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

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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/C3), 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.

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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 (γ-Glu-Cys-)n-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
Cysteine + Glutamate

\[ \gamma\text{-Glutamylcysteine Synthase (GshI)} \]

\[ \gamma\text{-Glutamylcysteine} \]

\[ \text{Glutathione Synthetase (GshII)} \]

\[ \text{Glutathione} \]

\[ \text{Phytochelatin Synthase (PCS)} \]

\[ \text{Phytochelatin} \]

**Figure 1.** Pathway showing enzymatic synthesis of phytochelatins by phptoche-latin 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 key 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 lacP2ΔM15]) (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 Δars::cam dam dcm supE44 hsdR17 thi leu rpsL lacY galK galT ara tonA thr tsx Δ(lac-proAB), Cm*) (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-GshII*-GlpF expressing SpPCS and GshI* or SpPCS, GshII* 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 GCCGGGAATTC AAGGAGATATACATCAGAAGGAGATATACATGATGAGTCAGAAGGAGATATACATGATGAGTCAGAAGGAGATATACATGATGAGTCA and the reverse primer TATGGAATTC GTCGACTCTAGAGCTTTCATAGTTAACATGGTTAGTGTATGGT. The PCR product was digested with *BamHI* and ligated into *BamHI*-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 (OD600) from overnight grown seed cultures. When the OD600 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, 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 phosphatase 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 MgCl2). 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, 110A; 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.

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,
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
engineered arsenic accumulators can be used as arsenic biore- sorbents.

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**References**


