaken for 1 hour at 37 °C, harvested by centrifugation at 4 °C and washed three times with ice-cold LB-chloramphenicol or phosphate buffer containing 0.5 mM of EDTA. Induced cells contained about 25% of their total protein as the GST-PMT fusion protein (Chen & Wilson, unpubl.).

To determine the effect of the presence of other metal ions on the bioaccumulation of Hg^{2+}, IPTG-induced *E. coli* cells were suspended in phosphate buffer (pH

7.0) or MOPS buffer (40 mM MOPS, pH 7.0, adjusted with KOH) containing 5 μM Hg^{2+} and either sodium chloride (0–400 mM), magnesium chloride (0–100 mM), or cadmium ion (0–0.1 mM) (1000 ppm cadmium reference standard solution, Fisher Chemical, USA). The desired pH of the phosphate buffer was obtained by adjusting the ratio of tribasic sodium phosphate to dibasic sodium phosphate, or dibasic sodium phosphate to monobasic sodium phosphate, and then fine tuned with phosphoric acid or sodium hydroxide. 0.5 mM EDTA or sodium citrate was added to cell suspensions to determine the effect of metal chelators on the bioaccumulation of Hg^{2+}.

Hg^{2+} determination

The Hg^{2+} content in the cell pellets was determined by inductively coupled plasma (ICP) atomic emission spectroscopy or by cold vapor atomic absorption spectroscopy and converted to amount of Hg^{2+} per 8×10^8 cells (assuming an optical density of 1 is equivalent to 8×10^8 cells). To prepare samples for ICP atomic emission spectroscopy, cell pellets were freeze dried and then digested in 70% nitric acid for 24 hours at 42 °C (Drapeau et al. 1983). Samples were prepared as previously described (Hatch & Ott 1968) for cold vapor atomic absorption spectroscopy.

Results and discussion

Hg^{2+} bioaccumulation by genetically engineered E. coli

Our previous studies (Chen & Wilson, unpubl.) showed that expression of yeast MT or the GST fusion protein of yeast or pea MT in *E. coli* cells protected cells from toxic effects of Hg^{2+} (e.g. cell lysis) and significantly increased the bioaccumulation of Hg^{2+} transported by MerT and MerP. Induced JM109 cells containing pSUTP and pGPMT3 accumulated approximately four-fold more Hg^{2+} from a 5 μM Hg^{2+} solution than cells expressing MerT and MerP alone while cells without a mercury transport system gave no significant accumulation of Hg^{2+}. In this study, we determined the maximum bioaccumulation capacity of one of the genetically engineered *E. coli* strains, JM109-pSUTP-pGPMT3, and tested its ability to accumulate Hg^{2+} over a range of Hg^{2+} solutions of various concentrations. As shown in Figure 1, the genetically engineered *E. coli* accumulated Hg^{2+} effectively at a range of low concentrations of Hg^{2+} (0–20 μM) and the percentage of total Hg^{2+} accumulated was about 80% of the initial amount added. The highest bioaccumulation level was approximately 30 nmol/8×10^8 cells or 88 μmol/g cell dry weight, which falls in the range of the maximum biosorption capacity of most reported microorganisms (15–290 μmol/g cell dry weight) (Gadd 1988). Wild type *E. coli* had no significant accumulation of Hg^{2+} (data not shown), therefore, its bioaccumulation was not included in Figure 1.

It was also shown in the previous studies (Chen & Wilson, unpubl.) that Hg^{2+} bioaccumulation by the genetically engineered *E. coli* is fast, as 95% of the final accumulation level was reached within the first 10 minutes of incubation, and the addition of nutrients such as glucose did not increase the Hg^{2+} bioaccumulation level, as even in deionized water the cells accumulated comparable amount of Hg^{2+}.

Effect of ionic strength on Hg^{2+} bioaccumulation

Chang and Hong (1994) have reported that cells of a Hg^{2+} resistant strain *Pseudomonas aeruginosa* PU21(Rip64) adsorb Hg^{2+} more strongly than an ion exchange resin. However, the biosorbent is still highly sensitive to the presence of sodium ion, e.g. its biosorption capability at low concentrations of Hg^{2+} is reduced by over 90% in the presence of 150 mM sodium chloride. To determine the effect of ionic strength on Hg^{2+} bioaccumulation by the genetically engineered *E. coli* cells, bioaccumulation was performed at various concentrations of sodium chloride. JM109 cells containing pGPMT3 and pSUTP were grown, induced, and harvested as described in Materials and Methods and incubated with 5 μM of Hg^{2+} in the presence of various concentrations of sodium chloride. Figure 2 shows that the addition of up to 400 mM NaCl to the cell suspension did not change the Hg^{2+} bioaccumulation level. It is apparent that in contrast to the adsorption of Hg^{2+} using biosorbents or ion exchange resins, the intracellular bioaccumulation process is not inhibited by elevated ionic strength.

Effect of Mg^{2+} on Hg^{2+} bioaccumulation

In addition to alkaline metal ions, alkaline-earth metal ions such as magnesium and calcium are often found in contaminated waters. The presence of these divalent cations may also reduce the efficiency of ion exchange resins or biosorbents by elevating the ionic strength and/or by their competitive binding to the active sites

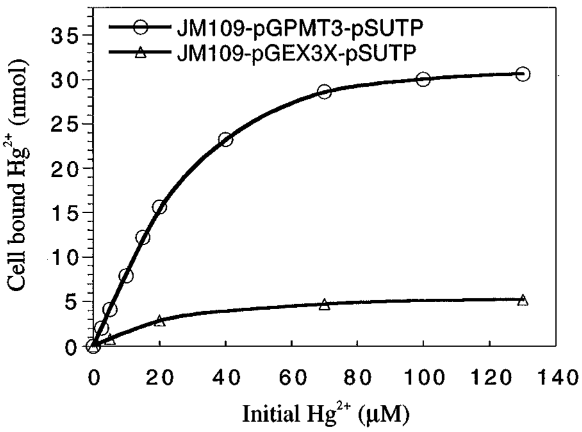

Figure 1. Hg^{2+} bioaccumulation at various concentrations by JM109 cells expressing GST-PMT and MerT-MerP or the cells expressing MerT-MerP only. *E. coli* cultures were grown and induced as described in Materials and Methods. The induced cells were harvested and resuspended in phosphate buffers containing the indicated concentrations of Hg^{2+}. The cells were harvested and washed after one hour of incubation at 37 degC. X axis represents the initial concentrations of Hg^{2+} in solutions and y axis represents the Hg^{2+} amount (nmol) accumulated by 8×10^8cells.

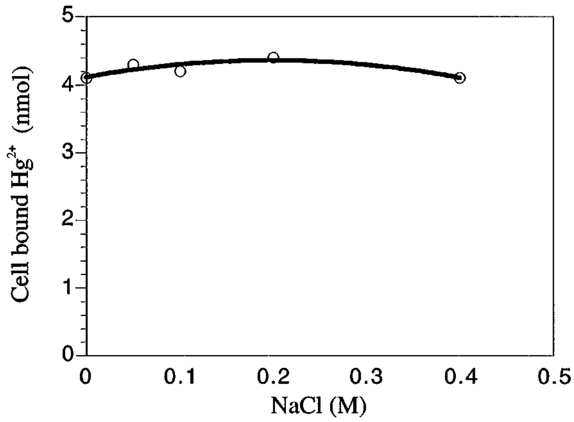

Figure 2. Effect of sodium ion on the bioaccumulation of Hg^{2+} by *E. coli* JM109 cells expressing GST-PMT and MerT-MerP. JM109 cultures with pSUTP and pGPMT3 were grown and induced as described in Materials and Methods. The induced cells were harvested and resuspended in phosphate buffers containing 5 μM Hg^{2+} and the indicated concentrations of NaCl. The cells were harvested and washed after one hour of incubation at 37 °C. X axis represents the initial concentrations of Hg^{2+} in solutions and y axis represents the Hg^{2+} amount (nmol) accumulated by 8×10^8cells.

of the ion exchange resins or biosorbents. It has been shown that one of the most selective biosorbents, Algasorb, loses 30% of its copper-binding capacity in the presence of 40 mM magnesium ion (Darnall et al. 1986). To determine the effect of alkaline-earth metal ions on Hg^{2+} bioaccumulation, the bioaccumulation assay was carried out in MOPS buffers containing 5 μM Hg^{2+} and different concentrations of magnesium chloride. In the presence of 0, 30, 60, 100, 150, or 200 mM magnesium chloride, the genetically engineered *E. coli* cells accumulated approximately the same amount of Hg^{2+} (4.1 μmol/8 $\times$ 10^8 g cell dry weight), suggesting that the Hg^{2+} bioaccumulation process is resistant to the presence of alkaline-earth metal ions.

Effect of Cd^{2+} on Hg^{2+} bioaccumulation

With solid waste disposal costs increasing, more attempts will be made to recycle heavy metals after clean-up processes. Specificity in metal removal may be required for new biological processes in order to recycle the desired heavy metal(s). The first generation of metal recovery systems using natural biomass, like existing ion exchange resins, lack specificity in metal binding. The second generation of microorganisms and biosorbents optimized by genetic or/and protein engineering would improve the specificity of biological metal clean-up processes. The Hg^{2+} transport system in the cells allows bioaccumulation to be specific for Hg^{2+}. To further test its selectivity over other heavy metals, bioaccumulation experiments were performed in the presence of various concentrations of cadmium ion (0–200 μM). Cd^{2+} was selected because of its high affinity to MT and its toxicity to microorganisms. Bioaccumulation assays carried out in phosphate buffers containing 5 μM Hg^{2+} showed that the presence of 5, 20, and 50 μM Cd^{2+}had little effect on Hg^{2+} bioaccumulation (accumulation level remained at about 4.1 μmol/8 $\times$ 10^8 g cell dry weight), and the genetically engineered *E. coli* cells still retained about 90% of their Hg^{2+} bioaccumulation activity (3.6 μmol/8 $\times$ 10^8 g cell dry weight) in the presence of 200 μM, a 20-fold excess of Cd^{2+}.

Effect of pH on Hg^{2+} bioaccumulation

pH variation in contaminated water often affects metal clean-up processes. pH may influence the speciation and/or the mobility of heavy metals (Darimont & Frenay 1990). A high pH may result in the formation of stable metal complexes, e.g. hydroxides, oxides, and carbonates, making the heavy metals less available to ion exchange resins or biosorbents. A low pH may increase the mobility of heavy metals and

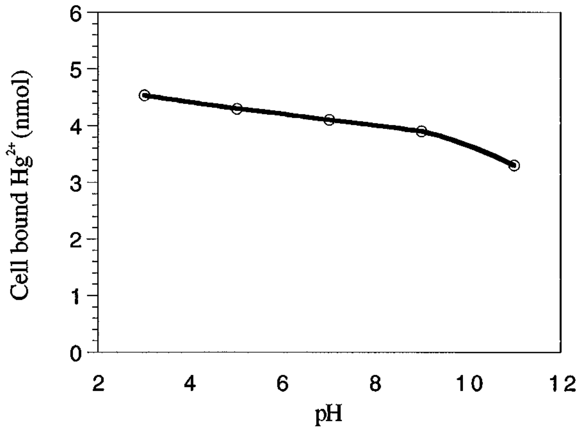

Figure 3. pH profile of Hg^{2+} bioaccumulation by *E. coli* JM109 cells expressing GST-PMT and MerT-MerP. JM109 cultures with pSUTP and pGPMT3 were grown and induced as described in Materials and Methods. The induced cells were harvested and resuspended in phosphate buffers containing 5 μM Hg^{2+} and ranging in pH from 3 to 11. The cells were harvested and washed after one hour of incubation at 37 °C. X axis represents the initial concentrations of Hg^{2+} in solutions and y axis represents the Hg^{2+} amount (nmol) accumulated by 8×10^8cells.

therefore may enhance their availability. On the other hand, cation competition due to the presence of excess protons often dramatically decreases the adsorption of heavy metals to ion exchange resins or biosorbents.

The effect of pH on the biosorption of Hg^{2+} have been investigated by Chang and Hong (1994). Their results demonstrated that biosorption of Hg^{2+} by non-living *Pseudomonas aeruginosa* PU21(Rip64) was still highly dependent on pH, e.g. one unit deviation from the optimal pH (pH 7.4) causes a 50% reduction of Hg^{2+} biosorption though the biosorbent shows a higher binding capacity and affinity for Hg^{2+} than an ion exchange resin. Pazirandeh et al. (1995) have reported on the expression of a MT in the periplasm of *E. coli* cells to absorb heavy metals and have tested the ability of the recombinant *E. coli* to remove Cd^{2+} at various pHs. The Cd^{2+} biosorption of the recombinant bacteria attains its maximum at pH 7.0 but diminishes to 75% of the maximum at pH 9.0 and falls below 15% at pH 3.0. These results further indicate the dependence of heavy metal biosorption on pH.

Figure 3 shows the pH profile of Hg^{2+} bioaccumulation by recombinant *E. coli* cells expressing GST-MT and MerP-MerT. Hg^{2+} bioaccumulation remained at the same level within a pH range of 3 to 9 and dropped about 20% at pH 11. These results demonstrate that in contrast to biosorbents or ion exchange resins, the Hg^{2+} bioaccumulation system was resistant to pH over a broad range. The results further suggest that Hg^{2+} is accumulated inside the *E. coli* cells and preferentially bound to MT and therefore the intracellular accumulation process is less sensitive to spontaneous desorption and the presence of competing cations than ion exchange or biosorption.

Effect of metal chelators on Hg^{2+} bioaccumulation

Metal chelators or complexing agents are frequently involved in metal contamination. Soil, sediment or particulates are often found to adsorb heavy metals in the environment. The presence of these substances may cause contamination to be resistant to common treatments such as ion exchange or biosorption because the formation of complex compounds or the adsorption of metal ions on soil or sediment decreases the availability or mobility of the heavy metals.

Metal chelators are found not to inhibit the metal uptake activity of some metal transport systems in microorganisms (Kusano et al. 1990; Archibald & Duong 1984) and to even play a role in a few microbial metal transport processes (Crosa 1989). To investigate whether the Hg^{2+} bioaccumulation system is resistant to the presence of metal chelators, 0.5 mM EDTA or citrate was added to a cell suspension to determine the bioaccumulation of Hg^{2+}. Hg^{2+} bioaccumulation level in the presence of EDTA and citrate was respectively 4.3 and 3.9 μmol/8×10^8cells. Over 95% of the Hg^{2+} bioaccumulation activity was retained in the presence of the metal chelators, indicating that the Hg^{2+} bioaccumulation system has a high affinity for Hg^{2+}. This is in accordance with the finding that a pair of cysteine residues in MerP is involved in sequestering and transport of Hg^{2+} and provides the Hg^{2+} transport process a high affinity for Hg^{2+} (Sahlman & Granstroem Skaerfstad 1993; Morby et al. 1995).

The resistance of the Hg^{2+}-bioaccumulating system to the presence of metal chelators raises the possibility of applying such a system for the removal of Hg^{2+} from soil, sediment or particulates on which Hg^{2+} strongly adsorbs. This could be done by washing Hg^{2+} contaminated soil, sediment or particulates with a chelator and regenerating the chelator by passing the solution through a reactor containing one of the Hg^{2+}-accumulating strains. The regenerated chelator solution would be returned to the soil or particulates in a recycle stream where it could again extract more Hg^{2+} from the soil or particulates.

Conclusions

E. coli JM109 was genetically engineered to simultaneously express GST-PMT and a Hg^{2+} transport system. The strain was capable of accumulating Hg^{2+} from dilute solutions (0–20 μM). The presence of up to 400 mM sodium chloride or 200 mM magnesium chloride did not appear to change the effectiveness of the Hg^{2+}-accumulating system, indicating that it is highly selective over sodium and magnesium ions. A 20 fold excess of Cd^{2+} failed to inhibit Hg^{2+} bioaccumulation by the strain, suggesting that the process is specific for Hg^{2+}. The Hg^{2+}- bioaccumulating system also showed resistance to pH over a broad range (pH 3–11). The presence of EDTA and citrate in cell suspensions did not have a significant effect on Hg^{2+} bioaccumulation. Ion exchange resins or biosorbents for Hg^{2+} removal generally lack specificity and have been found sensitive to pH and ionic strength. It was demonstrated in our experiments that in contrast to biosorbents or ion exchange resins, the genetically engineered *E. coli* strain was capable of dealing with a broad range of perturbations in ambient condition and furthermore its Hg^{2+} bioaccumulation process was specific for Hg^{2+}. Such genetically engineered microorganisms have potential for the removal and recovery of Hg^{2+} from contaminated water, soil, or sediment.

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