ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Systematic Engineering of Phytochelatin Synthesis and Arsenic Transport for Enhanced Arsenic Accumulation in *E. coli*

Shailendra Singh,[1,2] Seung Hyun Kang,[1] Wonkyu Lee,[1]
Ashok Mulchandani,[1] Wilfred Chen[1]

[1]Department of Chemical and Environmental Engineering, University of California, Riverside, California 92521; telephone: 951-827-2473: fax: 951-827-5696; e-mail: wilfred@engr.ucr.edu

[2]Cell Molecular & Developmental Biology Graduate Program, University of California, Riverside, California 92521

Received 25 August 2009; revision received 1 October 2009; accepted 5 October 2009

Published online 20 October 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.22585

ABSTRACT: Phytochelatin (PC) is a naturally occurring peptide with high affinity towards arsenic (As). In this article, we demonstrated the systematic engineering of PC-producing *E. coli* for As accumulation by addressing different bottlenecks in PC synthesis as well as As transport. Phytochelatin synthase from *Schizosaccharomyces pombe* (SpPCS) was expressed in *E. coli* resulting in 18 times higher As accumulation. PC production was further increased by co-expressing a feedback desensitized γ-glutamylcysteine synthetase (GshI*), resulting in 30-fold higher PC levels and additional 2-fold higher As accumulation. The significantly increased PC levels were exploited further by co-expressing an arsenic transporter GlpF, leading to an additional 1.5-fold higher As accumulation. These engineering steps were finally combined in an arsenic efflux deletion *E. coli* strain to achieve an arsenic accumulation level of 16.8 μmol/g DCW, a 80-fold improvement when compared to a control strain not producing phytochelatins.

Biotechnol. Bioeng. 2010;105: 780–785.

KEYWORDS: heavy metals; arsenic; bioremediation; glutathione; phytochelatins

Introduction

Arsenic (As), which ranks 1st on the Environmental Protection Agency's (EPA) priority list of drinking water contaminant, affects the health of millions of people worldwide (Nriagu and Pacyna, 1988; Smith et al., 2000). Arsenate [As (V)] and arsenite [As (III)] are the most common forms with As (V) being the major form in surface water and As (III) in ground water. Arsenic exposure has been associated with many diseases such as increased risk of hypertension (Chen et al., 1995), cancer (Karagas et al., 1998) and hyperkeratosis (Cebrian et al., 1983).

In recent years, bioremediation has been suggested as an environmentally benign and effective way for As removal (Bae et al., 2001; Mejare and Bulow, 2001). Engineered microbes producing metal-binding peptides have been exploited for this purpose with varying degrees of success (Ma et al., 2001; Say et al., 2003; Singh et al., 2008a,b). One particularly promising approach was recently reported using an engineered *E. coli* strain expressing the arsenic-specific *Fucus vesiculosus* metallothionein (fMT) and an As transporter GlpF. The whole cell As level of 8.1 μmol/g dry cell weight (DCW) is three times higher than other reported values using engineered *E. coli*.

Phytochelatins (PCs) are natural occurring peptides found in plants and fungi that are known to bind arsenite with high affinity (Schmoger et al., 2000). PCs have a general structure of $(\gamma\text{-Glu-Cys-})_n\text{-Gly}$ ($n = 1$–11) and have been shown to chelate heavy metals with higher affinity and binding capacity than MTs. PCs are synthesized (Fig. 1) by phytochelatin synthase (PCS) via the transfer of γ-Glu-Cys from glutathione (GSH) to another GSH or other PCs (Mehra and Winge, 1991; Vatamaniuk et al., 1999; Zenk, 1996) and can be easily fine-tuned for high-level production (Kang et al., 2007).

Here we demonstrate a systematic cellular engineering approach for enhanced whole-cell As accumulation based on controllable PC production and intracellular As

S. Singh's present address is One MedImmune Way, Gaithersburg, MD 20878.
Wonkyu Lee's present address is Department of Molecular Science and Technology, Ajou University, Suwon 442-7491, South Korea.
Correspondence to: W. Chen
Contract grant sponsor: NSF
Contract grant number: BES0422791; BES0329482

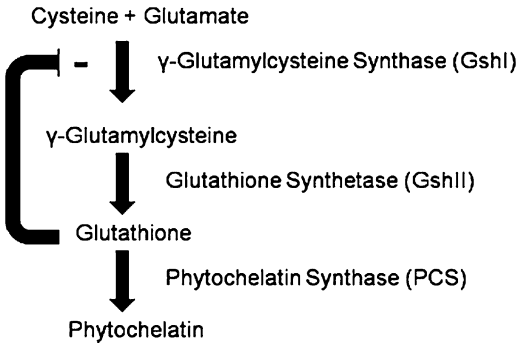

Figure 1. Pathway showing enzymatic synthesis of phytochelatins by phtochelatin synthase.

transport. In *E. coli*, biosynthesis of GSH proceeds by γ-glutamylcysteine synthetase (GshI) with the conversion of glutamate and cysteine into γ-glutamylcysteine (γ-EC), which is then converted to GSH by GSH synthetase (GshII) (Meister, 1995). Two keys enzymes, GshI and PCS, involved in the PC production pathway are functionally expressed for optimized PC production. The improved intracellular chelation when combined with the simultaneous over-expression of an arsenic transporter GlpF (Sanders et al., 1997) and deletion of the arsenic efflux pump ArsAB (Dey et al., 1994) lead to significantly higher arsenic accumulation levels.

Materials and Methods

Bacterial Strains and Cell Culture

E. coli strain JM109 (*e14-(McrA-) recA1 endA1 gyrA96 thi-1 hsdR17* (rK- mK+) *supE44 relA1 Δ(lac-proAB)* [F' *traD36 proAB lacI^{q}ZΔM15*]) (Stratagene, La Jolla, CA) was used for expression of SpPCS, GSHI*, and GlpF. *E. coli* strain JM109 was used for plasmid cloning and propagation. *E. coli* strain AW10 (JM110 Δ*ars*::*cam dam dcm supE44 hsdR17 thi leu rpsL lacY galK galT ara tonA thr tsx* Δ(*lac-proAB*), Cm^r) (Kuroda et al., 1997) with the As efflux pump deleted was used as indicated.

Plasmid pQE60 (Qiagen, Valencia, CA) was used in control experiments. Plasmid pQE-SpPCS containing an ampicillin marker was used to express SpPCS. Plasmids pQE-SpPCS-GshI*, and pQE-SpPCS-GshI*-GlpF expressing SpPCS and GshI* or SpPCS, GshI* and GlpF, in an operon under an inducible T5/*tac* promoter were used. Luria-Bertani (LB) medium (Sambrook et al., 2001) containing 100 μg/mL of ampicillin (Sigma, St. Louis, MO) was used for cell culture or protein expression.

Plasmid Construction and Transformation

Construction of plasmids pQE-SpPCS & pQE-SpPCS-GshI* has been reported previously (Kang et al., 2007). For constructing pQE-SpPCS-GshI*-GlpF, the *GlpF* gene was amplified from pMT-GlpF (Singh et al., 2008b) with the forward primer GCCG**GGATCC** *AAGGAGATATACAT*-CAGAAGGAGATATACATATGAGTCA and the reverse primer TA**GGATCC** *GTCGAC*TCTA*GAGCTC*TTCAAG-TTAATGGTGATGGT

GATGGT. The PCR product was digested with *Bam*HI and ligated into *Bam*HI-digested pQE-SpPCS-GshI* yielding pQE-SpPCS-GshI*-GlpF. Plasmids were transformed into JM109 or AW10 using the heat shock method as described in Sambrook et al. (2001).

Heavy Metal Accumulation

Cells were inoculated into 25 mL of LB medium containing the appropriate antibiotics in a 125-mL flask at optical density 0.1 at 600 nm (OD_{600}) from overnight grown seed cultures. When the OD_{600} reached 0.5, expression of SpPCS, GSHI*, GlpF, and GSHII was induced with 0.8 mM IPTG followed by the addition of 10 μM sodium arsenite. After 3 h incubation, cell densities were measured and 1 mL samples were taken for metal analysis. For whole-cell metals contents, cells were washed with 5 mM HEPES buffer containing 0.8% NaCl three times before drying at 65°C for 24 h. The dried cell pellets were digested with 100 μL of concentrated nitric acid for 2 days (modified from Sriprang et al., 2003). The total arsenic contents were measured using atomic adsorption spectroscopy (AAnalyst 800, Perkin Elmer, Inc., Waltham, MA). Arsenic was determined at 193.7 nm in a graphite furnace.

Protein Expression Analysis

The expression of various enzymes and proteins was verified by Western blotting (Sambrook, 2001). Cells were harvested, washed, concentrated to an optical density at 600 nm of 10 with SDS gel-loading buffer and lysed by boiling at 95°C for 10 min. The proteins of cell-free supernatants were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. The nitrocellulose membrane was incubated with a monoclonal anti-HIS tag antibody or anti-FLAG tag antibody (Novagen, San Diego, CA) in Tris-buffered saline (TBS) overnight and washed with TBS containing 0.05% (v/v) tween 20 three times and subsequently incubated with alkaline phophatase conjugated goat anti-mouse immunoglobulinG for 1 h. After washing three times with TBS containing 0.05% (v/v) tween 20, blots were developed with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidin in alkaline phosphate substrate buffer (100 mM Tris–HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$). Prestained protein standard marker (Bio-Rad, Hercules, CA) was used to determine protein molecular weights.

GSH, γ-EC, and PC Analysis

Cells were cultured and induced as described above. The derivatization procedure with monobromobimane (mBBr) using fluorescence detection was adapted from Sneller et al. (2000). Cells were harvested, washed with 5 mM HEPES buffer containing 0.8% NaCl, and freeze-dried. The lyophilized samples were resuspended in 1 mL of 6.3 mM diethylenetriamine pentaacetic acid (DTPA) with 0.1% TFA. Thio-containing peptides, γ-glutamylcysteine (γ–EC), GSH and PCs, were extracted by repeating eight to ten times of vortexing with 0.3 mL of glass beads for 30 s and putting on the ice for 30 s. The homogenates were centrifuged and supernatants were filtered. Two hundred fifty microliters of PC samples was mixed with 450 μL of 200 mM 4-(2-hydroxy-ethyl)-piperazine-1-propane-sulfonic acid buffer (pH 8.2) containing 6.3 mM DTPA and 10 μL of 25 mM mBBr. After 30 min of derivatization at 45°C in the dark, 1 M of methane sulfonic acid was added for stopping reaction. The peptides were separated on a reverse phase Gemini C18 column (pore size, 110Å; particle size, 5 μm; dimension, 4.6 mm × 150 mm, Phenomenex, Torrance, CA) by binary linear gradient elution program using methanol with 0.1% (v/v) trifluoroacetic acid (TFA) and water with 0.1% (v/v) TFA. The column was equilibrated with 12% methanol with 0.1%TFA and eluted by gradient from 12% to 100% methanol over 60 mins at a flow rate of 0.5 mL/min. Fluorescence was monitored by Agilent 1200 Series HPLC fluorescence detector. Excitation wavelength was 380 nm and emission wavelength was 470 nm. GSH, γ-EC and PC2 standards are commercially available from Sigma.

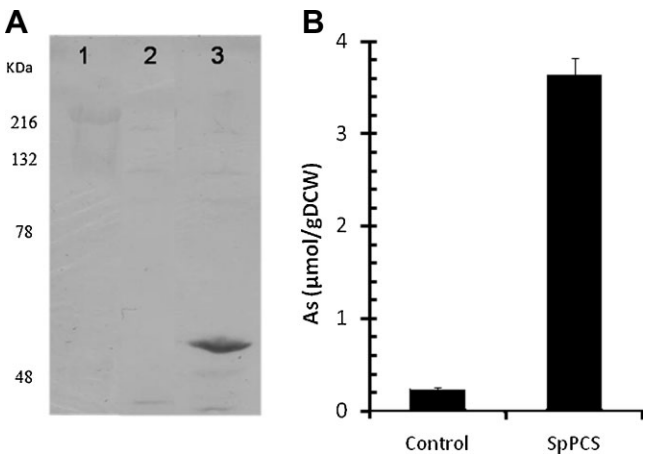

Figure 2. Characterization of *E. coli* cells expressing SpPCS. **A**: Expression of SpPCS detected by Western Blot using an anti-His-tag antibody. **Lane 1**: molecular weight marker; **lane 2**: *E. coli* cells containing the control vector; **lane 3**: *E. coli* cells expressing SpPCS. **B**: Arsenic accumulation in *E. coli* cells harboring the control vector (control) or expressing SpPCS. The As content was measured by atomic absorption spectrometry.

Results

Expression of SpPCS on Arsenic Accumulation and Non-Protein Thiol Levels

Previously, we demonstrated that expression of phytochelatin synthase from *Schizosaccharomyces pombe* (SpPCS) in *E. coli* improved Cd accumulation by fivefold via PC-mediated intracellular chelation (Kang et al., 2007). One of the most unique features of PCs is their ability to chelate As(III), therefore, experiments were conducted to investigate whether similar enhancements in As(III) accumulation could be observed. A protein band of the expected size was detected for cells expressing SpPCS using anti-His tag antibody, while no band was detected for the control cells (Fig. 1). As expected, cells expressing SpPCS accumulated 18-fold more As (Fig. 2B) as compared to cells harboring the control vector. This increase in As accumulation was accompanied by the production of PCs in growing cells (mostly PC2) (Fig. 3B), indicating that PCs are solely responsible for the increased As accumulation. Although the intracellular PC content in the presence of As(III) was only 25% of that in the presence of cadmium (Kang et al., 2007), production of PCs still resulted in the disappearance of GSH and accumulation of γ-EC (Figs. 2C and 3D). This result suggests a highly coupled process between GSH and PC synthesis since the production of PC alone cannot explain the consumption of all GSH. Rather, it is likely that the relaxed feedback inhibition of the first enzyme (GshI) involved in GSH synthesis and the inhibition of the second enzyme (GshII) in the presence of As resulted in a net accumulation of γ-EC (Kelly et al., 2002).

Engineering GSH Synthesis and As Transport for Improved Accumulation

To increase the supply of GSH, a feedback desensitized GshI (GshI*) (Rui et al., 2004), was over-expressed along with SpPCS. Co-expression of GshI* and SpPCS resulted in not only improved levels of GSH but also a 30-fold increase in PC2 content (Fig. 3B). Unfortunately, the increase in As accumulation was only an extra twofold, signifying that uptake may become limiting (Fig. 3A) as observed with other heavy metals (Kang et al., 2007).

We have previously reported (Singh et al., 2008b) that over-expression of an arsenic transporter GlpF improves the arsenic uptake by *E. coli*. Therefore, a similar approach was exploited to investigate if this could enable more As complexation by the intracellular PCs. Cells expressing SpPCS, GshI* and GlpF accumulated 1.5-folds higher arsenic (Fig. 4) as compared to cells only expressing SpPCS & GshI*. The resulting As level of 10.0 μmol/g DCW is 20% higher than our previous reported value of 8.1 μmol/g DCW using cells expressing GlpF and fMT. The PC levels did not significantly alter (data not shown) in this case,

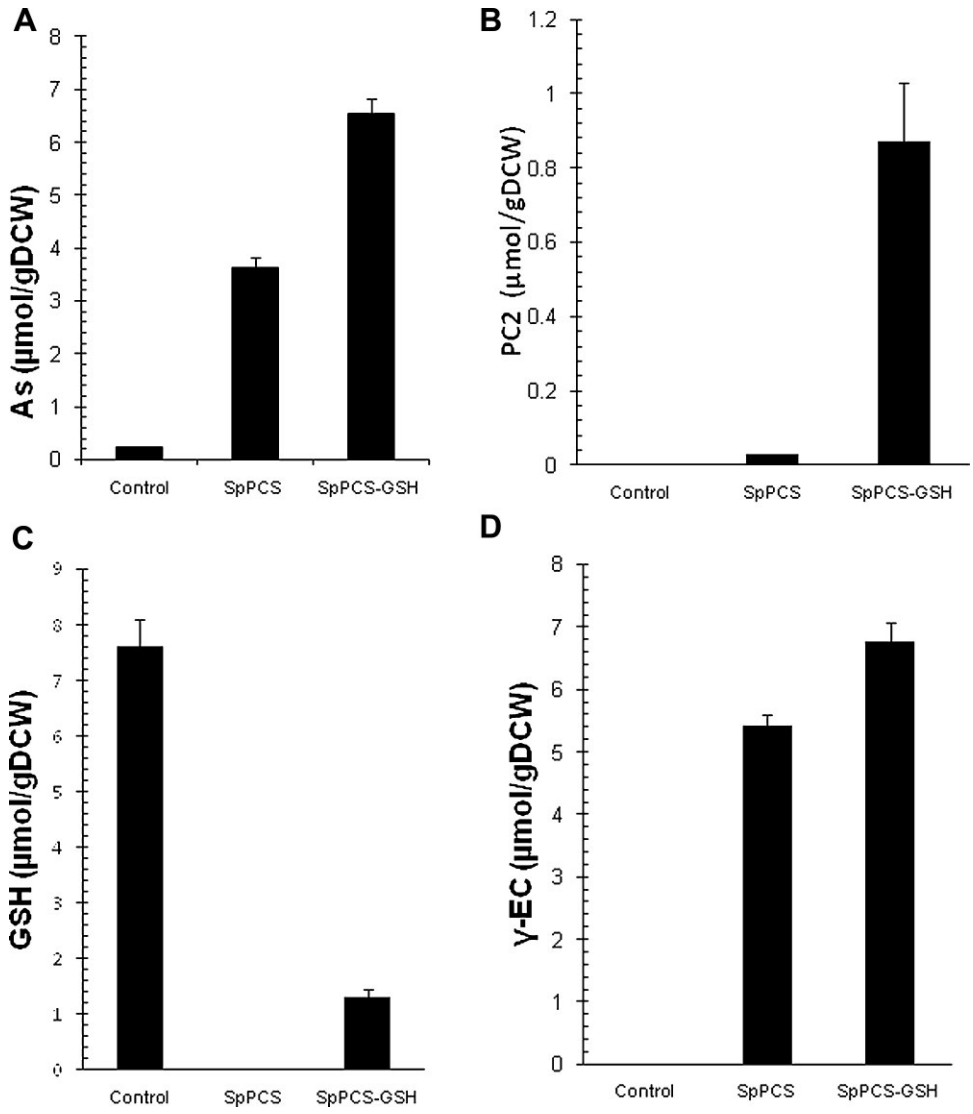

Figure 3. Intracellular thiols determination and As accumulation. Intracellular As (**A**), PC2 (**B**), GSH (**C**), and γ-EC (**D**) contents of *E. coli* strain JM109 harboring either the control vector, pQE-SpPCS (SpPCS), or pQE-SpPCS-GshI* (SpPCS-GSH). Data shown are the mean values (standard deviations) obtained from three independent experiments.

confirming that the improved arsenic accumulation is solely the result of an increase in uptake.

Enhanced Arsenic Accumulation by Arsenic Pump Deletion

It is interesting to note that the level of improvement in As (III) accumulation is still lower than the improvement in PC production. In *E. coli*, As(III) is actively excluded from cells using an ArsAB efflux pump, and eliminating this efflux mechanism may increase the amount of intracellular As(III) available for PC chelation. To examine this possibility, similar experiments were conducted in an ArsAB deletion strain Aw10 (Kuroda et al., 1997); the resulting cellular As(III) content of 16.8 µmol/g DCW was 1.5-fold higher than that in JM109 expressing SpPCS, GshI* and GlpF, where ArsAB is functional (Fig. 4). This level of As accumulation is 10-fold higher than those observed with cells expressing either AtPCS or ArsR (Kostal et al., 2004; Sauge-Merle et al., 2003) and is twofold higher than the fMT-expressing cells (Singh et al., 2008b).

Discussion

PCs represent a natural candidate for arsenic remediation due to their high affinity for arsenite (Schmoger et al., 2000; Sauge-Merle et al., 2003; Singh et al., 2008a). In this article, we reported the systematic engineering of PC-producing

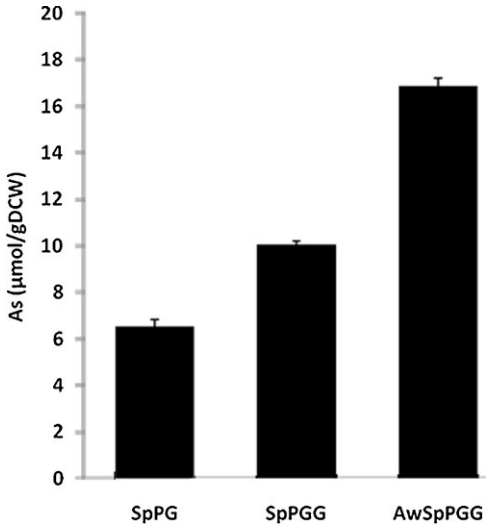

Figure 4. Intracellular As contents of *E. coli* strain JM109 harboring either pQE-SpPCS-GshI* (SpPG) or pQE-SpPCS-GshI*-GlpF (SpPGG) and *E. coli* strain Aw10 harboring pQE-SpPCS-GshI*-GlpF (AwSpGG). Data shown are the mean values (standard deviations) obtained from three independent experiments.

E. coli for As accumulation by addressing various bottlenecks in PC synthesis and arsenic transport. Although expression of SpPCS resulted in PC production and 18-fold higher arsenic accumulation, PC production appeared to be limited by the supply of GSH. This bottleneck was eliminated by expression of a feedback desensitized GshI* to enhanced the supply of GSH, resulting in 30-fold improved PC levels but only 2-fold higher arsenic accumulation. This significant decoupling between the increase in PC level and arsenic accumulation suggests a possible limitation in the As transport. This possibility was confirmed by co-expressing an arsenic uptake transporter GlpF along with SpPCS and GshI*, which resulted in 1.5-fold further improvement in As accumulation while maintaining the PC level. However, this marginal increase in As accumulation points to other mechanisms responsible for limiting the amount of intracellular As for PC chelation. In *E. coli*, active efflux of As(III) is the main detoxification mechanism in reducing the intracellular As content. We reasoned that by deleting the active arsenic efflux pump ArsAB (Kuroda et al., 1997) would allow higher retention of As inside the cells. This was indeed the case as the As level was further improved by 1.5-fold in an ArsAB deletion strain. By carefully coordinating PC synthesis with intracellular As availability, we have successfully increased the intracellular As content by almost fivefold from 3.6 μmol/g DCW for SpPCS-expressing cells to 16.8 μmol/g DCW. We believe this is the first report for such a systematic approach in engineering an arsenic accumulating bacteria and may be adaptable in generating similar engineered strains for other heavy metals removal. Such engineered arsenic accumulators can be used as arsenic biosorbents.

This work was supported by a grant from NSF (BES0422791 and BES0329482). We thank Thomas K. Wood, Texas A&M University, for plasmid pMMB277-Ptac-isoILR1-gshI* carrying gshI* gene. Acknowledgments are also due for Peter Agre, Duke University School of Medicine, for providing plasmid pTrc10HisGlpF carrying GlpF gene and to B.P. Rosen, Wayne State University, for providing strain AW10.

References

Bae W, Mehra R, Mulchandani A, Chen W. 2001. Genetic engineering of *Escherichia coli* for enhanced bioaccumulation of mercury. Appl Environ Microbiol 67:5335–5338.

Cebrian ME, Albores A, Aguilar M, Blakely E. 1983. Chronic arsenic poisoning in the north of Mexico. Hum Toxicol 2:121–133.

Chen CJ, Hsueh YM, Lai MS, Shyu MP, Chen SY, Wu MM, Kuo TL, Tai TY. 1995. Increased prevalence of hyper tension and long-term arsenic exposure. Hypertension 25:53–60.

Dey S, Dou D, Rosen BP. 1994. ATP-dependent arsenite transport in everted membrane vesicles of *Escherichia coli*. J Biol Chem 269: 25442–25446.

Kang SH, Singh S, Kim JY, Lee W, Mulchandani A, Chen W. 2007. Bacteria metabolically engineered for enhanced phytochelatin production and cadmium accumulation. Appl Environ Microbiol 73:6317–6320.

Karagas MR, Tosteson TD, Blum J, Morris JS, Baron JA, Klaue B. 1998. Design of an epidemiologic study of drinking water arsenic exposure and skin and bladder cancer risk in a U.S. population. Environ. Health Perspect 106(Suppl. 4):1047–1050.

Kelly BS, Antholine WE, Griffith W. 2002. *Escherichia coli* γ-glutamylcystein synthetase. J Biol Chem 277:50–58.

Kostal J, Yang R, Wu CH, Mulchandani A, Chen W. 2004. Enhanced arsenic accumulation in engineered bacterial cells expressing ArsR. Appl Environ Microbiol 70:4582–4587.

Kuroda M, Dey S, Sanders OI, Rosen BP. 1997. Alternate energy coupling of ArsB, the membrane subunit of the Ars anion-translocating ATPase. J Biol Chem 272:326–331.

Ma LQ, Komar KM, Tu C, Zhang W, Cai Y, Kennelley ED. 2001. A fern that hyperaccumulates arsenic. Nature 409:579–579.

Mehra RK, Winge DR. 1991. Metal-ion resistance in fungi—Molecular mechanisms and their regulated expression. J Cell Biochem 45:30–40.

Meister A. 1995. Glutathione biosynthesis and its inhibition. Method Enzymol 252:26–30.

Mejare M, Bulow L. 2001. Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals. Trends Biotechnol 19:67–73.

Nriagu JO, Pacyna JM. 1988. Quantitative assessment of worldwide contamination of air, water and soils by trace metals. Nature 333:134–139.

Rui L, Kwon YM, Reardon KF, Wood TK. 2004. Metabolic pathway engineering to enhance aerobic degradation of chlorinated ethenes and to reduce their toxicity by cloning a novel glutathione S-transferase, an evolved toluene o-monooxygenase, and γ-glutamylcysteine synthetase. Environ Microbiol 6:491–500.

Sambrook J, Fritsch EF, Maniatis T. 2001. Molecular cloning: A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanders OL, Rensing C, Kuroda M, Mitra B, Rosen BP. 1997. Antimonite is accumulated by the glycerol facilitator GlpF in *Escherichia coli*. J Bacteriol 179:3365–3367.

Sauge-Merle S, Cuine S, Carrier P, Lecomte-Pradines C, Luu DT, Peltier G. 2003. Enhanced toxic metal accumulation in engineered bacterial cells expressing *Arabidopsis thaliana* phytochelatin synthase. Appl Environ Microbiol 69:490–494.

Say R, Yilmaz N, Denizli A. 2003. Biosorption of cadmium, lead, mercury, and arsenic ions by the fungus *Penicillium purpurogenum*. Sep Sci Technol 38:2039–2053.

Schmoger MEV, Oven M, Grill E. 2000. Detoxification of arsenic by phytochelatins in plants. Plant Physiol 122:793–801.

Singh S, Lee W, DaSilva NA, Mulchandani A, Chen W. 2008a. Enhanced arsenic accumulation and removal by engineered yeast cells expressing *Arabidopsis thaliana* phytochelatin synthase. Biotechnol Bioeng 99: 333–340.

Singh S, Mulchandani A, Chen W. 2008b. Highly selective and rapid arsenic removal by metabolically engineered *E. coli* expressing *Fucus vesiculosus* metallothionein. Appl Environ Microbiol 74:2924–2927.

Smith AH, Lingas EO, Rahman M. 2000. Contamination of drinking water by arsenic in Bangladesh: A public health emergency. Bull World Health Organ 78:1093–1103.

Sneller FEC, Heerwaarden LM, Koevoets PLM, Vooijs R, Schat H, Verkleij JAC. 2000. Derivatization of phytochelatins from *Silence vulgaris*, induced upon exposure to arsenate and cadmium: Comparison of derivatization with ellman's reagent and monobromobimane. J Agric Food Chem 48:4014–4019.

Sriprang R, Hayashi M, Ono H, Takagi M, Hirata K, Murooka Y. 2003. Enhanced accumulation of $Cd2^{+}$ by a *Mesorhizobium* sp. transformed with a gene from *Arabidopsis thaliana* coding for phytochelatin synthase. Appl Environ Microbiol 69:1791–1796.

Vatamaniuk OK, Mari S, Lu YP, Rea PA. 1999. AtPCS1, a phytochelatin synthase from *Arabidopsis*: Isolation and *in vitro* reconstitution. Proc Natl Acad Sci USA 96:7110–7115.

Zenk MH. 1996. Heavy metal detoxification in higher plants: A review. Gene 179:21–30.