

Co-Expression of *Arabidopsis thaliana* Phytochelatin Synthase and *Treponema denticola* Cysteine Desulphydrase for Enhanced Arsenic Accumulation

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Received 5 August 2011; revision received 29 August 2011; accepted 31 August 2011

Published online 13 September 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.23325

ABSTRACT: Arsenic is one of the most hazardous pollutants found in aqueous environments and has been shown to be a carcinogen. Phytochelatins (PCs), which are cysteine-rich and thio-reactive peptides, have high binding affinities for various metals including arsenic. Previously, we demonstrated that genetically engineered *Saccharomyces cerevisiae* strains expressing phytochelatin synthase (AtPCS) produced PCs and accumulated arsenic. In an effort to further improve the overall accumulation of arsenic, cysteine desulphydrase, an aminotransferase that converts cysteine into hydrogen sulfide under aerobic condition, was co-expressed in order to promote the formation of larger AsS complexes. Yeast cells producing both AtPCS and cysteine desulphydrase showed a higher level of arsenic accumulation than a simple cumulative effect of expressing both enzymes, confirming the coordinated action of hydrogen sulfide and PCs in the overall bioaccumulation of arsenic.

Biotechnol. Bioeng. 2012;109: 605–608.

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KEYWORDS: arsenic; phytochelatin; cysteine desulphydrase; yeast

Arsenic is one of the most hazardous pollutants found in the aqueous environment, and has been shown to be a carcinogen, affecting the health of millions throughout the world (Smedley and Kinniburgh, 2002). Due to its

extreme toxicity, the US Environmental Protection Agency reduced the maximum contaminant level for arsenic in drinking water from 50 to 10 ppb in 2006 (U.S.EPA, 2001).

Arsenic occurs in several oxidation states. Among them As(III) is generally considered to be more mobile and more toxic than As(V) (Liu et al., 2001). Several treatment technologies have been applied in laboratory- and/or field-scale testing for the removal of arsenic from drinking water including coagulation, filtration, ion exchange, adsorption, and reverse osmosis (DeMarco et al., 2003; Martin et al., 1995; Zouboulis and Katsoyiannis, 2002). However, these technologies are either too expensive, due to the need to oxidize As(III) to As(V), or ineffective for As(III) treatment especially at low arsenic concentration. Therefore, technologies showing economic competitiveness, environmental amenability, and high selectivity are need for effective removal of arsenite.

Bioremediation processes are considered to be cost-effective and environmental-friendly way for heavy metals removal (Singh et al., 2008). In nature, microorganisms have developed several strategies for detoxification of heavy metals such as biotransformation, biomineralization, or biosorption (Barkay and Schaefer, 2001). These different microbial detoxifying mechanisms can potentially be combined to design an efficient bioremediation process (Tsai et al., 2009).

Phytochelatins (PCs) are small enzymatically synthesized cysteine-rich peptides widely found in plants and yeasts, and have been shown to efficiently bind heavy metals such as cadmium, mercury, lead, and arsenite (Kim et al.,

Correspondence to: W. Chen

Contract grant sponsor: NSF

Contract grant number: CBET0422791; CBET0903894

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2005; Maitani et al., 1996; Schmoger et al., 2000). We have reported enhanced accumulation of arsenite by an engineered *S. cerevisiae* strain expressing the *Arabidopsis thaliana* phytochelatin synthase (AtPCS) due to complexation with PCs (Singh et al., 2008).

Sulfide plays an important role in many metal detoxification mechanisms such as mineralization, which reduces the toxicity of heavy metals by forming insoluble metal precipitates (Barkay and Schaefer, 2001). However, most of the naturally occurring sulfate-reducing bacteria produce hydrogen sulfide only under strictly anaerobic conditions (White and Gadd, 1998) and therefore they are not suitable for applications under aerobic conditions. Wang et al. (2001) functionally expressed a cysteine desulfhydrase (CysDes) from *Treponema denticola* in *E. coli* for the aerobic sulfide production and heavy metal precipitation using cysteine as the substrate. Apart from the formation of insoluble arsenic trisulfide (Newman et al., 1997), the formation of soluble arsenic sulfide complexes (thioarsenics) is also a well-known phenomenon (Hollibaugh et al., 2005; Stauder et al., 2005). Although little is known about the bioavailability of thioarsenics, they are reported to be less toxic than arsenite (Rader et al., 2004). In some eukaryotes, incorporation of sulfide to form a more stable high molecular weight PC-metal-sulfide complex in the vacuole has also been demonstrated (Mendoza-Cozatl et al., 2005, 2006). Given these studies, the goal of this work was to co-express cysteine desulfhydrase and AtPCS in *S. cerevisiae* to elevate the intracellular accumulation of arsenic via the formation of PC-arsenic-sulfide complexes.

We first investigated whether cysteine desulfhydrase expression (BY4742/CysDes) could result in enhanced sulfide formation in yeast as this has not been previously demonstrated. Samples were taken after 15 h to determine the cell density (OD) and the intracellular levels of sulfide and As(III). As clearly shown in Figure 1A, expression of cysteine desulfhydrase resulted in elevated production of sulfide and the level of enhancement increased with the amount of cysteine added. The enhanced sulfide production also resulted in higher levels of arsenite sequestration in a sulfide-dependent manner (Fig. 1B). Unfortunately, a significant growth inhibition was observed in the presence of cysteine (Table I), even though the presence of cysteine desulfhydrase partially reduced the level of growth inhibition. While cells expressing cysteine desulfhydrase accumulated 25% more arsenite than the wild-type strain BY4742 even in the absence of any added cysteine, this is likely the most desirable condition in practice when combined with the expression of AtPCS as it saves the additional cost of adding cysteine into the medium.

Although expression of AtPCS has been shown to enhance arsenite accumulation in *S. cerevisiae* when grown in a rich medium (6.7 g/L yeast nitrogen base without amino acid, 5 g/L casamino acids, 20 g/L of galactose) (Singh et al., 2008), we tested whether a similar enhancement in arsenite accumulation could be obtained using a defined medium without cysteine supplementation as cysteine is one of the

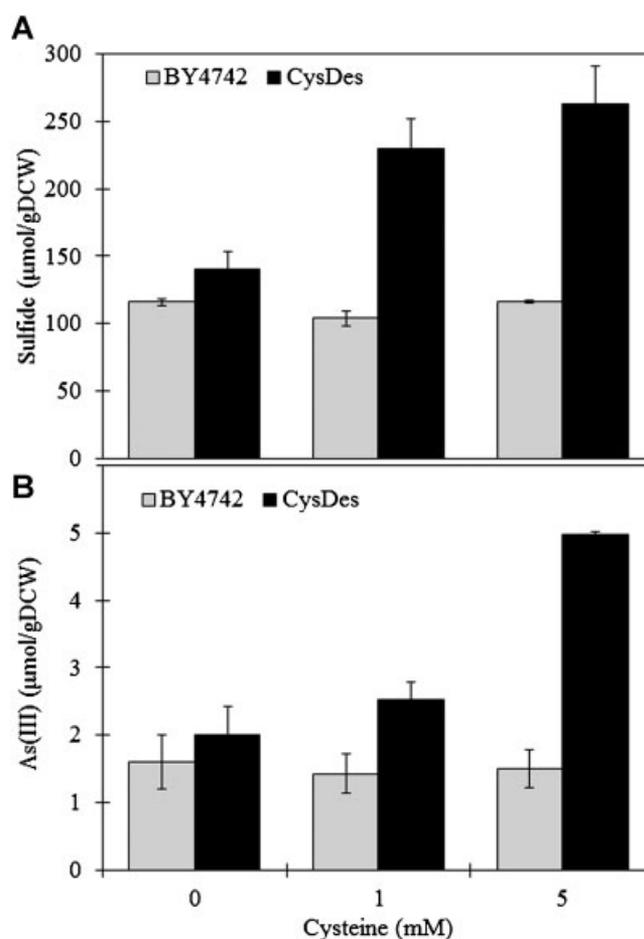


Figure 1. A: Sulfide production and (B) intracellular As(III) accumulation from the engineered *S. cerevisiae* strain BY4742 expressing cysteine desulfhydrase at various cysteine concentrations. Cells were grown in 200 mL defined medium containing 6.7 g/L of yeast nitrogen base without amino acids, 20 g/L of galactose, 20 mg/L of adenine sulfate, L-tryptophan, L-histidine-HCl, L-arginine-HCl and L-methionine, 30 mg/L of L-tyrosine, L-isoleucine, L-lysine-HCl and L-phenylalanine, 100 mg/L of L-glutamic acid and L-aspartic acid, 150 mg/L of L-valine, 200 mg/L of threonine and 400 mg/L of serine at 30°C. Twenty milligrams per liter of uracil and/or 30 mg/L of L-leucine was also added if required. DCW, dry cell weight. Results were obtained from three independent experiments.

precursors for PC synthesis. As shown in Figure 2, cells producing PC showed up to a threefold increase in the intracellular arsenite content over the control BY4742 strain. The level of As accumulation correlated well with the

Table I. Cell densities (OD) of the yeast strain BY4742 with or without cysteine desulfhydrase expression after 15 h cultivation at various cysteine concentrations.

Cysteine conc. (μM)	Cell growth	
	BY4742	CysDes
0	0.975 \pm 0.05	1.142 \pm 0.01
1	0.634 \pm 0.05	0.639 \pm 0.08
5	0.262 \pm 0.02	0.455 \pm 0.05

Data shown below were the average from three independent experiments.

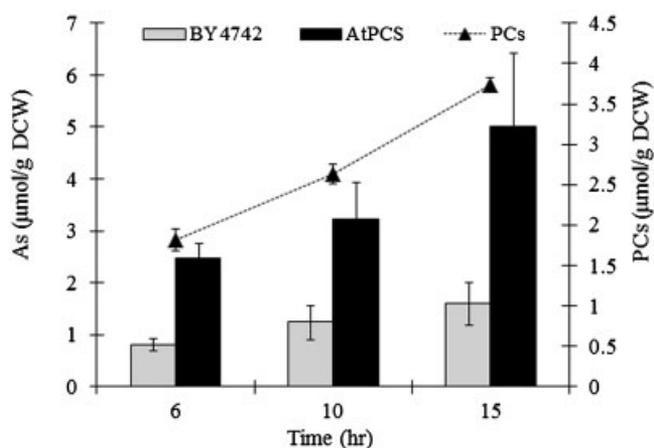
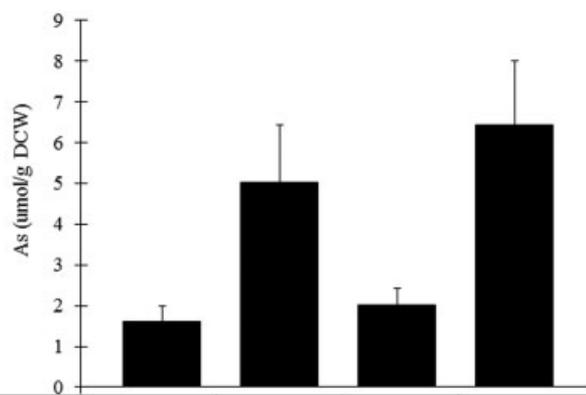


Figure 2. PC production and intracellular As(III) accumulation from the engineered *S. cerevisiae* strain BY4742 expressing AtPCS. DCW, dry cell weight. Results were obtained from three independent experiments. Culture conditions were the same as described in Figure 1.

increasing intracellular PC content, confirming again PC is solely responsible for the enhanced As accumulation.

Since the separate expression of cysteine desulfhydrase or AtPCS could both elevate the intracellular arsenic content, it is interesting to explore whether co-expression of both enzymes could further improve As(III) accumulation in an additive manner. Cells co-expressing both enzymes showed the highest intracellular As content, and the overall level of enhancement was 25% higher than a simple addition of the two individual contributions (Fig. 3). This is surprising as the intracellular PC level for the co-expression strain was 10% lower than the AtPCS-expressing strain. This can be attributed to the fact that glutathione, which is a precursor for PC synthesis, is also a good substrate for cysteine desulfhydrase for sulfide synthesis. In addition, the enzyme GSH1, which is responsible for glutathione precursor (γ -EC) synthesis, is known to be inhibited by glutathione (Pócsi et al., 2004). Therefore, the increased consumption of glutathione by both cysteine desulfhydrase and PCS resulted in higher glutathione production and a corresponding 30% higher intracellular sulfide content (Chu et al., 1997). The improved arsenite binding is also likely the result of the formation of high molecular weight PC–As–S complexes as observed in many plants and fungi (Mendoza-Cozatl et al., 2005, 2006).

In conclusion, engineered *S. cerevisiae* strains expressing cysteine desulfhydrase and/or AtPCS were created for enhanced accumulation of arsenic. Cells expressing both AtPCS and cysteine desulfhydrase showed a higher level of arsenic accumulation than a simple cumulative effect of expressing both enzymes, confirming the importance of coordinated action of hydrogen sulfide and PCs in the overall bioaccumulation of arsenic.



Strain	BY4742	AtPCS	CysDes	AtPCS/CysDes
PCs Conc. (μmol/gDCW)	ND	3.8±0.1	ND	3.4±0.2
Sulfide Conc. (μmol/gDCW)	83.9±1.5	87.1±4.4	90.1±0.6	121.1±1.6

Figure 3. Intracellular As(III) accumulation from engineered *S. cerevisiae* strains expressing either cysteine desulfhydrase, AtPCS, or both enzymes. Data shown in the table were the average from three independent experiments. The difference of intracellular As(III) content between AtPCS and AtPCS/CysDes are statistically significant with 95% confidence. The corresponding PC and sulfide concentrations are also shown. DCW, dry cell weight.

Materials and Methods

Plasmid pYES3-atPCS1::FLAG was kindly donated by Dr. Rea at University of Pennsylvania, Philadelphia (Vatamaniuk et al., 1999). The AtPCS gene was under the control of a constitutive yeast promoter PGK and flanked by a FLAG tag. The gene coding for cysteine desulfhydrase was amplified from the plasmid pCysdesulf/LacI2/Rock, a gift from Dr. Keasling at University of California, Berkeley (Wang et al., 2001), by PCR and cloned into the Not1 and BamH1 restriction sites of the multiple copy yeast shuttle vector pYES3 (Vatamaniuk et al., 1999). The entire cassette (PGKp-CysDes::His-PGKt) of cysteine desulfhydrase gene was then amplified by PCR and further bluntly inserted into the Sma1 site of plasmid YEplac181 (Gietz and Sugino, 1988) containing a LEU2 selection marker to form p181CysDes in order to co-transform with pYES3-atPCS1::FLAG carrying a URA3 selection marker. The gene for cysteine desulfhydrase was under the control of strong yeast promoter PGK and flanked by a His tag. Plasmid maps of the two constructs are given in supplementary materials. *S. cerevisia* strain BY4742 (MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0) (Singh et al., 2008) was used for study and plasmid transformation was carried out by the lithium acetate method (Ito et al., 1983).

To measure the levels of PC, samples were taken periodically from growing cultures (arsenite enriched media), and PCs were extracted and analyzed by HPLC (Agilent Tech., Palo Alto, CA). Briefly cells were pelleted down from the samples, washed with 5 mM HEPES buffer

containing 0.8% NaCl twice before freezing at -80°C for 24 h. PCs were extracted with diethylenetriamine pentaacetic acid (DTPA) with 0.1% TFA and derivatized by reacting with 4-(2-hydroxy-ethyl)-piperazine-1-propane-sulfonic acid buffer (pH 8.2) containing DTPA and monobromobimane (mBrB). The PC content was measured using an excitation wavelength at 380 nm and an emission wavelength at 470 nm.

For arsenic measurements, cells were harvested, 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 internal arsenic content was measured using atomic adsorption spectroscopy (Perkin Elmer, Inc., Waltham, MA). Arsenic was determined at 193.7 nm in a graphite furnace.

The sulfide content was determined by a colorimetric assay as described by Aiking et al. (1982). Briefly, culture samples were centrifuged and the cell pellet was resuspended in 1 mL of 0.75 M NaOH after washing twice with 5 mM HEPES buffer containing 0.8% NaCl. This suspension was transferred to a microcentrifuge tube and incubated at 95°C for 15 min. The suspension was then mixed with 375 μL of 0.75 M NaOH and 250 μL of 2.6% zinc acetate dihydrate, 125 μL of 0.1% *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride in 5 M HCl (freshly prepared) was added, and the solution was vortexed until clear. Next, 50 μL of 11.5 mM FeCl_3 in 6 M HCl was added and the solution was vortexed and incubated at room temperature for 30 min. After appropriate dilution and centrifugation, the OD of the sample was determined at 670 nm.

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