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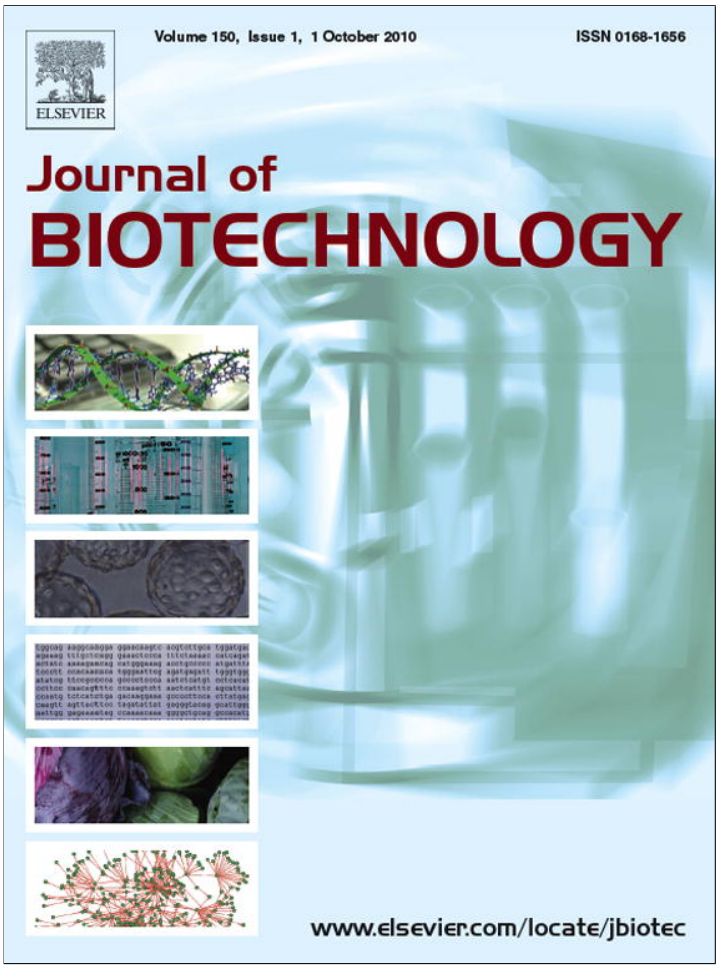

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Enhanced arsenic accumulation in *Saccharomyces cerevisiae* overexpressing transporters Fps1p or Hxt7p

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ABSTRACT

Arsenic contamination of ground water affects the health of millions of people worldwide. Bioremediation has the potential to lower contaminant levels in cases where physical methods are either ineffective or cost prohibitive. The yeast *Saccharomyces cerevisiae* was engineered for enhanced arsenite accumulation by overexpression of transporters responsible for the influx of the contaminant. The transporter genes *FPS1* and *HXT7* were cloned under the control of the late-phase *ADH2*-promoter. This allowed for protein production at high biomass levels without the addition of inducer. Following the transfer of stationary phase cells to buffer, the engineered strains were capable of 3–4-fold greater arsenic uptake as compared to control cells. Further, at trace levels of the metalloid, the cells overexpressing the Fps1p transporter removed ca. 40% more arsenite from the extracellular medium than the controls. Arsenic uptake was also evaluated in cells overexpressing the transporters coupled with high-level production of cytosolic As sequestors (phytochelatins or bacterial ArsRp) to act as an intracellular sink. This led to an up to 4-fold increase in As accumulation in the resting cell culture as compared to native cells. The results demonstrate important steps needed to engineer a yeast biosorbent with enhanced accumulation capabilities for this metalloid.

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1. Introduction

Arsenic (As) is an extremely toxic metalloid that is naturally present in the Earth's crust. Groundwater contamination arises from natural (igneous activity) and human (e.g., mining runoff) activities (Nriagu, 1994; Macy et al., 1996; National Research Council, 1977). The health hazards of arsenic range from neurological and cardiovascular diseases to various malignancies due to chronic exposure, and can prove fatal at acute doses (Karagas et al., 1998). Arsenic exists as arsenite (As(III)) and arsenate (As(V)) with the former being the more potent form (Tamas and Wysocki, 2001; Rosen, 2002). *In vivo*, arsenite reacts with protein thiol groups and generates reactive oxygen species (ROS) which can damage nearly all cellular components (Liu et al., 2001). Further, arsenate is reduced *in vivo* to the more toxic arsenite (Tamas and Wysocki, 2001; Rosen, 2002). In Bangladesh alone, an estimated 50 million people drink water contaminated with high levels of As (Pearce, 2003). Similar to the guidelines set by the World Health Organization (WHO), the United States Environmental Protection Agency has reduced the maximum allowable contaminant level from 50 to 10 ppb (EPA, 2006). This change has affected over 4000 water supply systems serving 13 million people across the United States (EPA, 2006).

Typically, physical methods such as a co-precipitation, membrane or sorption technique are used to decontaminate water (Clifford et al., 1986; Driehaus et al., 1995). However, these methods can prove to be too costly, impractical to apply over large scales, or unable to remove trace quantities of the metalloid (Chwirka et al., 2000; Driehaus et al., 1995; Wilkie and Hering, 1998). Bioremediation offers a cost-effective and comparatively innocuous alternative to physical methods for heavy-metal decontamination (Mejare and Bulow, 2001). Arsenic transport pathways exist and have been characterized in a number of lower and higher order organisms (Tamas and Wysocki, 2001; Rosen, 2002), and can be exploited for As removal. Although organisms have been isolated from As contaminated sites (Ma et al., 2001; Canovas et al., 2003), their use as biosorbents for As removal has not been reported. Many studies on biosorbents have focused on nonspecific binding to cell walls; such biosorbents, however, generally lack the high affinity and specificity desired (Say et al., 2003).

There are several advantages to working with the yeast *Saccharomyces cerevisiae* including its ability to grow rapidly on various carbon sources, well developed fermentation methods, a fully

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sequenced genome, a large number of available genetic tools, and generally recognized as safe (GRAS) status by the United States Food and Drug Administration (Romanos et al., 1992). The details of the *S. cerevisiae* As detoxification pathway have also been elucidated (Bobrowicz et al., 1997; Tamas and Wysocki, 2001). Arsenite is transported across the plasma membrane by the action of the aquaglyceroporin, Fps1p, and the hexose transporters, Hxt7p and Hxt9p (Wysocki et al., 2001; Liu et al., 2004). The intracellular arsenite is either transported out of the cell by the action of the As extrusion protein Acr3p or sequestered in internal vacuoles by the action of Ycf1p (in an ATP-mediated process) (Ghosh et al., 1999). Arsenate is transported into the cell by the phosphate transporter, Pho84p, and reduced to arsenite by arsenate reductase, Acr2p (Berhe et al., 1995; Mukhopadhyay et al., 2000). Extrusion or sequestration then occurs.

Other prokaryotic and eukaryotic organisms have also developed novel defense mechanisms. In the bacterium *Escherichia coli*, the genes of the *ars* operon encode proteins necessary to resist As insult (Carlin et al., 1995). The regulatory protein, ArsRp, binds specifically to trivalent As (and antimony) and can effectively discriminate against carbonate, nitrate, phosphate, and sulfate (Scott et al., 1997). Recently, engineered *E. coli* cells expressing ArsRp as a protein fusion were evaluated for bioremediation purposes (Kostal et al., 2004). All plants and some worms and fungi produce short peptides known as phytochelatins (PCs) in response to metalloid stress (Gekeler et al., 1989; Mehra et al., 1988; Vatamaniuk et al., 2002). These peptides contain glutamic acid and cysteine repeats and can bind trivalent As to retain the metalloid in a non-toxic form in the cell with the vacuoles being the putative end point of the complex (Grill et al., 1985; Schmoger et al., 2000). Expression of PCs in growing *S. cerevisiae* cells has been shown to increase arsenite accumulation 6-fold (Singh et al., 2008).

The objective of this work was to engineer a yeast biosorbent with enhanced arsenite uptake and accumulation capabilities. Arsenic accumulation was evaluated in a resting *S. cerevisiae* cell system after overexpression of the As(III) transporters, Fps1p and Hxt7p, using the late-phase *ADH2*-promoter. Intracellular accumulation was studied at both high metalloid concentrations and low concentrations (similar to those encountered in groundwater) to evaluate the effect of transporter overexpression. Arsenic uptake was further studied by expressing the transporter Fps1p in conjunction with PCs or ArsRp. Plasmid stabilities were measured for the single and dual-plasmid systems and compared with the observed As uptake.

2. Materials and methods

2.1. Strains and plasmids

S. cerevisiae strains 4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and 15616 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 acr3::kan*) were obtained from Open Biosystems (Huntsville, AL). Strain 15616 has the gene encoding the As extrusion protein Acr3p deleted, but is otherwise isogenic to strain 4742. *Escherichia coli* strain XL-1 Blue (Stratagene, La Jolla, CA) was used for cloning and plasmid maintenance.

Plasmids YEplac195 and YEplac181 are 2μ-based vectors containing the uracil and leucine markers, respectively (Gietz and Sugino, 1988). Plasmid pKOS12-122c-NdeI (Kosan Biosciences, CA) contains the *ADH2*-promoter and terminator sequences. Genes for the arsenite transporters, *FPS1* and *HXT7*, were amplified using PCR with strain 4742 as template, KOD Hot Start DNA Polymerase (EMD Biosciences, San Diego, CA), and the primers shown in Table 1. The *FPS1* gene was inserted into the multiple cloning site between the *ADH2*-promoter and terminator of pKOS12-122c-NdeI. The P_{ADH2}-*FPS1*-T_{ADH2} cassette was then inserted between the *Bam*HI and *Sal*I sites in YEplac195 and YEplac181 resulting in p2m-ADH2-FPS1 and p2m-ADH2-FPS1-Leu, respectively. The *HXT7* gene was inserted into plasmid pCR4-TOPO using the TA Cloning kit (Invitrogen, Carlsbad, CA) forming vector pCR4-HXT7. The *Sal*I and *Pme*I sites on p2m-ADH2-FPS1 were used to replace the *FPS1* gene with the *HXT7* gene to create plasmid p2m-ADH2-HXT7. The *FPS1* and *HXT7* genes were double-pass sequenced (Genewiz, NJ) to ensure no errors were incorporated during PCR amplification. The construction of plasmid pYES-AtPCS1::FLAG has been described previously (Singh et al., 2008). This plasmid contains the 2μ origin, the *Arabidopsis thaliana* phytochelatin synthase (PCS) under the control of the *S. cerevisiae PGK*-promoter, and the *URA3* gene as a selection marker. The *arsR* gene was amplified from plasmid pet-ArsR (Kostal et al., 2004) using the primers shown in Table 1. The amplification product was digested with *Not*I and *Bam*HI and inserted into similarly digested pYES-AtPCS1::FLAG resulting in plasmid p2m-PGK-ArsR.

2.2. Media and cell cultivation

Complex, non-selective YPD and YPG media contain yeast extract (10 g/l, Difco Laboratory, MI), peptone (20 g/l, Difco) and dextrose or galactose (10 g/l), respectively. Selective semi-defined SDC and SGC media contain yeast nitrogen base (6.7 g/l, Difco), casamino acids (5 g/l, Difco), adenine (100 mg/l), and dextrose or galactose (10 g/l), respectively. Minimal SD(His/Lys) medium contains yeast nitrogen base (6.7 g/l, Difco), dextrose (10 g/l), histidine (100 mg/l) and lysine (150 mg/l). Zinc chloride (100 μM) was added when expressing the *AtPCS* gene cassette. Uracil (100 mg/l) was supplemented when needed. Plates included Bacto-agar (20 g/l, Difco).

Seed cultures (3 mL medium in 15 mL tubes) were inoculated from −80 °C frozen cultures and placed in an air shaker (Model G25, New Brunswick Scientific, NJ) at 30 °C and 250 rpm for 36 h. Batch cultures (20 mL medium in 125 mL flask or 3 mL medium in 15 mL tube) were inoculated at approximately 1% (v/v) and cultivated in a water bath shaker (Model G76D, New Brunswick Scientific) or air shaker at 30 °C and 250 rpm. Following batch growth, cells were washed three times with and resuspended in sodium buffer (Na-buffer, 50 mM TRIS, 150 mM NaCl, pH 7.4). Trivalent arsenic (Ricca Chemical Company, TX) was added at specific concentrations. Samples were collected at various time points and prepared as described below.

Table 1
Primers used for the amplification of genes *FPS1*, *HXT7* and *arsR*. The capitalized letters are base pairs homologous to the gene, and underlined base pairs are the introduced restriction enzyme site. A His-tag (italics) was added to the 3′ end of ArsR.

Gene	Primer	Primer sequence	Restriction Site
FPS1	FPS1F (forward)	gcagtcgacgacATGAGTAATCCTCAAAAAGCTC	*SalI*
	FPS1R (reverse)	tttcgtttaaactttcTCATGTTACCTTCTTAGCATTACC	*PmeI*
HXT7	HXT7F (forward)	ttttactcgagaaATGTCACAAGACGCTGCTATTG	*XhoI*
	HXT7R (reverse)	atgaattgtttaaactgttcgcaaaTTATTTGGTGCTG	*PmeI*
arsR	ARSRF (forward)	agcatttagcggccgcATGGGTCCAGGTGTTGGC	*NotI*
	ARSRR (reverse)	attcggatccTTA*GTGGTGGTGGTGGTGGT*GACTGCAAATGTTCTTACTGTC	*BamHI*

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2.3. Plasmid stability

Cells were spread onto YPD plates (~200 cells/plate) and incubated at 30 °C for 2–3 days until individual colonies appeared. Colonies (200–400) were transferred to selective SDC(A), SD(His/Lys) or SD(His/Lys/Ura) plates. Plasmid stability was calculated as the percentage of cells that grew on the selective plate relative to the total number transferred.

2.4. Sample preparation and measurement

Samples (1 mL) were centrifuged at 13,000 rpm (Microfuge 18, Beckman Coulter, CA). To measure extracellular As concentrations, the supernatant was collected and diluted in 1% nitric acid to be within the linear range of the atomic absorption spectrophotometer (AAS). To measure intracellular arsenic concentrations, the cells were washed three times with Na-buffer and dried at 65 °C for a minimum of 12 h (modified from Singh et al., 2008). Repeated wash steps were used to remove any arsenic loosely bound to the cell surface. Concentrated nitric acid (100 μl, Fisher Scientific, CA) was added and the cells were allowed to stand for a minimum of 2 days. Water (900 μl) was then added to the cells to bring the final volume to 1 mL. The samples were diluted in 1% nitric acid to be within the linear range of the AAS.

Arsenic concentrations were measured using atomic adsorption spectrophotometry (AAnalyst 800 or SIMAA 6000, Perkin Elmer, Wellesley, MA) at 193.7 nm using a graphite furnace tube. Palladium (0.054%) and magnesium (0.011%) were added as matrix modifier to the samples prior to loading. Each sample was measured in triplicate.

3. Results and discussion

3.1. Arsenite uptake with FPS1 and HXT7 overexpression

Exposing growing cells to high concentrations of arsenic can hamper growth and result in suboptimal accumulation. Thus, it would be advantageous to accumulate biomass prior to exposing the cells to the metalloid. The *S. cerevisiae ADH2*-promoter offers a convenient method to induce protein production after significant cell growth has occurred. Transcription from this promoter is initiated in the late exponential–early stationary phases of batch culture growth without the addition of an inducer (Gancedo, 1998). The *ADH2*-promoter also allows strong expression and growth during cultivation in non-selective complex medium (Lee and Da Silva, 2005).

Arsenic uptake was evaluated under resting cell conditions following overexpression of the *FPS1* or *HXT7* transporter genes from the *ADH2*-promoter. Strains 4742 and 15616 (*ACR3* deletion) were transformed with plasmid p2m-ADH2-FPS1, p2m-ADH2-HXT7, or YEplac195 (control). These strains were cultivated in complex YPD medium for 36 h, then centrifuged and transferred to Na-buffer. No further cell growth was observed. Sodium arsenite was added to a final concentration of 20 or 100 μM. Samples were collected at different time points and processed to determine intracellular arsenic concentrations. Initial results are shown in Figs. 1 and 2 for the Fps1p and Hxt7p transporters, respectively.

Overexpression of Fps1p clearly increased arsenite uptake in the cells. Specific and volumetric uptake in strains transformed with the *FPS1* plasmid were approximately 2-fold higher than in strains harboring the blank plasmid at arsenite concentrations of 20 and 100 μM (Fig. 1). Intracellular As levels increased monotonically over the 10 h after As addition. On average, specific uptake was 3-fold higher in Fps1p overexpressing cells at 100 μM than at 20 μM. Very similar results were observed for the Hxt7p transporter. Specific uptake increased on average 2-fold in cells overexpressing Hxt7p as compared to the strains carrying the blank plasmid at arsenite concentrations of 20 and 100 μM (Fig. 2A and B). Volumetric uptake was 2–3-fold higher in *HXT7*-overexpressing cells compared to the control cells (Fig. 2C and D). Specific uptake in *HXT7*-overexpressing cells was approximately 4-fold higher at 100 μM than at 20 μM.

Uptake was also compared for the two strains 4742 and 15616 (*ACR3 deletion*) in Figs. 1 and 2. The latter strain cannot produce the Acr3p protein required to extrude arsenite back into the medium.

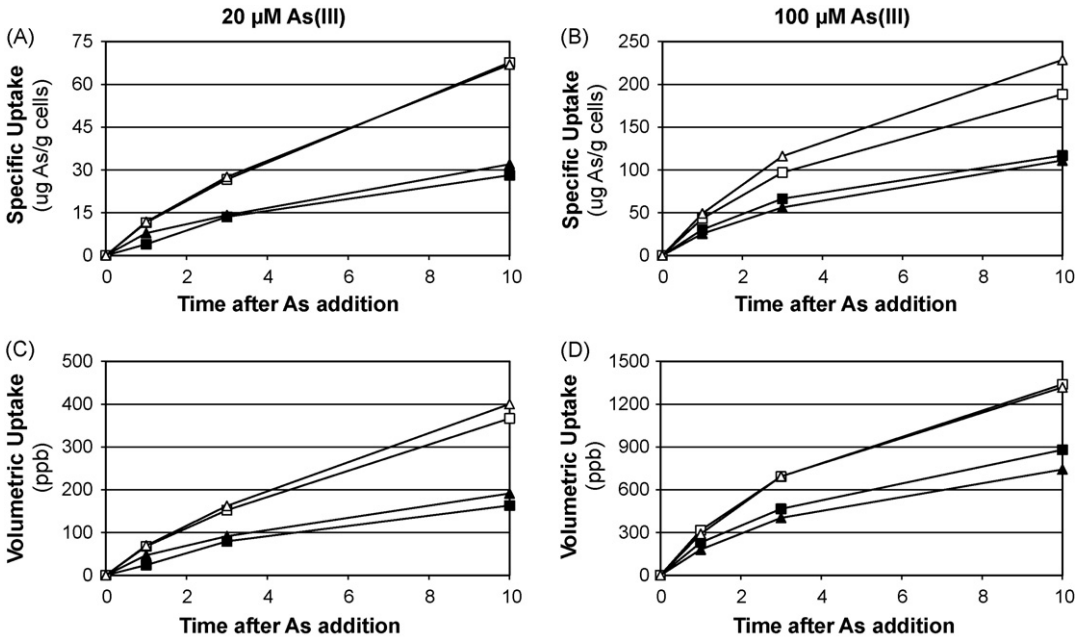

Fig. 1. Intracellular arsenite accumulation in cells overexpressing the Fps1p transporter. Cells were grown in YPD medium for 36 h, transferred to Na-buffer and As was added at the specified concentrations. Specific accumulation is shown for arsenite concentrations of (A) 20 μM and (B) 100 μM. Volumetric uptake is shown for arsenite concentrations of (C) 20 μM and (D) 100 μM. (□, ■) represent strain 4742. (△, ▲) represent the *ACR3* deletion strain 15616. Open and closed symbols represent strains harboring plasmid p2m-ADH2-FPS1 and YEplac195 (control), respectively.

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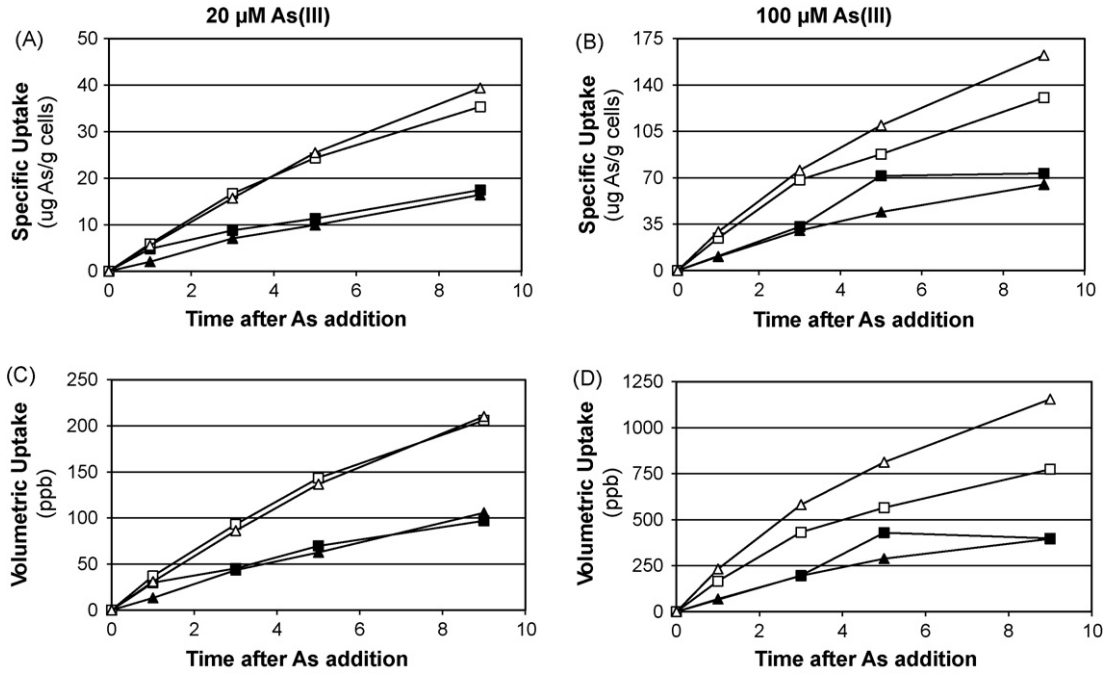

Fig. 2. Intracellular arsenite accumulation in cells overexpressing the Hxt7p transporter. Cells were grown in YPD medium for 36 h, transferred to Na-buffer and As was added at the specified concentrations. Specific accumulation is shown for arsenite concentrations of (A) 20 μM and (B) 100 μM. Volumetric uptake is shown for arsenite concentrations of (C) 20 μM and (D) 100 μM. (□, ■) represent strain 4742. (△, ▲) represent the *ACR3* deletion strain 15616. Open and closed symbols represent strains harboring plasmid p2m-ADH2-HXT7 and YEplac195 (control), respectively.

Specific uptake was comparable between the 4742 and 15616 strains in all cases. This result was expected and can be attributed to *ACR3* regulation. The *ACR3* gene is expressed in response to metalloid stress (Bobrowicz et al., 1997). In our experiments, the cells were grown in complex media for 36 h without any arsenite present. Hence, when transferred to Na-buffer containing As, there was no expressed Acr3 protein present to transport As(III) out of the cell and no resources for the cells to make the efflux protein. Therefore, the *ACR3* knockout provided no additional benefit in the resting cell system.

To further evaluate arsenic uptake in cells overexpressing the Fps1p or Hxt7p transporters, strain 15616 harboring the p2m-ADH2-FPS1, p2m-ADH2-HXT7 or YEplac195 (control) plasmid was cultivated in either YPD or YPG complex media for 36 h. At the end of batch growth, cells were transferred to Na-buffer, arsenite was added at 100 μM and uptake was measured at the 10 h time point. The results are shown in Fig. 3.

Arsenic uptake in cells overexpressing the Fps1p or Hxt7p transporter in YPD (glucose medium) was approximately 3–4-fold higher than in control cells at 10 h. As uptake was approximately 30% higher in cells overexpressing the Hxt7p transporter as compared to cells overexpressing the Fps1p transporter. These results are in general agreement with those in Figs. 1 and 2. Plasmid stability was also measured for these cells. Strain 15616 harboring plasmid YEplac195, p2m-ADH2-FPS1 or p2m-ADH2-HXT7 was cultivated in complex YPD medium for 36 h, and cells were transferred onto YPD plates and subsequently onto selective SDC(A) plates. Plasmid stability was 74%, 61% and 67% for cells carrying the YEplac195, p2m-ADH2-FPS1 and p2m-ADH2-HXT plasmids, respectively, and thus was similar for all three strains.

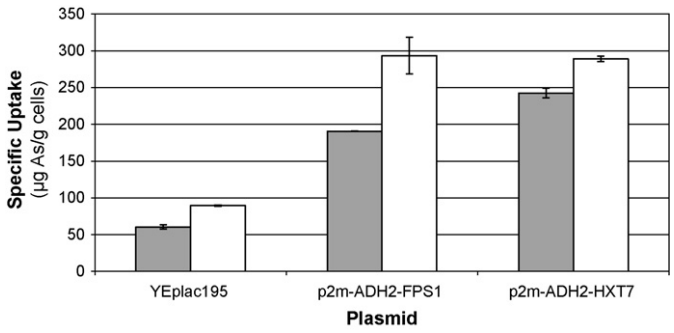

Fig. 3. Specific intracellular arsenite accumulation in cells overexpressing the Fps1 or Hxt7p transporter. Cells were grown in YPD (gray bars) or YPG (white bars) for 36 h, transferred to Na-buffer and 100 μM As was added. The error bars indicate the standard deviation from duplicate experiments.

In complex medium containing galactose (YPG), uptake was approximately 3-fold higher in cells expressing either the Fps1p or Hxt7p transporters relative to the control (Fig. 3). Interestingly, uptake was 20–50% higher when cells were cultivated in galactose as compared to glucose. Uptake in galactose medium is important for the studies combining overexpression of transporters and sequestors described below. Previous work evaluating arsenic accumulation in cells expressing phytochelatins was performed in galactose medium (Singh et al., 2008).

Prior results (Liu et al., 2004) demonstrated Hxt7p to be the dominant transporter of As. However, in our work, uptake was similar between the two transporters. This likely is due to differences in experimental conditions. In the present system, the genes are overexpressed, resulting in a greater number of transporters available on the cell surface. In addition, As uptake was evaluated with a resting cell system (similar to final application conditions). In contrast, the prior studies focused on a comparison in the absence of overexpression and following induction with arsenic in exponential phase. Given the similarity we observed, our further experiments focused on only Fps1p.

3.2. Arsenite uptake at trace concentrations

A recent survey in Bangladesh showed that ground water concentrations of arsenic in a large fraction of tube-wells were above

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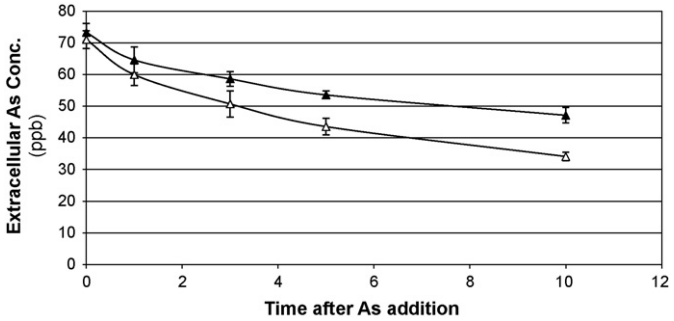

Fig. 4. Uptake of trace quantities of As in 15616 cells overexpressing the Fps1p transporter. Cells were grown in complex YPD medium for 36 h, concentrated and transferred to Na-buffer containing 75 ppb As. Extracellular arsenic concentrations were monitored over time. Open and closed symbols represent the strain harboring plasmid p2m-ADH2-FPS1 and YEplac195, respectively. The error bars indicate the standard deviation from triplicate experiments.

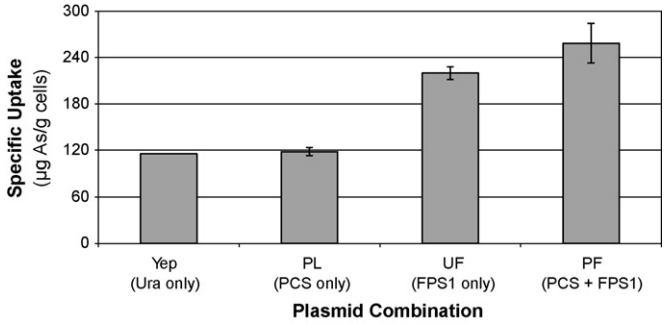

Fig. 5. Specific intracellular accumulation of arsenic in complex YPG medium. Strain 15616 was cultivated in batch culture medium for 36 h, transferred to Na-buffer and arsenic (100 μM) was added. Samples were collected at the 10 h time point. The error bars represent the standard deviation from duplicate experiments. Yep: plasmid YEplac195 only (negative control); PL: plasmids pYES-AtPCS1::FLAG and YEplac181 (chelator only); UF: plasmids p2m-ADH2-FPS1-Leu and YEplac195 (transporter only); PF: plasmids pYES-AtPCS1::FLAG and p2m-ADH2-FPS1-Leu.

the WHO mandated limit of 10 ppb and ranged from 50 to 100 ppb (Smith et al., 2000). Therefore, the ability of the engineered yeast to remove trace quantities of arsenic was evaluated. Strains 4742 and 15616 harboring plasmid p2m-ADH2-FPS1 or YEplac195 were cultivated in YPD for 36 h and transferred to Na-buffer at a final concentration of ca. 12 g cells/l (approximately 2-fold higher than stationary phase). Arsenite was then added to a final concentration of 1 μM (75 ppb). The extracellular As concentration was measured for 10 h following addition of As; the results are shown for strain 15616 in Fig. 4. Cells harboring the blank plasmid YEplac195 were able to reduce the extracellular As concentration to approximately 47 ppb in 10 h (37% As removed). Within the same time, cells overexpressing the Fps1p transporter reduced the extracellular metalloid concentration to approximately 35 ppb (53% As removed). As observed previously (Fig. 1), the uptake was comparable between the 4742 (wild-type) and 15616 (*Acr3*) strains harboring the same plasmid (data not shown). Increasing cell concentrations should further increase arsenite removal. In related work on the uptake of arsenate, increasing cell concentration resulted in higher volumetric uptake of arsenate while maintaining identical level of specific uptake per unit mass of cells (data not shown).

3.3. Arsenic uptake in cells co-expressing a transporter and sequestor

Enhanced arsenic accumulation has been reported in yeast cells expressing phytochelatins (Singh et al., 2008) and bacterial cells overexpressing the ArsRp protein (Kostal et al., 2004). Therefore, these two intracellular sequestors were synthesized to determine if this further increased arsenic accumulation in cells overexpressing the transporters.

Strain 15616 (Δ*ACR3*) was co-transformed with plasmids for overexpression of the Fps1p transporter (p2m-ADH2-FPS1-Leu) and for production of PCs or ArsRp (pYES-AtPCS1::FLAG or p2m-PGK-ArsR). The AtPCS1 and *arsR* genes were under the control of the native *PGK*-promoter to allow for production of the sequesters prior to exposure to arsenic. The *FPS1* gene remained under the control of the *ADH2*-promoter for maximum protein production at the end of batch growth. Controls included strain 15616 transformed with (a) plasmids p2m-ADH2-FPS1-Leu and YEplac195 (Fps1p transporter only); (b) plasmids pYES-AtPCS1::FLAG and YEplac181 (PC chelator only); (c) plasmids p2m-PGK-ArsR and YEplac181 (ArsR binding protein only); or (d) plasmid YEplac195 (negative control). For cells carrying AtPCS1, the batch culture medium contained zinc chloride for PCS activation during growth. The cells were then washed and transferred to buffer (Zn-free) prior to addition of the arsenite.

The combination of Fps1p and phytochelatin overexpression was evaluated after cultivation in complex YPG medium. Although it is non-selective, complex medium is optimum for expression from the *ADH2*-promoter (Lee and Da Silva, 2005). After 36 h of batch culture, cells were transferred to Na-buffer and sodium arsenite was added to a concentration of 100 μM. The results are shown in Fig. 5. Specific uptake in cells overexpressing the transporter increased approximately 2-fold over control cells expressing the blank plasmid YEplac195. Specific uptake did not increase in cells expressing phytochelatins only. However, specific uptake increased 2.4-fold in cells expressing the combination of Fps1p and PCs. The specific uptake in cells expressing both Fps1p and PCs was not significantly greater than for cells expressing the Fps1 transporter alone.

Plasmid stability was measured to determine if plasmid loss was responsible for the lack of improved uptake with expression of the PCS. The cells were cultivated for 36 h in non-selective YPG, transferred onto YPD plates and subsequently onto either selective SDC(A) or SD(His/Lys/Ura) plates to select for the FPS1 or AtPCS plasmid, respectively. For the control strain containing plasmid YEplac195, the percentage of plasmid-containing cells was approximately 82%. This represents the basal plasmid stability under conditions when no selection pressure is applied and no additional proteins are expressed. For plasmids p2m-ADH2-FPS1or pYES-AtPCS1::FLAG, 59% and 49% of the cells retained the plasmid, respectively (Table 2). The increase in arsenic uptake and the plasmid stability value observed for *FPS1* are consistent with those seen previously in the absence of the chelator. Lower stability of the AtPCS plasmid relative to the *FPS1* plasmid is expected as the *PGK*-promoter is induced from the beginning of batch growth. However, plasmid stability alone is clearly not responsible for the lack of improved uptake after introduction of AtPCS1. This is likely due to the conditions for this experiment. While a 6-fold

Table 2
Plasmid stability after 36 h in complex YPG medium (Fps1p transporter, PC chelator).

Strain	Percent plasmid-containing cells		
	FPS1 plasmid	PCS plasmid	Both
FPS1/Blank[a]	59	n.a.	n.a.
PCS/Blank[b]	n.a.	49	n.a.
FPS1/PCS[c]	58	40	23

[a] Strain contains p2m-ADH2-FPS1-Leu and YEplac195.
[b] Strain contains pYES-AtPCS1::FLAG and YEplac181.
[c] Strain contains p2m-ADH2-FPS1-Leu and pYES-AtPCS1::FLAG.

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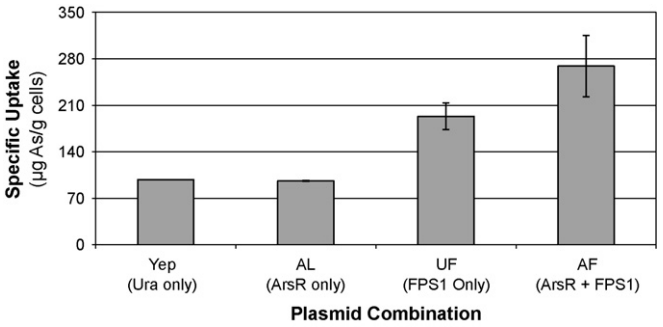

Fig. 6. Specific intracellular accumulation of arsenic in complex YPD medium. Strain 15616 was cultivated in batch culture medium for 36 h, transferred to Na-buffer and arsenic (100 μM) was added. Samples were collected at the 10 h time point. The error bars indicate the standard deviation from triplicate experiments. Yep: plasmid YEplac195 only (negative control); AL: plasmids p2m-PGK-ArsR and YEplac181 (binding protein only); UF: plasmids p2m-ADH2-FPS1-Leu and YEplac195 (transporter only); AF: plasmids p2m-PGK-ArsR and p2m-ADH2-FPS1-Leu.

increase in As uptake was previously seen (Singh et al., 2008), this was under exponential growth conditions, not in buffer following 36 h of cell mass accumulation. It is possible that under the current conditions, transport is the limiting process and increased chelation has little effect on As accumulation. The low percentage of cells containing both the *FPS1* and AtPCS1 plasmids (23%; Table 2) may limit any synergistic effects of the two in combination.

The effect of combining Fps1p overexpression and production of ArsRp was evaluated in the same manner. The results shown in Fig. 6 are similar to those for the Fps1p/PC combination (Fig. 5). In cells overexpressing *FPS1* only, specific uptake increased 2-fold over control cells harboring the blank plasmid YEplac195. Specific uptake did not increase in cells expressing the *arsR* gene under these cultivation conditions. However, cells expressing the dual cassette accumulated 2.5-fold more arsenic as compared to control cells. The plasmid stability results for these strains after cultivation in YPD medium for 36 h are shown in Table 3. The results are comparable to those observed with the Fps1p-AtPCSp combination above and do not explain the lack of effect of sequestor introduction alone. A synergistic improvement in uptake with both transporter and sequestor is more apparent for the *arsR/FPS1* combination (Fig. 6 relative to Fig. 5), and a larger percentage of cells (35%; Table 3) contains both plasmids. The results for this system also suggest a possible transport limitation during As uptake in a resting cell system.

Culturing cells containing two unique 2μ based plasmids (in addition to the native 2μ plasmid) presents a number of challenges. The variation in relative copy number and stability of the two plasmids can result in differing As uptake from one experiment to the next. This is further complicated by the need to use a complex, non-selective medium for the complete derepression of the late-phase *ADH2*-promoter. Strategies addressing these limitations should further increase As accumulation.

Table 3
Plasmid stability after 36 h in complex YPD medium (Fps1p transporter, ArsRp binding protein).

Strain	Percent plasmid-containing cells		
	FPS1 plasmid	ArsR plasmid	Both
FPS1/Blank[a]	40	n.a.	n.a.
ArsR/Blank[b]	n.a.	48	n.a.
FPS1/ArsR[c]	66	49	35

[a] Strain contains p2m-ADH2-FPS1-Leu and YEplac195.
[b] Strain contains p2m-PGK-ArsR and YEplac181.
[c] Strain contains p2m-ADH2-FPS1-Leu and p2m-PGK-ArsR.

4. Conclusions

In this study, enhanced arsenic accumulation was observed in cells overexpressing the As transporters Fps1p and Hxt7p under the control of the *S. cerevisiae ADH2*-promoter. At arsenite concentrations of 20 and 100 μM, cells overexpressing the transporters were capable of 3–4-fold greater As accumulation as compared to control cells. At trace concentrations of the metalloid (75 ppb), the engineered cells were able to remove 40% more As within the same time period.

This work demonstrates the advantage of transporter overexpression (with and without sequestor overexpression) for the uptake of arsenite. The next step is to optimize the uptake and sequestration process by balancing the expression of the transporters and PCs or ArsRp via gene integration and promoter choice. Similar strategies are currently being evaluated for increased uptake of pentavalent arsenic into the engineered yeast cells.

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References

Berhe, A., Fristedt, U., Persson, B.L., 1995. Expression and purification of the high-affinity phosphate transporter of *Saccharomyces cerevisiae*. Eur. J. Biochem. 227, 566–572.
Bobrowicz, P., Wysocki, R., Owsianik, G., Goffeau, A., Ulaszewski, S., 1997. Isolation of three contiguous genes, *ACR1*, *ACR2* and *ACR3*, involved in resistance to arsenic compounds in the yeast *Saccharomyces cerevisiae*. Yeast 13, 819–828.
Canovas, D., Duran, C., Rodriguez, N., Amils, R., de Lorenzo, V., 2003. Testing the limits of biological tolerance to arsenic in a fungus isolated from the River Tinto. Environ. Microbiol. 5, 133–138.
Carlin, A., Shi, W., Dey, S., Rosen, B.P., 1995. The *ars* operon of *Escherichia coli* confers arsenical and antimonial resistance. J. Bacteriol. 144 (4), 981–986.
Chwirka, J.D., Thomson, B.M., Stomp, J.M., 2000. Removing arsenic from groundwater. J. Am. Water Works Assoc. 92, 79–88.
Clifford, D., Subramonion, S., Sorg, T., 1986. Removing dissolved inorganic contaminants from water. Environ. Sci. Technol. 20, 1072–1080.
Driehaus, W., Seith, R., Jekel, M., 1995. Oxidation of arsenic(III) with manganese oxides in water treatment. Water Res. 29, 297–305.
EPA, 2006. Environmental Protection Agency. http://www.epa.gov/safewater/arsenic/index.html (accessed December 29th, 2006).
Gancedo, J., 1998. Yeast carbon catabolite repression. Microbiol. Mol. Biol. Rev. 62, 334–361.
Gekeler, W., Grill, E., Winnacker, E.L., Zenk, M.H., 1989. Survey of the plant kingdom for the ability to bind heavy-metals through phytochelatins. Z. Naturforsch. C: Biosci. 44, 361–369.
Ghosh, M., Shen, J., Rosen, B.P., 1999. Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. 96, 5001–5006.
Gietz, R.D., Sugino, A., 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74, 527–534.
Grill, E., Winnacker, E.L., Zenk, M.H., 1985. Phytochelatins—the principal heavy-metal complexing peptides of higher-plants. Science 230, 674–676.
Karagas, M.R., Tosteson, T.D., Blum, J., Morris, J.S., Baron, J.A., 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, 1047–1050.
Kostal, J., Yang, R., Wu, C.H., Mulchandani, A., Chen, W., 2004. Enhanced arsenic accumulation in engineered bacterial cells expressing ArsR. Appl. Environ. Microbiol. 70, 4582–4587.
Lee, K.M., Da Silva, N.A., 2005. Evaluation of the *Saccharomyces cerevisiae ADH2* promoter for protein synthesis. Yeast 22, 431–440.
Liu, S.X., Athar, M., Lippai, I., Waldren, C., Hei, T.K., 2001. Induction of oxyradicals by arsenic: implication for mechanism of genotoxicity. Proc. Natl. Acad. Sci. U.S.A. 98, 1643–1648.
Liu, Z.J., Boles, E., Rosen, B.P., 2004. Arsenic trioxide uptake by hexose permeases in *Saccharomyces cerevisiae*. J. Biol. Chem. 279, 17312–17318.
Ma, L.Q., Komar, K.M., Tu, C., Zhang, W.H., Cai, Y., Kennelley, E.D., 2001. A fern that hyperaccumulates arsenic. Nature 409, 579–1579.
Macy, J.M., Nunan, K., Hagen, K.D., Dixon, D.R., Harbour, P.J., Cahill, M., Sly, L.I., 1996. *Chrysiogenes arsenatis* gen. nov., sp. nov., a new arsenate respiring bacterium isolated from gold mine wastewater. Int. J. Syst. Bacteriol. 46, 1153–1157.
Mehra, R.K., Tarbet, E.B., Gray, W.R., Winge, D.R., 1988. Metal-specific synthesis of two metallothioneins and γ-glutamyl peptides in *Candida glabrata*. Proc. Natl. Acad. Sci. U.S.A. 85, 8815–8819.

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Mejare, M., Bulow, L., 2001. Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals. Trends Biotechnol. 19, 67–73.
Mukhopadhyay, R., Shi, J., Rosen, B.P., 2000. Purification and characterization of Acr2p, the *Saccharomyces cerevisiae* arsenate reductase. J. Biol. Chem. 275, 21149–21157.
National Research Council, 1977. Arsenic: Medical and Biologic Effects of Environmental Pollutants. National Academy of Sciences, Washington, DC.
Nriagu, J.O., 1994. Arsenic in the environment. Part II: human health and ecosystem effects. In: Nriagu, J.O. (Ed.), Advances in Environmental Science and Technology. John Wiley and Sons, Inc., New Jersey.
Pearce, F., 2003. Arsenic's fatal legacy grows. New Sci., 2407.
Romanos, M.A., Scorer, C.A., Clare, J.J., 1992. Foreign gene-expression in yeast—a review. Yeast 8, 423–488.
Rosen, B.P., 2002. Biochemistry of arsenic detoxification. FEBS Lett. 529, 86–92.
Say, R., Yilmaz, N., Denizli, A., 2003. Biosorption of cadmium, lead, mercury, and arsenic ions by the fungus *Penicillium purpurogenum*. Separ. Sci. Technol. 38 (9), 2039–2053.
Schmoger, M.E.V., Oven, M., Grill, E., 2000. Detoxification of arsenic by phytochelatins in plants. Plant Physiol. 122, 793–801.
Scott, D.L., Ramanathan, S., Shi, W.P., Rosen, B.P., Daunert, S., 1997. Genetically engineered bacteria: electrochemical sensing systems for antimonite and arsenite. Anal. Chem. 69, 16–20.
Singh, S., Lee, W., Da Silva, N.A., Mulchandani, A., Chen, W., 2008. Enhanced arsenic accumulation by engineered yeast cells expressing *Arabidopsis thaliana* phytochelatin synthase. Biotechnol. Bioeng. 99, 333–340.
Smith, A.H., Lingas, E.O., Rahman, M., 2000. Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. Bull. World Health Organ. 78 (9), 1093–1103.
Tamas, M.J., Wysocki, R., 2001. Mechanisms involved in metalloid transport and tolerance acquisition. Curr. Genet. 40, 2–12.
Vatamaniuk, O.K., Bucher, E.A., Ward, J.T., Rea, P.A., 2002. Worms take the 'phyto' out of 'phytochelatins'. Trends Biotechnol. 20, 61–64.
Wilkie, J.A., Hering, J.G., 1998. Rapid oxidation of geothermal arsenic(III) in streamwaters of the eastern Sierra Nevada. Environ. Sci. Technol. 32, 657–662.
Wysocki, R., Chery, C.C., Wawrzycka, D., Van Hulle, M., Cornelis, R., Thevelein, J.M., Tamas, M.J., 2001. The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*. Mol. Microbiol. 40, 1391–1401.