SIV-2

A Molecular Biotechnology For Removal of Toxic Heavy Metals

Sang-Weon Bang[1], Douglas S. Clark[2], and Jay D. Keasling[2]
Department of Life Science, National Research Laboratory for Water Environmental Ecology & Restoration, Hanyang University, Seoul 133-791, Korea[1]
Department of Chemical Engineering, University of California, Berkeley, CA 94720-1462, U.S.A.

ABSTRACT

The thiosulfate reductase gene (*phsABC*) from *Salmonella typhimurium* was expressed in *Escherichia coli* in order to produce sulfide from inorganic thiosulfate and precipitate metals as metal sulfide complexes. A 5.1-kb DNA fragment containing the native *phsABC* and a 3.7-kb DNA fragment, excluding putative promoter and regulatory regions were inserted into expression vectors pTrc99A and pJB866, respectively. Upon expression of *phsABC*, *E. coli* DH5α harboring the *phsABC* constructs showed higher thiosulfate reductase activity and produced significantly more sulfide than the control strain (*E. coli* DH5α) under both aerobic and anaerobic conditions. Among the four constructs, *E. coli* DH5α harboring pSB74 produced the highest level of thiosulfate reductase and removed most of heavy metals from solution under anaerobic conditions. In a mixture of 100 μM each of cadmium, lead, and zinc, the strain could remove 99% of the total metals from solution within 10 hours. Cadmium was removed first, lead second, and zinc last. In contrast, a negative control did not produce any measurable sulfide and removed very little metals from solution. These results have important implications for removal of metals from wastewater contaminated with several metals.

INTRODUCTION

Biological methods for heavy metal removal include bioaccumulation and biosorption, o xidation and reduction, methylation and demethylation, metal-organic complexation, ligand d egradation, and phytoremediation (Bolton Jr. & Gorby 1995, White *et al.* 1997, White & Ga dd 1998).

Many soluble metals can form insoluble complexes with hydroxides, carbonates, phosphates, and sulfides (Speight 1996). One of the best-known natural metal precipitation mechanisms is due to sulfide production from sulfate by sulfate-reducing bacteria (SRB) found in anoxic sediments containing high concentrations of lead and mercury (Ford et al., 1995, Webb *et al.* 1998, White *et al.* 1998). Recently, several genetically engineered microorganisms have been developed for biop rotection and remediation of wastestreams laden with metals and/or organics (Springael *et al.* 19 94, Stephen & Macnaughton 1999). One example is the genetic modification of metal-binding peptides for heavy metal chelation and removal. These genetically engineered microorga nisms may be particularly useful for remediation of heavy metals for which no natural organisms exists (Mejàre *et al.* 1998, Pazirandeh *et al.* 1998, Kotrba *et al.* 1999).

Among several bacterial hydrogen sulfide generating systems, we chose the thiosulfate reductase gene (*phsABC*; *phs* represents production of hydrogen sulfide) from *Salmonella typhimurium* to overproduce hydrogen sulfide. Thiosulfate reductase catalyzes the dissimilatory reduction of inorganic thiosulfate to hydrogen sulfide and sulfite (Clark and Barrett, 1987). Sequence analyses of the chromosomal *phsABC* region from *S. typhimurium* revealed a functional operon with three open reading frames, designated *phsA*, *phsB*, and *phsC* (Heinzinger *et al.*, 1995). Amino acid sequence analyses revealed that PhsA has significant similarity to the sequence of molybdoprotein oxidoreductases and PhsB to that of the iron-sulfur protein of the reductases. PhsC does not show any significant homology to any sequences in GenBank but it has characteristics similar to the hydrophobic subunits of the reductases (Heinzinger *et al.*, 1995). Single-copy *phs-lac* translational fusions required both anaerobiosis and thiosulfate for full

expression, whereas multicopy *phs-lac* translational fusions responded to either thiosulfate or anaerobiosis, suggesting that oxygen and thiosulfate control of the *phs* operon involves negative regulation (Heinzinger *et al.*, 1995).

The primary focus of this study was to develop a genetically-engineered bacterium capable of producing sulfide under aerobic, microaerobic, or anaerobic conditions for heavy metal precipitation. There are several potential advantages of using thiosulfate and thiosulfate reductase for heavy metal remediation: (1) thiosulfate is a relatively inexpensive source of sulfur for sulfide production; (2) thiosulfate is a weak metal chelator that facilitates mobilization of heavy metals in contaminated soils and is effective at reducing metal toxicity from some common metals in aquatic environments (Hockett and Mount, 1996); (3) as thiosulfate reductase catalyzes the stoichiometric production of hydrogen sulfide and sulfite from thiosulfate (Clark and Barrett, 1987), the sulfite may be further reduced to sulfide by a group of bacteria, providing another equivalent of sulfide for metal precipitation; and (4) it should be possible to engineer sulfide-dependent metal removal by transferring the recombinant thiosulfate reductase system to certain environmental bacteria lacking in dissimilatory sulfate reduction pathway.

MATERIALS AND METHODS

Strains, plasmids and molecular techniques. The bacterial strains and plasmids used in this study are listed in Table 1. All plasmids were transformed into *E. coli* DH5α, and the thiosulfate reductase gene was expressed in the presence of thiosulfate. The primer sequences derived from *phsABC* region were 5'-tcagcgaattctaataacaggagg-3'(forward) and 5'-cattattttatggatccgctcagac-3' (reverse), and 5'-tcagctggatccaataacaggagg-3' (forward) and 5'-cattattttatgaattcgctcagac-3' (reverse). Restriction sites for *Bam*HI and *Eco*RI were inserted in the sequences for the purpose of directional cloning and are underlined. Restriction fragments containing the native and PCR-amplified *phsABC* region were ligated into pTrc99A and pJB866 expression vectors. The constructed plasmids were then transformed into *E. coli* DH5α by the procedure of Hanahan (1983).

Culture conditions. Culture conditions and heavy metal removal experiments were described previously (Bang *et al*, 2000[1], 2000[2]). A modified morpholinopropane sulfonate (MOPS)-buffered minimal medium was used in all experiments in this study. To prevent abiotic metal precipitation with phosphate, the K_2HPO_4 component of the original MOPS medium (Neidhardt *et al.*, 1974) was replaced with glycerol 2-phosphate (1.32 mM). *E. coli* DH5α cells were pre-adapted to the MOPS minimal medium by several serial subcultures. The *E. coli* DH5α cells harboring various *phsABC* genetic cassettes were inoculated on a Luria agar (Miller's LB agar) plate supplemented with appropriate antibiotics (100 μg/ml ampicillin for the cells harboring pTrc99A derivatives or 12.5 μg/ml tetracycline for the cells harboring the pJB866 derivatives). The cells were incubated overnight at 37°C. A half inoculating loop of the cells from the plate was transferred into fresh MOPS medium (50 ml) supplemented with 10 mM glucose, 1 μg/ml thiamine, appropriate antibiotics and inducers (3 mM IPTG for the cells harboring pTrc99A derivatives or 1 mM *m*-toluate for the cells harboring the pJB866 derivatives). The cells were incubated overnight at 37°C with aeration and agitation (200 rpm). Five ml each of the overnight-grown cultures were transferred into 50 ml of fresh MOPS medium supplemented with the same components under the same conditions as above. The cells were harvested by centrifugation at 6,000 x g for 10 min. The cell pellets were resuspended in various volumes of fresh MOPS medium to achieve the same cell density (OD_{600}= 1.0) and used as an inoculum for further experiments. Aliquots (0.5 ml) of the cell suspensions were transferred to 50 ml of fresh MOPS medium supplemented with the same components plus 3 mM $Na_2S_2O_3 \cdot 5H_2O$. For heavy

metal removal experiments, various concentrations of $CdCl_2$, $PbCl_2$, and $ZnCl_2$ were added to the medium. The cultures were grown at 37 ^{o}C without agitation to minimize the loss of sulfide from

Table 1. Bacterial strains and plasmids.

Strain or plasmids	Relevant characteristics[a]	Source or Reference
E. coli DH5α	F', *ϕ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17*(r_k^-, m_k^+) *phoA supE44 λ⁻ thi-1 gyrA96 relA1*	BRL[b]
pTrc99A	Expression vector; P_{trc}, *lacI*^{q}, *rrnBT1T2* and Amp^r	Amann *et al.*, 1988
pJB866	Expression vector; P_m, *xylS839*, *oriT* and Tc^r	Blatny *et al.*, 1999
pEB40	pUC19 with 5,136 bp *Eco*RI-*Sal*I DNA fragment containing native *phs* regulatory and structural genes from *Salmonella typhimurium* LT2	Fong *et al.* 1993
pSB74	pTrc99A with 5,136 bp *Eco*RI-*Sal*I DNA fragment containing native *phs* regulatory and structural genes from *Salmonella typhimurium* LT2	Bang *et al.* 2000[1]
pSB77	pJB866 with 5,136 bp *Eco*RI-*Sal*I DNA fragment containing native *phs* regulatory and structural genes from *Salmonella typhimurium* LT2 (*Xho*I compatible ends)	Bang *et al.* 2000[1]
pSB103	pTrc99A with 3,653 bp PCR amplified DNA fragment containing only the structural genes of *phsA*, *phsB* and *phsC* from pSB74	Bang *et al.* 2000[1]
pSB107	pJB866 with 3,653 bp PCR amplified DNA fragment containing only the structural genes of *phsA*, *phsB* and *phsC* from pSB74	Bang *et al.* 2000[1]

[a] Abbreviations: P_{trc}, hybrid *trp-lac* promoter ; *lacI*^{q}, gene encoding the *lac* repressor protein; *rrnBT1T2*, transcription terminators; Pm, *m*-cleavage pathway promoter of the TOL plasmid; *xylS839*, gene encoding the repressor protein that controls P_m; *oriT*, origin of transfer; Amp^r, ampicillin resistant; Tc^r, tetracycline resistant; *phs*, gene for the production of hydrogen sulfide from thiosulfate.
[b] BRL, Gibco BRL, Inc., Gaithersburg, MD.

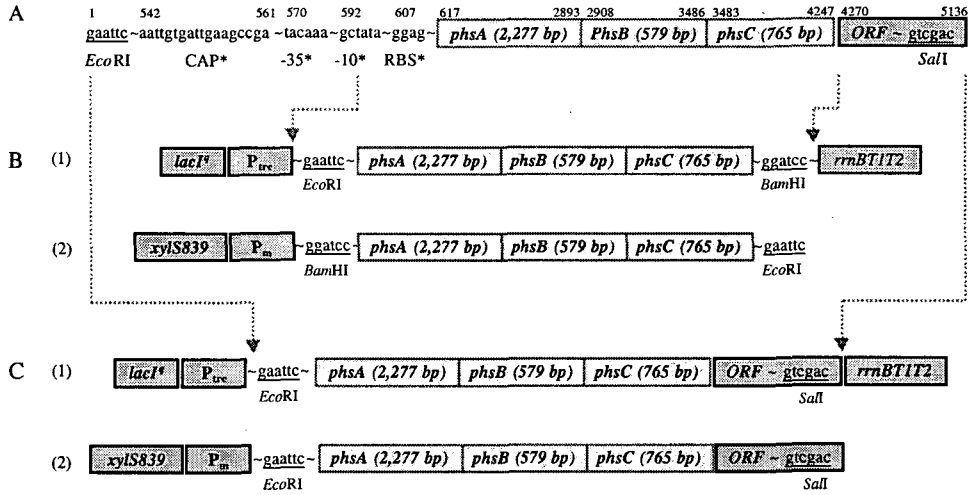
the culture medium. A redox-indicator, resazurin, was added into a separate series of cultures to indicate the reduction state of the culture.

Thiosulfate reductase activity. *E. coli* DH5α harboring the *phsABC* constructs were grown in MOPS medium as described above. After several serial subcultures, the cells were inoculated into a fresh medium and grown at 37^{o}C with shaking (for aerobic culture) or without shaking (for anaerobic culture). At an OD_{600} of 0.7, the cells were harvested, washed twice with ice-cold 0.1 M Tris-acetate buffer (pH 9.0), and resuspended in the same buffer to the same cell density (approximately 3.5 X 10^9 cells/ml). One ml each of the cultures was disrupted on ice by sonication (10 pulses at 10% duty cycle at a power setting of 2 on a Sonifier [model S-450, Branson Ultrasonic Co., Danbury, Conn.]). After centrifugation (14,000 x g for 30 min. at 4^{o}C), the supernatants were collected and used for enzyme assay. Thiosulfate reductase activity in the cell extracts was determined by reacting the sulfite product with pararosaniline (Chauncey *et al.*, 1987). One unit of thiosulfate reductase activity is defined as the production of 1 μmol of sulfite in 1 min. Mean values of three replicate experiments are reported. The relative activity is the ratio of the thiosulfate reductase activity in the cell-free extracts from *E. coli* harboring *phsABC* constructs grown aerobically or anaerobically to that in the control cells grown aerobically.

Sulfide production. For a simple sulfide detection assay, a semi-solid LB agar medium (0.2% noble agar in Luria broth) containing 2.5 mM $FeCl_2 \cdot 4H_2O$ and 3 mM $Na_2S_2O_3 \cdot 5H_2O$ with appropriate antibiotics and inducers was used. The formation of black precipitate (FeS) in the medium was considered to be an indication of sulfide production. Direct measurement of sulfide was performed with a Sure-Flow Combination silver/sulfide electrode (model 9616, Orion, Inc., Beverly, Mass.). Cell cultures of the same cell density (OD_{600} = 0.5) were prepared in 250 ml of fresh MOPS medium supplemented with 10 mM glucose, 1 μg/ml thiamine, appropriate antibiotics and inducers, and 3 mM $Na_2S_2O_3 \cdot 5H_2O$. A fraction of each culture (10 ml) was transferred into 25-ml serum vials and capped with a rubber stopper and aluminum seal. The vials were incubated at 37^{o}C without shaking. At time intervals, the samples (2 ml) were withdrawn, filtered to collect cell free supernatant, and added to an equal volume of sulfide antioxidant buffer (SAOB), which was prepared according to the suggestions of the electrode manufacturer (Orion, Inc., Beverly, Mass.). The sulfide concentrations in the samples were measured using the sulfide

probe. The samples were analyzed in triplicate and mean values are reported. Sulfide concentration less than 50 μM were not detectable using the electrode.

Figure 1. Construction and genetic structure of the *phsABC* cassettes.



(A) The 5,136 bp *Eco*RI-*Sal*I DNA fragment containing the native *phs* regulatory and structural genes from *Salmonella typhimurium* LT2 (pEB40). **(B)** Structure of 3,653 bp PCR amplified DNA fragment containing only the structural *phsABC* cloned into pTrc99A creating pSB103 (1) and into pJB866 creating pSB107 (2). **(C)** Structure of the native *phsABC* operon cloned into pTrc99A creating pSB74 (1) and into pJB866 creating pSB77 (2). The numbers represent the locations of nucleotide bases. An asterisk (*) indicates putative sequences. Abbreviations: *phsABC*, thiosulfate reductase gene; CAP, catabolite activator protein-binding site; RBS, ribosome biding site; ORF, open reading frame (truncated); P_{trc}, hybrid *trp-lac* promoter ; *lacI*^{q}, gene encoding the *lac* repressor protein; *rrnBT1T2*, transcription terminators; P_m, *m*-cleavage pathway promoter of the TOL plasmid; *xylS839*, gene encoding the repressor protein that controls P_m.

Heavy metal removal. For individual heavy metal removal studies, cadmium, lead, and zinc were added separately to the medium to final concentrations between 100 and 500 μM. For mixed heavy metal removal experiments, combinations of the metals were added to the medium at concentrations of 100 μM each. All cultures were grown at 37 ^{o}C without agitation. At time intervals, samples (1.0 ml) of culture medium were withdrawn and OD_{600} was measured. After centrifugation (10,000 x g for 15 minutes), 0.1 ml of the supernatant was filtered (Millipore MF membrane, 0.45 μm pore size) and transferred to 9.9 ml of 10% nitric acid solution to measure the metal concentration remaining in the medium. All culture and sample preparations were

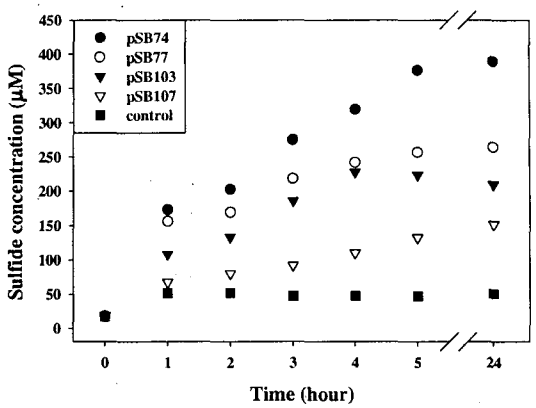
Table 2. Thiosulfate reductase activity by cell-free extracts of *E. coli* DH5α harboring *phsABC* constructs

	Aerobic culture		Anaerobic culture	
Plasmids	Enzyme activity[a] (U/g dry wt)	Relative activity[b]	Enzyme activity[a] (U/g dry wt)	Relative activity[b]
control[d]	138 ± 26	1	218 ± 22	1.6
pSB74	324 ± 49	2.3	684 ± 40	5.0
pSB77	209 ± 31	1.5	627 ± 27	4.5
pSB103	191 ± 27	1.4	529 ± 27	3.8
pSB107	187 ± 22	1.4	498 ± 31	3.6

[a] One unit is equivalent to 1 μmol of sulfite produced/ min in 1.0 ml assay system. Mean values of three replicate experiments were reported.

[b] The relative activity is the ratio of the thiosulfate reductase activity in the cell-free extracts from *E. coli* harboring *phsABC* constructs grown aerobically or anaerobically to that in the control cells grown aerobically.

Figure 2. Sulfide production by the *E. coli* DH5α harboring the *phsABC* constructs.



The sulfide concentration was determined using a sulfide electrode. The reported values are the means of triplicate measurements; the standard errors were less than ± 6%. Symbols: filled circles, pSB74; open circles, pSB77; filled triangles, pSB103; open triangles, pSB107; filled squares, pTrc99A.

performed simultaneously to reduce individual sample error. The metal concentration was determined by inductively coupled plasma optical emission spectrometry (ICP-OES). All ICP-OES operating settings were selected according to the recommendations of the manufacturer (Perkin Elmer, Newark, CT). The concentrations of the metals in a sample were obtained by computer analysis. Cadmium, lead and zinc standards for ICP-OES analysis were prepared by dilution to obtain the desired concentrations. Metal concentrations in the solutions were determined in triplicate and the values reported are the means. The standard errors in metal removal experiments were less than ±5%.

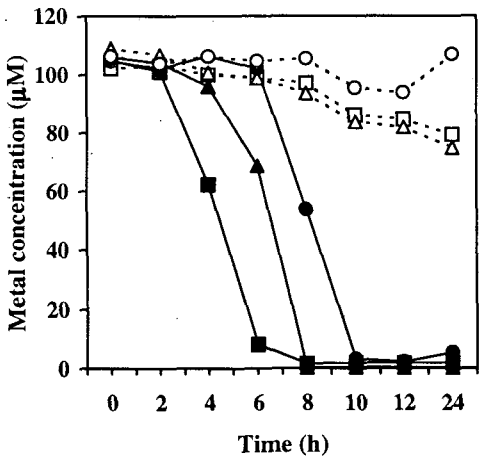
RESULTS AND DISCUSSION

Construction of phsABC genetic cassettes. A 5.1-kb *Eco*RI-*Sal*I digested DNA fragment from *Salmonella typhimurium* containing the native *phsABC* fragment encompassing the structural and putative regulatory regions was inserted into the pTrc99A and pJB866 expression vectors, resulting in plasmids pSB74 and pSB77, respectively (figure 1, table 1). The DNA fragment also contains a truncated open reading frame (ORF) of unknown function downstream of *phsABC*. A 3.7-kb PCR-amplified *phsABC* fragment, designed to eliminate the putative promoter, regulatory region, and the ORF of unknown function downstream of *phsABC*, was inserted into the pTrc99A and pJB866 expression vectors, resulting in plasmids pSB103 and pSB107, respectively.

Thiosulfate reductase activity of cell-free extracts. *E. coli* DH5α harboring the native *phsABC* constructs had higher thiosulfate reductase activity under both aerobic and anaerobic conditions than *E. coli* DH5α harboring the engineered *phsABC* constructs (table 2). In general, the high-copy plasmid constructs had more activity than their medium-copy counterparts, and cells grown anaerobically had higher activity than cells grown aerobically.

Hydrogen sulfide production from thiosulfate. All four cultures turned black (due to FeS precipitation) when grown in the sulfide detection medium supplemented with 2.5 mM $FeCl_2 \cdot 4H_2O$ and 3 mM $Na_2S_2O_3 \cdot 5H_2O$. In contrast, *E. coli* cells harboring the pTrc99A vector alone did not turn black in 24 hours. This result was an indication that *E. coli* DH5α harboring the *phsABC* cassettes produced a functional thiosulfate reductase and generated sulfide from inorganic thiosulfate. Resazurin indicator revealed that the cultures remained aerobic for the first hour, and then became microaerobic and anaerobic thereafter. *E. coli* DH5α harboring pSB74

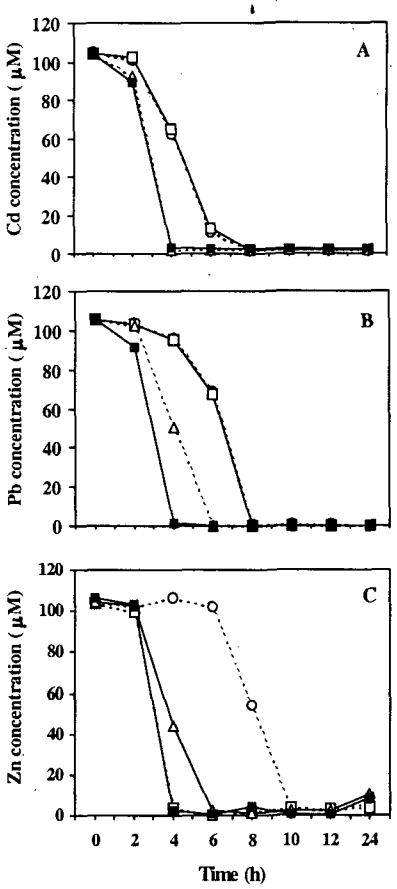
Figure 3. Mixed metal removal by *E. coli* DH5α harboring pSB74.



The cells were grown in the presence of 100 μM each of $CdCl_2$, $PbCl_2$,and $ZnCl_2$. At two-hour time intervals, the samples were withdrawn and the concentrations of the metals remaining in solution were determined. Filled symbols indicate metal removal by *E. coli* DH5α harboring pSB74; open symbols indicate metal removal by *E. coli* DH5α harboring pTrc99A alone. Squares, cadmium; triangles, lead; circles, zinc.

(A) Cadmium removal from medium containing 100 μM each ofcadmium, lead, and zinc (open circles), cadmium and lead (open squares), cadmium and zinc (open triangles), and cadmium alone (filled squares). (B) Lead removal from medium containing 100 μM each of cadmium, lead, and zinc (open circles), cadmium and lead (open squares), lead and zinc (open triangles), and lead alone (filled squares). (C) Zinc removal from medium containing 100 μM each of cadmium, lead, and zinc (open circles), cadmium and zinc (open squares), lead and zinc (open triangles), and zinc alone (filled squares).

Fig.4. Metal removal in the presence of various combinations of cadmium, lead and zinc.



(native *phsABC* operon inserted into pTrc99A) produced the most sulfide of all strains tested under such conditions: 173 μM in one hour, 377 μM in 5 hours, and 389 μM in 24 hours, respectively (figure 2). The second highest sulfide production was observed with the cells harboring pSB77 (native *phsABC* operon in pJB866): 156 μM in one hour and 257 μM in 24 hours. *E. coli* DH5α harboring pSB103 and pSB107 (modified *phsABC* operon on pTrc99A and pJB866) generated 210 μM and 152 μM sulfide in 24 hours, respectively. Sulfide production by *E. coli* DH5α harboring pTrc99A (a control) remained below detection limits of the sulfide electrode (approximately 50 μM).

Heavy metal removal by E. coli harboring phsABC. When 50 μM $CdCl_2$ was present in the culture medium, all four strains harboring the *phsABC* constructs removed nearly all of cadmium within 24 hours (data not shown). A bright yellow precipitate developed in all four cultures, an indication of CdS precipitation. In contrast, the negative control (*E. coli* DH5α harboring pTrc99A) removed less than one quarter of total cadmium and did not turn yellow. In general, the percentage of cadmium removed from solution decreased as the cadmium concentration in the medium increased. *E. coli* DH5α pSB74 outperformed all other constructs: it removed nearly all of the cadmium at 100 and 150 μM $CdCl_2$ and a majority at 200 μM. At the high cadmium concentrations of 300 and 400 μM, it removed 46% and 25%, respectively (data not shown). The cells harboring pSB77 removed nearly all of the cadmium at concentrations up to 100 μM and

slightly less than *E. coli* DH5α pSB74 at all other concentrations. All other constructs removed significantly less cadmium than *E. coli* DH5α pSB74 at cadmium concentrations of 100 μM and higher. There was little difference in the growth of the strains at any particular cadmium concentration (data not shown).

In order to investigate removal of the heavy metals from a mixed-metal solution, 100 μM each of $CdCl_2$, $PbCl_2$, and $ZnCl_2$ were added to the medium. *E. coli* DH5α harboring pTrc99A alone was used as a negative control. *E. coli* DH5α pSB74 removed 99% of both cadmium and lead in 8 hours and 99% of zinc in 10 hours; cadmium was removed first, then the lead, and finally the zinc (figure 3). This order of metal removal correlates relatively well to the solubility product of metal sulfides (Lide 1998, Weast 1978), although there is some difference in the solubility of metal sulfides depending on the reaction and temperature tested: 3.6×10^{-29} for CdS and 3.4×10^{-24} for PbS (Weast 1978) versus 8×10^{-7} for CdS and 3×10^{-7} for PbS (Lide 1998). In contrast, the control strain removed little metal from mixtures in 24 hours under the same conditions.

To investigate the individual and combined effect of cadmium, lead, and zinc on the metal removal, *E. coli* DH5α pSB74 was grown in the medium containing various combinations of metal species. A mixture of heavy metals can show three possible types of removal behavior: (a) synergism, in which the effect of one heavy metal is increased by the presence of another; (b) antagonism, in which the effect of one heavy metal is neutralized by the presence of another; and (c) non-interaction, in which the effect of one heavy metal is not influenced by the presence of another (Beyenal *et al.* 1997). In any combination of the metals, *E. coli* DH5α pSB74 removed almost all of the cadmium from solution (figure 4A). Alone or with the zinc, the cadmium was completely removed in 4 hours, indicating that cadmium removal was not affected by the presence of zinc (non-interaction). However, in the presence of lead, complete cadmium removal was delayed by 8 hours. This behavior suggests that lead competes with cadmium for sulfide and, as such, delays cadmium removal. *E. coli* DH5α pSB74 also removed all of the lead within 8 hours in any combination of the metals (figure 4B). When in solution alone, lead was completely removed in 4 hours. In combination with zinc, the lead was removed in 6 hours. However, with cadmium or cadmium and zinc, lead was not completely removed until 8 hours. This result indicates that lead removal was affected by the presence of both cadmium and zinc; however, cadmium inhibited lead removal more than zinc. Furthermore, the addition of zinc to cadmium and lead had no apparent effect on lead removal beyond that in the presence of cadmium. In the case of zinc, *E. coli* DH5α pSB74 completely removed zinc from all metal combinations within 10 hours (figure 4C). Alone or with cadmium, zinc was removed completely within 4 hours, suggesting that zinc removal was unaffected by the presence of cadmium. In combination with lead, the zinc was not completely removed until 6 hours. Moreover, in the presence of cadmium, lead, and zinc, the complete removal of zinc was delayed by 10 hours, suggesting the combined inhibitory effects of cadmium and lead in zinc removal.

In this study, we used recombinant DNA technology to engineer the thiosulfate reductase operon (*phsABC*) from *Salmonella typhimurium* to overproduce hydrogen sulfide from inorganic thiosulfate and precipitate cadmium as cadmium sulfide. The system developed in this study removed significant amounts of heavy metals alone as well as in mixtures. The combined effects of cadmium, lead and zinc on removal were also investigated This sulfide-producing genetic system could be transferred to certain bacteria (such as organic pollutant degraders that are sensitive to toxic metals) enabling them to tolerate or to remove heavy metals, in addition to mineralizing organic pollutants as a carbon source.

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