Enhanced Arsenate Uptake in *Saccharomyces cerevisiae* Overexpressing the Pho84 Phosphate Transporter

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Arsenate is a major toxic constituent in arsenic-contaminated water supplies. Saccharomyces cerevisiae *was engineered as a potential biosorbent for enhanced arsenate accumulation. The phosphate transporter, Pho84p, known to import arsenate, was overexpressed using a 2μ-based vector carrying* PHO84 *under the control of the late-phase* ADH2 *promoter. Arsenate uptake was then evaluated using a resting cell system. In buffer solutions containing high arsenate concentrations (12,000 and 30,000 ppb), the engineered strains internalized up to 750 μg of arsenate per gram of cells, a 50% improvement over control strains. Increasing the cell mass 2.5-fold yielded a proportional increase in the volumetric arsenate uptake, while maintaining the same level of specific uptake. At high levels of arsenate, loss from the intact cells to the medium was observed with time; knockouts of two known arsenic extrusion genes,* ACR3 *and* FPS1*, did not prevent this loss. At trace level concentrations (120 ppb), rapid and total arsenate removal was observed. The presence of 50 μM phosphate reduced uptake by approximately 15% in buffer containing 80 μM (6,000 ppb) arsenate. At trace levels of arsenate (70 ppb), the phosphate reduced the initial rate of uptake, but not the total amount removed.* PHO84 *mRNA levels were nearly 30 times higher in the engineered strains relative to the control strains. Uptake may no longer be a limiting factor in the engineered system and further increases should be possible by upregulating the downstream reduction and sequestration pathways.* © 2012 American Institute of Chemical Engineers *Biotechnol. Prog.,* 28: 654–661, 2012
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Introduction

Drinking water supplies contaminated with arsenic are a major health risk to greater than 60 million people worldwide, as chronic exposure to toxic levels of arsenic causes a number of human diseases including cancer and organ failure.[1,2] To mitigate the health risks, both the World Health Organization and the US Environmental Protection Agency have defined 10 parts per billion of arsenic in water as the safe limit.[2,3] Arsenic in contaminated water supplies comes from both natural sources, such as leaching of bedrock deposits, and anthropogenic activities, e.g., runoff from mining, application of arsenic containing wood preservatives, and pesticides, and primarily exists in one of two oxide forms: arsenite [As(III)] and arsenate [As(V)].[4–7] The arsenite [As(III)] species is considered to be more oxidizing and toxic, and disrupts synthesis of proteins and DNA. The toxicity of arsenate [As(V)] stems from its interference in cellular phosphate metabolism, and due to its reduction to arsenite [As(III)] once inside the cell.[8–10]

Novel technologies based on engineered microorganisms have been proposed for arsenic remediation. It is known that arsenic enters many plant and microbial cells via a number of membrane transporters and the cells have evolved mechanisms to expel or sequester internalized toxic arsenic compounds.[11–13] One proposed approach is to engineer these pathways to create a microbial "arsenic biosorbent" that is capable of removing high levels of arsenic from its environment by internalizing and storing high levels of arsenic. Several studies using engineered *Escherichia coli* and *Saccharomyces cerevisiae* strains overexpressing native or heterologous sequestration and transport proteins specifically targeting arsenite [As(III)] have shown heightened internalization of this species of arsenic oxide.[14–18]

Arsenate [As(V)] is one of the two major species in arsenic contaminated water supplies and often represents more than 50% of the total arsenic depending on location and

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water chemistry,[6,9] e.g., arsenate levels in groundwater supplies in various areas of Bangladesh are found in the range of 20–1,100 ppb.[9] Inhibition of phosphate metabolism by arsenate and its reduction to the toxic arsenite species by intracellular reducing agents are both harmful to human health. Therefore, novel microbial tools effective in removing arsenate can offer significant benefits.

Native pathways in *S. cerevisiae* have been elucidated to show the mechanisms by which arsenate gains entry into the yeast cells and is subsequently neutralized and removed for detoxification (Figure 1).[19–22] The pathway suggests arsenate enters the intracellular matrix via membrane embedded phosphate transporters because of its chemical similarity to phosphate. The internalized arsenate is subsequently reduced to arsenite by Acr2 reductase to curtail its displacement of phosphate in vital energy production. Arsenite is then extruded via membrane embedded Acr3 transporters or packaged into the vacuole by Ycf1 proteins to prevent arsenite toxicity. Therefore, a *S. cerevisiae* based "arsenate biosorbent" can be engineered to maximize the internalization of arsenate from an aqueous environment through enhancing arsenate uptake and minimizing or eliminating extrusion.

The yeast high affinity phosphate transporter Pho84p has been shown to co-transport phosphate and arsenate into the cell interior.[21,23,24] In the current study, a novel resting yeast cell system with overexpression of Pho84p was constructed and evaluated for enhanced uptake of arsenate. *S. cerevisiae* strains, with and without deletion of the *ACR3* extrusion gene, were grown to stationary phase to accumulate cell mass and to overexpress Pho84p under the control of the strong late-phase *ADH2* promoter.[25,26] The resting cells were tested in aqueous buffer solutions containing various concentrations of arsenate to quantify levels of specific and volumetric uptake in the engineered yeast cells. Further studies evaluated the effects of cell density and the presence of phosphate (a competitor for transport) on arsenate uptake. Real-time PCR was used to assess *PHO84* transcription levels in both the wild type and engineered strains.

Materials and Methods

Strains and vectors

S. cerevisiae strains BY4742 (MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and BY15616 (MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 acr3::kan*) (Open Biosystems, Huntsville, AL) were used for *PHO84* expression. The latter strain is derived from BY4742 and has a deletion in the gene for the Acr3p arsenic extrusion protein. *E. coli* strain XL1 Blue (Stratagene, La Jolla, CA) was used for plasmid maintenance and amplification.

YEp*lac*195 is a 2μ-based vector with a *URA3* selection marker.[27] Plasmid pKOS12-122C carrying the *ADH2* promoter and *ADH2* terminator cassette was a gift from Kosan Biosciences (Hayward, CA). The *PHO84* gene was PCR amplified from the BY4742 *S. cerevisiae* genome using primers: PHO84F (5′-GAAGTCGACCCAAATGAGTTCCGT CAATAAAGATAC-3″) and PHO84R (5′-GCATCC TAGGGGCTTTTATGCTTCATGTTGAAGTTGAGATG-3″). The *PHO84* PCR product was inserted into *Sal*I and *Avr*II sites between the *ADH2* promoter and terminator on pKOS122-122C. The P_{ADH2}-*PHO84*-T_{ADH2} cassette was then excised and inserted between *Bam*HI and *Hind*III sites on pYEp*lac*195 forming plasmid p2μ-PHO84. BY4742 and BY15616 were transformed with either YEp*lac*195 (p2μ) or p2μ-PHO84 to create four strains: two control strains (BY4742 and BY15616 each with p2μ) and two engineered strains (BY4742 and BY15616 each with p2μ-PHO84).

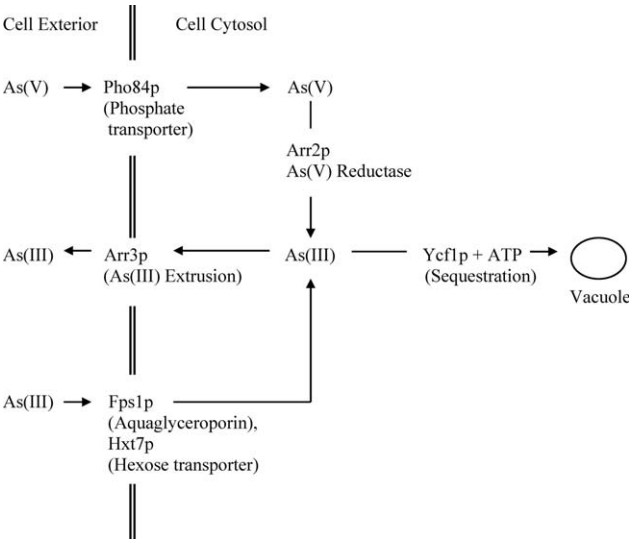

Figure 1. Arsenic uptake and processing pathways in *S. cerevisiae*.

Vector constructions were performed following standard protocols.[28] The TSS method[29] was used for *E. coli* transformation. Yeast transformations were carried out using a lithium acetate protocol.[30]

For *FPS1* gene deletion in BY15616, a knockout cassette containing the *LEU2-d8* marker was generated by nested PCR using the pXP222 plasmid[31] as template and the following primers: FPS1KOfwd (5′-CCAAGTACGCTCGA GGGTACATTCTAATGCATTAAAAGACGCATGCCTGCA GGTCGACTC-3′), FPS1KOrev (5′-CTATCAGTCTATATT ATTTGTTTCTTTTTCTTGTCTGTTTTCGAATTCGAGCTC GGTACCCGGG-3′), FPS1NESTEDKOfwd (5′-GTCCCAAT AAGCGTCGGTTGTTCTTCTTTATTATTTTACCAAGTAC GCTCGAGGGTAC-3′), and FPS1NESTEDKOrev (5′-ACC GGCGGTAGTAAGCAGTATTTTTTTCTATCAGTCTATAT TATTT G-3′). Strain BY15616 was transformed with the PCR-generated knockout cassette and plated onto leucine-deficient SD plates. Deletion of the *FPS1* open reading frame (ORF) occurred by double crossover recombination of the 70-mer homologous sequences on the knockout cassette and those flanking the *FPS1* ORF. Replacement of the *FPS1* ORF was verified using primers: FPS1KOCheckFwd(5′-TGT GAATCCGGAGACGGCAAGATT-3′) and FPS1KOCheck-Rev (5′-TAGGT GACCAGGC TGAGTTCATGT-3′).

Media

E. coli XL1 Blue was grown in Luria-Bertani (LB) medium,[28] supplemented with 0.1 mg/mL of ampicillin for selection of plasmid-containing strains. YPD media (1% dextrose, 1% Bacto yeast extract, and 2% Bacto peptone) and SDC media (1% dextrose, 0.67% yeast nitrogen base, 0.5% Bacto casamino acid, 20 mg/L L-tryptophan, and 100 mg/L adenine) were used for cultivating yeast strains. YPD and SDC agar plates contained 2% Bacto agar.

Arsenate uptake and measurement

For the arsenic uptake studies, *S. cerevisiae* strains were inoculated into 3 mL of SDC media from frozen glycerol

stocks and cultured for 18 h in a 30°C air shaker (New Brunswick Scientific, Model G-25) at 250 rpm. The cultures were used to inoculate 20 mL of YPD (1% glucose) at an initial optical density $(OD)_{600}$ of 0.02, and placed in a 30°C water bath shaker (New Brunswick Scientific, Model G76D) at 250 rpm. At 36 h, the cells were centrifuged and resuspended two times with 20 mL of sterile 25 mM Tris-succinate buffer, pH 4.5, and then diluted to OD_{600} 5.0–6.0 with buffer. Sterile arsenate standard (Fisher Scientific) was added to 20 mL of the cell/buffer mixture. To study the effect of phosphate on arsenate uptake, 25 mM Tris-succinate buffer was supplemented with 50 μM of K_2PO_4. For arsenate uptake at high cell density, cells were grown in several flasks in YPD (1% glucose) to stationary phase, centrifuged, and resuspended with 20 mL Tris-succinate buffer in a single flask resulting in an OD_{600} of 19.0–21.0. Cell OD_{600} were measured using a Pharmacia Novaspec II spectrophotometer, with OD_{600} of 1.0 equating to a dry cell weight (DCW) of approximately 0.6 g/L.

At various time points, 1 mL samples were taken and centrifuged, and the supernatant was stored separately. The cell pellets were washed twice with fresh 25 mM Tris-succinate buffer and then dried at 65°C for 2 days. Dried cell pellets were then digested with 100 μL of concentrated nitric acid for 2 days followed by addition of ddH_2O to reconstitute to a 1 mL volume (modified from Singh et al., 2008). Cell pellets from culture flasks without arsenate addition were similarly treated with nitric acid and reconstituted with water. This arsenic free "cell debris" solution was used in mixing arsenic standards for the atomic adsorption spectroscopy (AAS) analysis and for dilution of samples with high arsenic readings to provide a consistent background for all samples. Total elemental arsenic in the cell pellet (intracellular) and supernatant (medium) samples was measured using AAS with a graphite furnace (Perkin Elmer, Waltham, MA) at 193.7 nm wavelength.

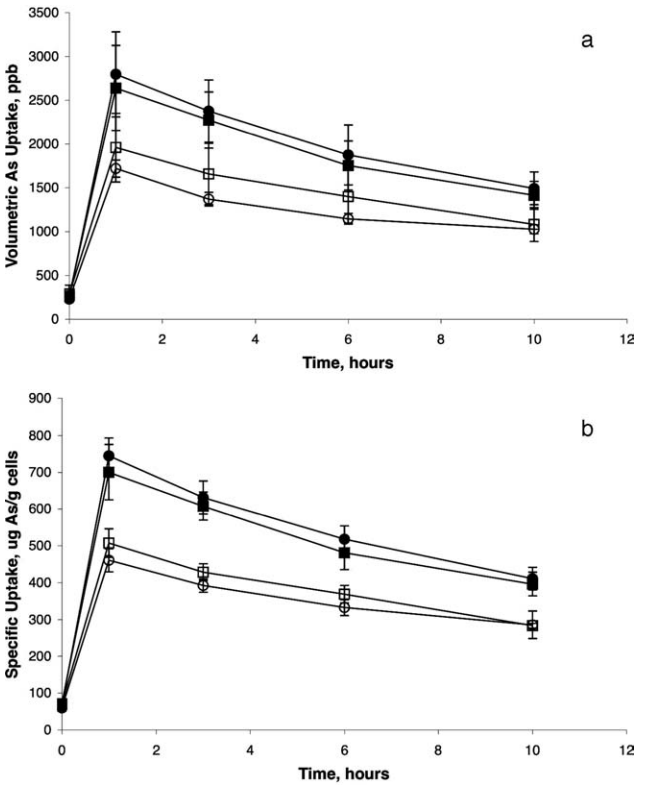

Figure 2. Arsenate uptake in buffer containing 12,000 ppb of As(V).

Cells were grown in YPD for 36 h to stationary phase, and transferred to Tris-succinate buffer with arsenate added to the specified concentration. (a) Volumetric arsenate uptake and (b) specific arsenate uptake by strains: 4742:p2μ (□), 15616:p2μ (○), 4742:p2μ-PHO84 (■), and 15616:p2μ-PHO84 (●). 4742:p2μ and 151616:p2μ are the controls. 15616 strains have the *ACR3* deletion. Data are mean values and standard deviations of triplicate experiments.

Plasmid stability

The segregational stability of the yeast plasmid was evaluated using replica plating. The 36-h yeast culture was diluted with sterile ddH_2O and plated onto YPD plates. Two hundred colonies were transferred to SDC plates (lacking uracil) and YPD plates (to verify viability). The percent plasmid containing cells was determined as the number of colonies on the SDC plates divided by the number of viable colonies transferred.

PHO84 *expression measurement using real-time PCR*

The yeast mRNA was isolated from cells using the Yeastar RNA Kit (Zymo Research, Orange, CA). The mRNA isolate was treated with the TURBO DNA-free Kit (Applied Biosystems/Ambion, Foster City, CA) to remove residual DNA. The cDNA was generated from mRNA using a High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The cDNA concentration for all samples was measured using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE) and diluted to identical concentrations using ddH_2O. Real-time PCR was performed using the iQ SYBR Green Kit (Biorad, Hercules, CA) on an Eppendorf RT-PCR cyler (Realplex4 Mastercycler), with primers: PHO84RTF (5′-TTACCGAAGGTGGTAACATGG CCT-3′) and PHO84RTR (5′-TTGTTGCCAACCGAAAC CTTCGTC-3′). Real-time PCR of the *S. cerevisiae* actin (*ACT1*) gene cDNA was used as the internal control for normalizing baseline expression levels of all samples. The actin primers used were: ActinFwd (5′-ATTCTGAGGTTGCT GCTTTGG-3′) and ActinRev (5′-TGTCTTGGTCTACC GACGATA-3′). Real-time PCR was conducted with the following conditions: 5 min at 95°C; 40 cycles of 20 s at 95°C, 15 s at 55°C, and 20 s at 72°C.

Results

Uptake at high extracellular arsenate concentration

The control strains (BY4742:p2μ and BY15616:p2μ) and engineered strains (BY4742:p2μ-PHO84 and BY15616:p2μ-PHO84) were each cultured in complex YPD media for 36 h to stationary phase. Aliquots were then harvested, washed, and resuspended in 20 mL of buffer to obtain cell densities of approximately 3.0–3.6 g/L (OD600 of 5.0–6.0). Arsenate was added to 12,000 ppb and samples were taken immediately after arsenate addition and at 1, 3, 6, and 10 h. Analysis of the intracellular samples showed rapid uptake by all strains; an average of 250 ppb of arsenate was removed immediately after the addition of arsenate (Figure 2a). After 1 h, the control strains removed between 1,700 and 1,900 ppb of arsenate, while the engineered strains overexpressing *PHO84* removed 2,600 to 2,800 ppb. Specific arsenate uptake (Figure 2b) was calculated by dividing volumetric arsenate uptake by the biomass concentration; this provides a

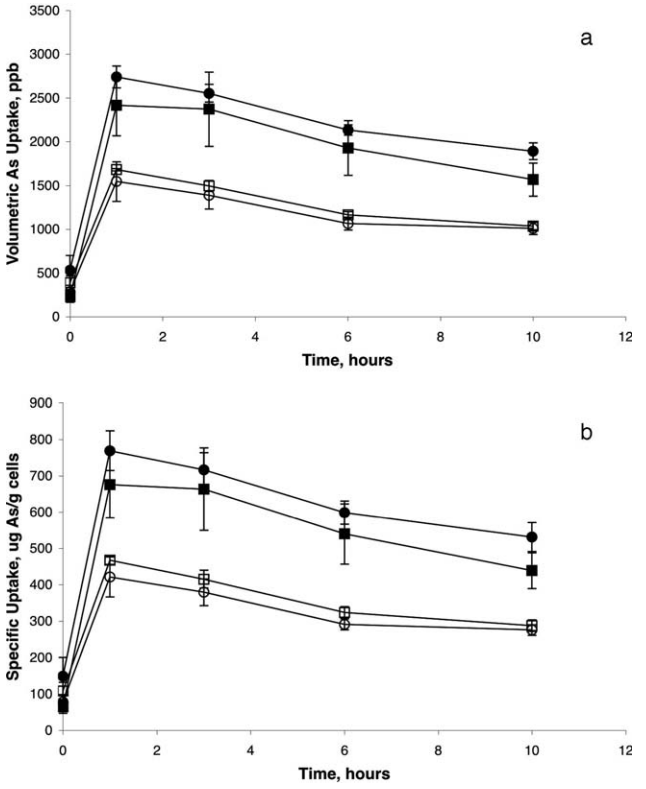

Figure 3. Arsenate uptake in buffer containing 30,000 ppb of As(V).

Cells were grown in YPD for 36 h to stationary phase, and transferred to Tris-succinate buffer with arsenate added to the specified concentration. (a) Volumetric arsenate uptake and (b) specific arsenate uptake by strains: 4742:p2μ (□), 15616:p2μ (○), 4742:p2μ-PHO84 (■), and 15616:p2μ-PHO84 (●). 4742:p2μ and 151616:p2μ are the controls. Data are mean values and standard deviations of duplicate experiments.

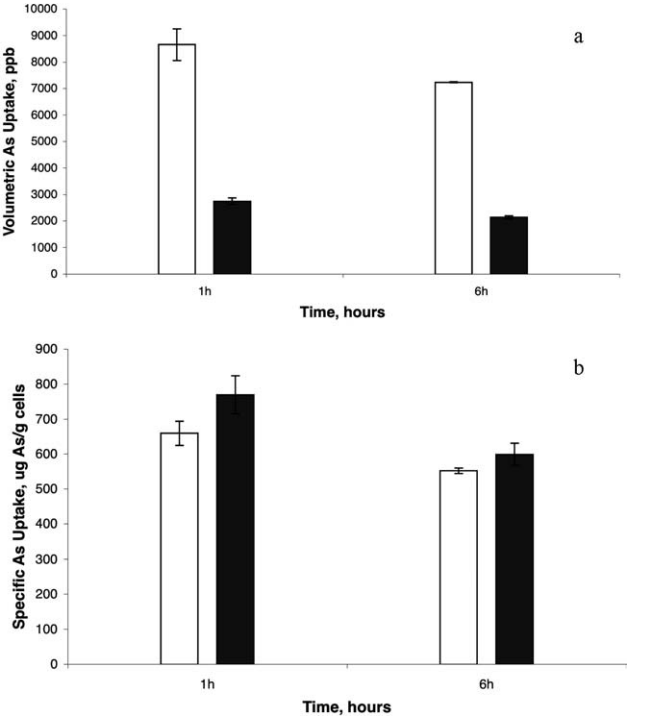

Figure 4. Comparison of arsenate uptake at high and low cell density.

BY15616:p2μ-PHO84 strain was cultured in YPD for 36 h to stationary phase and transferred to buffer containing 30,000 ppb of As(V). (a) Volumetric arsenate uptake and (b) specific arsenate uptake at high cell density (11.4–12.6 g/L) and low cell density (3.0–3.6 g/L) are shown with white bars and black bars, respectively. Data are average values and standard deviations of duplicate experiments.

more accurate comparison as it corrects for any variation in cell mass. Specific arsenate uptake levels for the control strains BY4742:p2μ and BY15616:p2μ were 507.1($\pm$44.6) μg(As)/g cells (DCW) and 460.3($\pm$53.9) μg(As)/g cells, respectively. Specific uptake levels for the engineered strains BY4742:p2μ-PHO84 and BY15616:p2μ-PHO84 were 699.8($\pm$99.0) μg(As)/g cells and 744.5($\pm$63.5) μg(As)/g cells. Therefore, overexpression of the *PHO84* gene resulted in the removal of up to 60% more arsenate on a per cell basis.

The results in Figure 2 show that the BY15616 strains (*ACR3* deletion) had comparable levels of arsenic accumulation as the BY4742 strains (intact *ACR3*). Acr3p is known to extrude internalized arsenite (Figure 1). However, prior studies indicate that transcription of *ACR3* is only induced in the presence of the arsenite [As(III)] metalloid.[32] No arsenite or arsenate was present during the batch culture of the yeast strains; arsenate was only added to the resting cells in the non-nutrient buffer mixture. Therefore, Acr3p should not have been expressed in either BY4742 or BY15616 leading to similar arsenic accumulation for the two strains, as observed.

To determine whether a maximum level of arsenate uptake was reached with the engineered yeast strains, experiments were conducted using 30,000 ppb of extracellular arsenate. The volumetric and specific arsenate uptake levels observed at 30,000 ppb were similar to those obtained for 12,000 ppb of arsenate (Figures 3a,b). The higher arsenate concentration in the solution did not result in increased arsenate uptake. Because we obtained comparable levels of arsenate internalization from experiments conducted with arsenate concentrations of 12,000 and 30,000 ppb, a maximum level of arsenate internalization has likely been reached in the resting yeast cell system.

As an additional verification, we conducted uptake experiments with an approximately 3.5-fold higher concentration of yeast in the buffer solution. Strain BY15616:p2μ-PHO84 was grown to stationary phase in several flasks and concentrated into a single flask to obtain a cell mass of 11.4–12.6 g/L. Arsenate was added to the high-density cell/buffer solutions to a concentration of 30,000 ppb. Samples were taken 1 h and 6 h after As(V) addition. The volumetric arsenate uptake for the higher cell density was on average 3.2 times higher than that measured for the lower cell density (Figure 4a). Therefore, increasing the cell mass resulted in a nearly proportional increase in volumetric uptake of arsenate. The specific uptake at the higher cell density was reduced by approximately 13% relative to the lower cell density (Figure 4b); this is not a significant deviation from the range of specific uptakes values observed for this strain. These results further validate our conclusion that a maximum level of uptake per cell had been reached in our resting cell system.

Uptake at low extracellular arsenate concentration

Arsenic contamination in water supplies often exceeds the safe limit of 10 ppb defined by the World Health

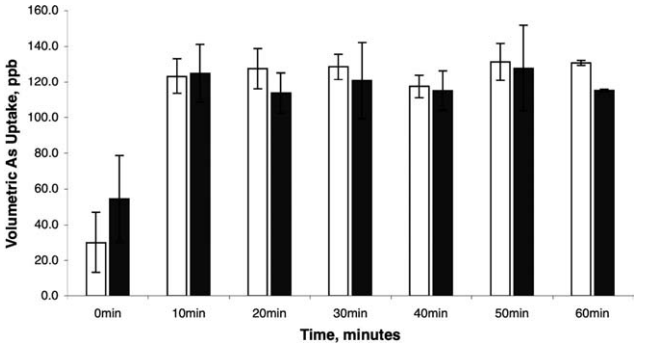

Figure 5. Volumetric arsenate uptake in buffer containing 120 ppb of As(V).

Cells were grown in YPD for 36 h to stationary phase and transferred to buffer with arsenate added to 120 ppb. Volumetric uptake for control BY15616:p2μ (white bars) and BY15616:p2μ-PHO84 (black bars) are shown. Data are the average values and standard deviations of duplicate experiments.

Organization.[4,33] Therefore, a *S. cerevisiae* based biosorbent should remove arsenate to below this threshold to be successful. To determine whether our engineered yeast strain is capable of removing arsenate to below the prescribed limit, BY15616:p2μ (control) or BY15616:p2μ-PHO84 (engineered) resting cells were suspended in buffer solutions containing 120 ppb of arsenate. Samples were taken at 10-min intervals over a 1-h period. AAS analysis of the intracellular samples showed that on average 30–55 ppb of arsenate were removed by the resting yeast cells immediately after arsenate addition (Figure 5). After 10 min, the control and engineered yeast strains had internalized all of the arsenate initially present in the buffer. To verify that the yeast cells had removed all extracellular arsenate, supernatant samples were measured and showed no detectable arsenate after 10 min. The strains were thus able to remove low levels of extracellular arsenate to concentrations below the 10 ppb limit defined by the World Health Organization. The very rapid rate of internalization of the extracellular arsenate demonstrates that *S. cerevisiae* cells have high affinity toward arsenate. This is desirable in a *S. cerevisiae*-based arsenic biosorbent.

Arsenate uptake in the presence of phosphate

Arsenate enters the yeast cell via the yeast Pho84 phosphate transporter because of its chemical similarity to phosphate. The arsenate uptake experiments described above were conducted using buffer solutions without phosphate. However, groundwater may contain an average of 30–50 μM of phosphate.[34,35] Therefore, it is essential to evaluate the effect that the presence of phosphate has on arsenate uptake, as both compounds compete for the phosphate transporter. The engineered strain BY15616:p2μ-PHO84 was cultivated for 36 h in YPD and was then resuspended in either Tris-succinate buffer without phosphate or Tris-succinate buffer with 50 μM of phosphate. Arsenate was added to the resting cell suspensions to a concentration of 80 μM (6,000 ppb). Samples were taken immediately following arsenate addition, and then at 2 h and at 4 h. Specific arsenate uptake in buffer solution without phosphate was measured at 567.1($\pm$52.9) μg(As)/g cell, while in the presence of 50 μM phosphate the arsenate uptake averaged 490.3($\pm$ 28.8) μg(As)/g cell, approximately 15% lower (Figure 6). The phosphate thus had a minor impact on the arsenate uptake capabilities of the engineered yeast in the current system.

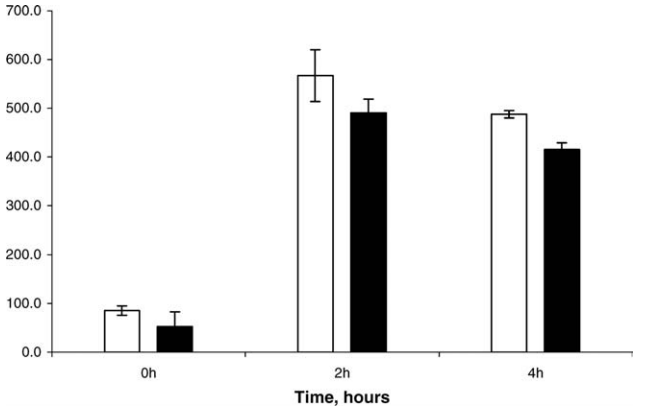

Figure 6. Comparison of specific arsenate uptake in buffer with and without phosphate.

BY15616:p2μ-PHO84 was grown in YPD for 36 h to stationary phase and transferred to buffer with or without 50 μM of phosphate. Arsenate was added to 80 μM (6,000 ppb). Specific arsenate uptakes in buffer without phosphate (white bars) and with phosphate (black bars) are shown. Data are the average values and standard deviations of duplicate experiments.

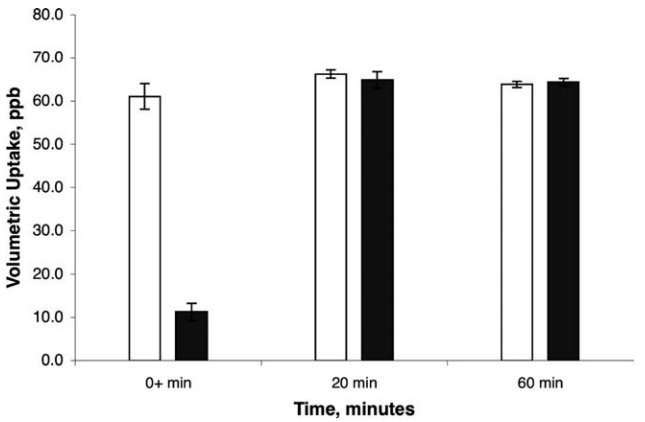

Figure 7. Volumetric arsenate uptake in buffer containing 70 ppb of As(V) with or without 50 μM of phosphate.

BY15616:p2μ-PHO84 was grown in YPD for 36 h to stationary phase and transferred to buffer prepared with or without 50 μM of phosphate. Arsenate was added to 70 ppb. Volumetric arsenate uptakes in buffer without phosphate (white bars) and with phosphate (black bars) are shown. Data are the average values and standard deviations of triplicate experiments.

The uptake of trace levels of arsenate was also evaluated in the presence of phosphate. In this study, BY15616:p2μ-PHO84 was placed in buffer solutions containing 70 ppb of arsenate, with or without 50 μM of phosphate. Samples were taken immediately after arsenate addition, and after 20 and 60 min. The immediate uptake was 6-fold lower in the phosphate-containing buffer compared to the phosphate-free buffer (Figure 7). However, within 20 min, the uptake was the same with or without phosphate and nearly complete removal of arsenate was observed. Therefore, 50 μM of phosphate inhibited the initial rate of arsenate uptake at trace concentrations but not the total amount removed. Similar results were observed with the BY15656:p2μ control strain. In previous studies, *S. cerevisiae* was shown to have a higher affinity and transport kinetics for phosphate relative to

Table 1. Plasmid Stability After 36 h of Cultivation in YPD

Strain	Percent plasmid-containing cells
4742 2μ (control)	98
4742 2μ-PHO84	54
15616 2μ (control)	90
15616 2μ-PHO84	63

arsenate and selenite in nutrient medium.[36,37] Therefore, the slight delay in arsenate uptake observed in our experiments may indicate the preferential initial uptake of phosphate.

Plasmid stability

2μ-Based plasmids are generally assumed to be maintained at 10–40 copies per cell in *S. cerevisiae*.[38] However, both copy number and segregational stability can vary depending on the gene product and expression level. In the current studies, the late-phase *ADH2* promoter was used for high-level synthesis of the Pho84 transporter prior to transfer to the buffer, and yeast cells were grown in a complex YPD media to maximize *ADH2* promoter induction.[26]

To determine the percentage of cells that retained the plasmid vector, plasmid stabilities were measured after 36 h of growth for the engineered and control strains. Plasmid stabilities were found to be 54% and 63% for BY4742:p2μ-PHO84 and BY15616:p2μ-PHO84, respectively; in comparison, both control strains (BY4742:p2μ and BY15616:p2μ) had plasmid stabilities of over 90% (Table 1). Therefore, the observed increases in arsenate uptake by the engineered strains were achieved with only 54–63% of the cell population retaining the *PHO84* expression cassette. While plasmid stability of the engineered strains may be improved in selective media, the induction from the late-phase *ADH2* promoter would be much lower.[26]

Determination of PHO84 *expression level with real-time PCR*

Real-time PCR was used to determine the transcription levels of the *PHO84* gene in both the control and engineered strains when grown to stationary phase. The ratio between *PHO84* mRNA and *ACT1* mRNA levels in each yeast strain was determined to gauge gene expression level. For the control strain BY4742:p2μ, the ratio of *PHO84* mRNA to *ACT1* mRNA was 0.030($\pm$0.005). In comparison, the engineered strain BY4742:p2μ-PHO84 had a *PHO84* mRNA to *ACT1* mRNA ratio of 0.868($\pm$0.057), 29 times higher than that measured in the control strain. Similarly, *PHO84* and *ACT1* mRNA ratios in the engineered strain BY15616:p2μ-PHO84 was 1.041($\pm$0.106), 28 times higher than the mRNA ratio of 0.036($\pm$0.001) measured in the control strain BY15616:p2μ. Given the plasmid stabilities for BY4742:p2μ-PHO84 and BY15616:p2μ-PHO84 (Table 1), the difference in mRNA levels between the engineered and control strains were even greater.

The real-time PCR data showed that the late-phase *ADH2* promoter provided very high levels of *PHO48* transcripts relative to the control strains. Although the level of transporter protein and insertion into the membrane were not directly quantified, the great excess of *PHO84* transcript indicates that Pho84p overexpression was responsible for the observed increases in arsenate uptake in the engineered strains (Figures 2 and 3). Based on these results, we hypothesize that arsenate transport may no longer be limiting arsenate removal in the engineered strains.

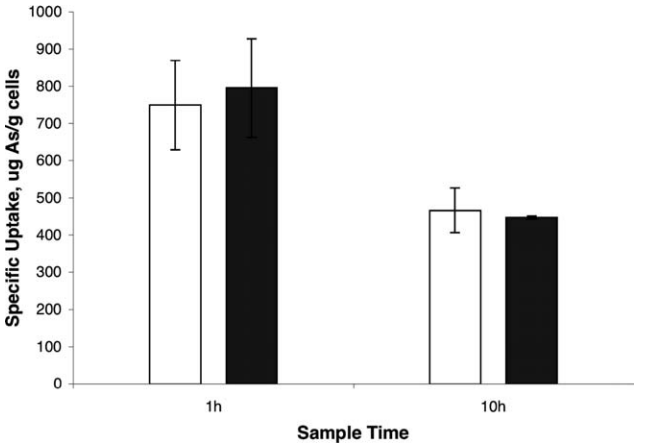

Figure 8. Comparison of specific arsenate uptake for BY15616 and BY15616Δ*FPS1* strains with Pho84 overexpression.

Cells were grown in YPD for 36 h to stationary phase and transferred to buffer with 7,000 ppb of arsenate. Specific arsenate uptake by BY15616:p2μ-PHO84 (white bars) and BY15616Δ*FPS1*:p2μ-PHO84 (black bars) are shown. Data are the average values and standard deviations of duplicate experiments.

Leakage of intracellular arsenate

In our studies at high arsenate concentrations, gradual decreases in intracellular arsenic level were observed over the course of the experiments (Figures 2 and 3). Similar results were seen for both BY4742 and BY15616 (*ACR3* deletion); therefore, the difference was not due to Acr3p-mediated extrusion.

To determine whether prolonged immersion in buffer with high levels of arsenate [As(V)] may have compromised cell health and integrity of the cell membranes, resulting in outward diffusion of internalized arsenic, cells from the 0-h and 10-h samples were treated with trypan blue and observed with the microscope. Yeast cells heated at 60°C for 20 min were also treated with trypan blue for comparison of cell membrane integrity. Microscope images of heat-treated yeast cells indicated entry of trypan blue into the cell interior through porous cell membranes damaged by the heat treatment. In comparison, the yeast cells exposed to arsenate showed essentially no entry of trypan blue. This implied that the cell membrane integrity was not compromised and likely not the reason for arsenic loss from the cells.

A recently published study[39] has concluded that the aquaglyceroporin Fps1p in the *S. cerevisiae* membrane is a bidirectional channel for the flow of arsenite. This diffusion of arsenite is believed to be concentration gradient driven. Following arsenate uptake, the reduction of the internalized arsenate to arsenite by Acr2p (Figure 1) creates a high intracellular arsenite concentration, which could drive the outward diffusion. To determine whether Fps1p is responsible for the observed leakage of internalized arsenic, we knocked out *FPS1* in BY15616 and transformed the strain with the p2μ-PHO84 plasmid. Arsenate uptake from buffer containing 7,000 ppb arsenate was then compared for BY15616 and BY15616Δ*FPS1* both overexpressing *PHO84*. Similar levels of uptake were observed. In addition, intracellular arsenic levels decreased between 1 h and 10 h for both BY15616 and the *FPS1* deletion strain (Figure 8). Therefore, an unidentified mechanism (possibly another bidirectional

transporter) must contribute to the export of the intracellular arsenic.

Discussion

Innovative microbial-based arsenic treatment technologies are potentially affordable alternatives to conventional physical filtration methods, and can be very beneficial in reducing arsenicosis in areas with arsenic contaminated water supplies. Encouraging results have been obtained in the removal of arsenite [As(III)], including the engineering of *E. coli* and *S. cerevisiae* for overexpression of arsenite transport and sequestration proteins.[15–18] However, given the prevalence of arsenate [As(V)], which can constitute more than 50% of total arsenic in contaminated water,[4] its disruption of phosphate metabolism, and its ready intracellular conversion to the more toxic As(III),[8] removal of this arsenic species is also essential. *S. cerevisiae* growth and cellular responses in nutrient media supplemented with arsenate and arsenite have been well characterized.[19–21,39] However, only limited investigations have quantified the level of arsenate uptake in growing cultures of *S. cerevisiae* or *E. coli*,[14,36] and the engineering of *S. cerevisiae* specifically for arsenate remediation has not been reported. In addition, arsenate removal from nutrient-deficient aqueous solutions is critical for application to groundwater. The current studies have addressed this by using a resting yeast cell system.

The *S. cerevisiae* Pho84 protein is a high affinity phosphate transporter that has been shown to cotransport arsenate; yeast strains lacking Pho84p expression demonstrate resistance to arsenate toxicity.[23] While it has been established that Pho84p transports arsenate, the capacity and rate at which resting yeast cells take up arsenate from aqueous solution has not been previously reported. Our studies have demonstrated that resting yeast cells are remarkably efficient at removing arsenate. In buffer containing 120 ppb of arsenate, both control and engineered strains were able to internalize all of the arsenate within the first 10 min, removing As(V) to levels below 10 ppb in the buffer solution. This is in clear contrast to the uptake of arsenite [As(III)]. In our studies on overexpression of the yeast arsenite transporters Fps1p and Hxt7p,[18] As(III) levels dropped from an initial 75 ppb to only 47 ppb after 10 h. Therefore, the As(V) transporter in *S. cerevisiae* is significantly more efficient relative to the As(III) transporters, resulting in considerably faster arsenic internalization. Competition for the transporters by phosphate was also found to have no overall detrimental effect on the level of arsenate uptake. Considering the arsenate uptake efficiency, it may be advantageous to convert (oxidize) the As(III) species to As(V) in water during pretreatment to maximize the use of the arsenate transporters in an engineered yeast biosorbent.

In addition to exhibiting a rapid rate of arsenate uptake, the yeast cells also displayed a high capacity for arsenate, with increased removal by the engineered strains relative to the control strains at high arsenate concentration. Furthermore, when a 3.5-fold higher cell concentration was used, the total volumetric As(V) increased proportionally while the specific uptake per cell remained the same. Therefore, the engineered yeast allows for rapid and high level uptake of arsenate, with immobilization of a larger density of engineered yeast cells translating to increased arsenic removal.

The real-time PCR measurements of *PHO84* gene expression in the engineered strains were found to be nearly 30 times higher than that of the control strains. Therefore, the transporter levels are likely no longer limiting and further arsenate removal may depend on upregulation of the downstream steps (Figure 1). The limit of arsenate uptake in the current engineered strains may be the result of significant buildup and toxicity of the internalized arsenate affecting other cell components or functions. Therefore, upregulation of arsenate reduction (via Acr2p) and subsequent sequestration for effective entrapment (e.g., overexpression of *Arabidopsis thalianas* phytochelatin[17]) may be essential to increase arsenate uptake, minimize the toxicity of free intracellular As(V) and As(III), and prevent leakage from the cells.

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Literature Cited

1. Ng JC, Wang J, Shraim A. A global health problem caused by arsenic from natural sources. *Chemosphere*. 2003;53:1353–1359.
2. World Health Organization Fact Sheet No. 210. *Arsenic in Drinking Water*. Geneva, 2001.
3. U.S. Environmental Protection Agency. *The Arsenic and Clarifications to Compliance and New Source Contaminants Monitoring Final Rule*. Washington DC, 2001.
4. Chatterjee A, Das D, Mandal BK, Chowdhury TR, Samanta G, Chakraborti D. Arsenic in ground water in six districts of West Bengal, India: the biggest arsenic calamity in the world. I. Arsenic species in drinking water and urine of the affected people. *Analyst*. 1995;120:643–650.
5. Mandal BK, Suzuki KT. Arsenic round the world: a review. *Talanta*. 2002;58:201–235.
6. Smedley PL, Kinniburgh DG. A review of the source, behavior and distribution of arsenic in natural waters. *Appl Geochem*. 2002;17:517–568.
7. Welch AH, Westjohn DB, Helsel DR, Wanty RB. Arsenic in ground water of the United States: occurrence and geochemistry. *Ground Water*. 2000;38:589–604.
8. Hughes MF. Arsenic toxicity and potential mechanism of action. *Toxicol Lett*. 2002;133:1–16.
9. Rossman TG. Mechanism of arsenic carcinogenesis, an integrated approach. *Mutat Res*. 2003;533:37–65.
10. Huang C, Ke Q, Costa M, Shi X. Molecular mechanisms of arsenic carcinogenesis. *Mol Cell Biochem*. 2004;255:57–66.
11. Mehra RK, Winge DR. Metal ion resistance in fungi—molecular mechanisms and their regulated expression. *J Cell Biochem*. 1991;45:30–40.
12. Zenk MH. Heavy metal detoxification in higher plants: a review. *Gene*. 1996;179:21–30.
13. Mukhopadhyay R, Rosen BP, Phung LT, Silver S. Microbial arsenic: from geocycles to genes and enzymes. *FEMS Microbiol Rev*. 2002;26:311–325.
14. Sauge-Merle S, Cuine S, Carrier P, Lecomte-Pradines C, Luu DT, Peltier G. Enhanced toxic metal accumulation in engineered bacterial cells expressing *Arabidopsis thaliana* phytochelatin synthase. *Appl Environ Microbiol*. 2003;69:490–494.
15. Kostal J, Yang R, Wu CH, Mulchandani A, Chen W. Enhanced Arsenic Accumulation in Engineered Bacterial Cells Expressing ArsR. *Appl Environ Microbiol*. 2004;70:4582–4587.
16. Singh S, Mulchandani A, Chen W. Highly selective and rapid arsenic removal by metabolically engineered *Escherichia coli* cells expressing *Fucus vesiculosus* metallothionein. *Appl Environ Microbiol*. 2008;74:2924–2927.
17. Singh S, Lee W, Da Silva NA, Mulchandani A, Chen W. Enhanced arsenic accumulation by engineered yeast cells expressing *Arabidopsis thaliana* phytochelatin synthase. *Biotechnol Bioeng*. 2008;99:333–340.

18. Shah D, Shen MWY, Chen W, Da Silva NA. Enhanced arsenic accumulation in *Saccharomyces cerevisiae* overexpressing transporters Fps1p or Hxt7p. *J Biotechnol*. 2010;150:101–107.
19. Bobrowicz P, Wysocki R, Owsianik G, Goffeau A, Ulaszewski S. Isolation of three contiguous genes, *ACR1*, *ACR2* and *ACR3*, involved in resistance to arsenic compounds in the yeast *Saccharyomyces cerevisiae*. *Yeast*. 1997;13:819–828.
20. Ghosh M, Shen J, Rosen BP. Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA*. 1999;96:5001–5006.
21. Mukhopadhyay R, Rosen BP. *Saccharomyces cerevisiae ACR2* gene encodes an arsenate reductase. *FEMS Microbiol Lett*. 1998;168:127–136.
22. Rosen BP. Biochemistry of arsenic detoxification. *FEBS Lett*. 2002;529:86–92.
23. Bunya M, Shikata K, Nakade S, Yompakdee C, Harashima S, Oshima Y. Two new genes, *PHO86* and *PHO87*, involved in inorganic phosphate uptake in *Saccharomyces cerevisiae*. *Curr Genet*. 1996;29:344–351.
24. Persson BL, Berhe A, Fristedt U, Martinez P, Pattison J, Petersson J, Weinander R. Phosphate permeases of *Saccharomyces cerevisiae*. *Biochim Biophys Acta*. 1998;1365:23–30.
25. Price V, Taylor W, Clevenger W, Worthington M, Young E. Expression of heterologous proteins in *Saccharomyces cerevisiae* using the *ADH2* promoter. *Methods Enzymol*. 1990;185: 308–318.
26. Lee KM, Da Silva NA. Evaluation of the *Saccharomyces cerevisiae ADH2* promoter for protein synthesis. *Yeast*. 2005;22: 431–440.
27. Gietz R, Sugino A. New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene*. 1988;74:527–534.
28. Sambrook J, Russell D. *Molecular Cloning: A Laboratory Manual*, 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001.
29. Chung CT, Niemela SL, Miller RH. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc Natl Acad Sci USA*. 1989;86:2172–2175.
30. Ito H, Fukuda Y, Murata K, Kimura A. Transformation of intact yeast cells treated with alkali cations. *J Bacteriol*. 1983;153: 163–168.
31. Fang F, Salmon K, Shen MWY, Aeling KA, Ito E, Irwin B, Tran UPC, Hatfield GW, Da Silva NA, Sandmeyer S. A vector set for systematic metabolic engineering in *Saccharomyces cerevisiae*. *Yeast*. 2011;28:123–136.
32. Wysocki R, Fortier PK, Maciaszczyk E, Thorsen M, Leduc A, Odhagen A, Owsianik G, Ulaszewski S, Ramotar D, Tamas M. Transcriptional activation of metalloid tolerance gene in *Saccharomyces cerevisiae* requires the AP-1-like proteins Yap1p and Yap8p. *Mol Biol Cell*. 2004;15:2049–2060.
33. Smith AH, Lingas EO, Rahman M. Contamination of drinking water by arsenic in Bangladesh: a public health emergency. *Bull World Health Organ*. 2001;78:1093–1103.
34. Duxbury JM. Remediation of arsenic for agriculture sustainability, food security and health in Bangladesh. *FAO Water*. 2007.
35. Hanrahan G, Salmassi TM, Khachikian CS, Foster KL. Reduced inorganic phosphorus in the natural environment: significance, speciation and determination. *Talanta*. 2005;66:435–444.
36. Jung C, Rothstein A. Arsenate uptake and release in relation to the inhibition of transport and glycolysis in yeast. *Biochem Pharmacol*. 1965;14:1093–1112.
37. Lazard M, Blanquet S, Fisicaro P, Labarraque G, Plateau P. Uptake of selenite by *Saccharomyces cerevisiae* involves the high and low affinity orthophosphate transporters. *J Biol Chem*. 2010;285:32029–32037.
38. Romanos MA, Scorer CA, Clare JJ. Foreign gene expression in yeast: a review. *Yeast*. 1992;8:423–488.
39. Maciaszczyk-Dziubinska E, Migdal I, Migocka M, Bocer T, Wysocki R. The yeast aquaglyceroporin Fps1p is a bidirectional arsenite channel. *FEBS Lett*. 2010;584:726–732.

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