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An Alternate Pathway of Arsenate Resistance in *E. coli* Mediated by the Glutathione S-Transferase GstB

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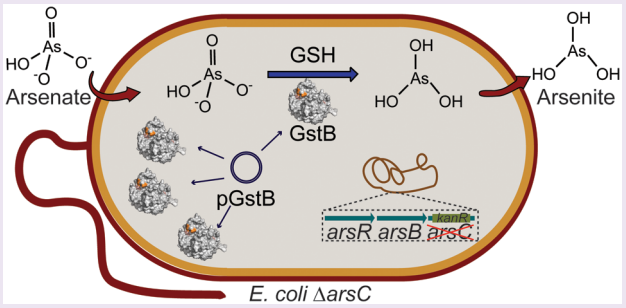
Supporting Information

ABSTRACT: Microbial arsenate resistance is known to be conferred by specialized oxidoreductase enzymes termed arsenate reductases. We carried out a genetic selection on media supplemented with sodium arsenate for multicopy genes that can confer growth to *E. coli* mutant cells lacking the gene for arsenate reductase (*E. coli* Δ*arsC*). We found that overexpression of glutathione S-transferase B (GstB) complemented the Δ*arsC* allele and conferred growth on media containing up to 5 mM sodium arsenate. Interestingly, unlike wild type *E. coli* arsenate reductase, arsenate resistance via GstB was not dependent on reducing equivalents provided by glutaredoxins or a catalytic cysteine residue. Instead, two arginine residues, which presumably coordinate the arsenate substrate within the electrophilic binding site of GstB, were found to be critical for transferase activity. We provide biochemical evidence that GstB acts to directly reduce arsenate to arsenite with reduced glutathione (GSH) as the electron donor. Our results reveal a pathway for the detoxification of arsenate in bacteria that hinges on a previously undescribed function of a bacterial glutathione S-transferase.



In nature, the toxic heavy metalloid, arsenic, exists predominately as oxyanions in either its pentavalent (arsenate, $H_2AsO_4^{1-}$) or trivalent oxidation states (arsenite, H_3AsO_3).[1] The natural abundance of both toxic species imposes a strong selection pressure for organisms to evolve arsenic detoxification pathways.[1,2] Detoxification of arsenite is achieved via sequestration or efflux from arsenite specific transporters.[3,4] Arsenate, on the other hand, is commonly exported only after reduction to arsenite.[1,5,6] This reductive step is catalyzed by a diverse class of enzymes termed arsenate reductases.[1]

Arsenate resistance seems to be a highly plastic process in which organisms can utilize a variety of pathways to perform the necessary reduction reaction. Five mechanistically and phylogenetically distinct classes of arsenate reductase enzymes have been identified so far.[1] While all families contain a catalytic redox-sensitive cysteine (Cys) residue, the specific mechanism of action can differ significantly between classes.[1] Arsenate reductases from Gram-positive bacteria accept electrons from thioredoxins, which are maintained in the reduced state by the NADPH-dependent enzyme thioredoxin reductase.[7] These enzymes display structural similarity to mammalian tyrosine phosphatase enzymes and exhibit phosphatase activity *in vitro*.[8,9] Conversely, arsenate reductases from Gram-negative bacteria, including *E. coli*, depend on the glutathione/glutaredoxin reductive pathway.[10,11] This class of reductases does not exhibit phosphatase activity.[12] The yeast *S. cerevisiae* protein, ACR2p, represents the third class of arsenate reductases. This eukaryotic class of enzymes is also dependent on reducing equivalents provided by glutaredoxin and glutathione,[13] but its active site is related to a different class of protein tyrosine phosphatases.[14] A fourth class has been identified in cyanobacteria, which express a glutaredoxin-dependent reductase enzyme with structural similarity to the thioredoxin class of reductases.[15] Finally, a fifth and unique class of reductive enzymes is part of anaerobic respiration in various Gram-positive and negative bacterial species, such as Gram-negative *C. arsenatis*, which utilize arsenate, rather than oxygen, as the terminal electron acceptor during respiration.[16]

In an earlier study, we used a genetic selection to identify alternate electron transfer pathways for the reduction of arsenate in *E. coli*. Selection for suppressor mutations capable of rescuing arsenate sensitivity in a glutathione (GSH) null *E. coli* mutant (Δ*gshA*) revealed an evolved pathway for glutathione synthesis via the *proAB* operon responsible for proline biosynthesis.[17] Suppressor mutations identified in the *proAB* genes restored electron transfer toward arsenate reduction by accumulating γ-glutamyl phosphate, which reacted spontaneously with L-cysteine to form γ-glutamyl cysteine, the reaction product for GshA.[17] These findings underscored the necessity for the glutaredoxin/glutathione system in providing

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reducing equivalents for arsenate reduction by the ArsC arsenate reductase.

Here, we asked whether *E. coli* encodes additional pathways capable of conferring resistance to lethal concentrations of arsenate. Using the ASKA library,[18−20] we selected for genes that, upon multicopy expression, allow for growth of arsenate reductase null mutants (*E. coli* Δ*arsC*) in media containing millimolar levels of arsenate. Surprisingly, we found that overexpression of the *gstB* gene, which encodes for the glutathione S-transferase GstB, conferred a high level of arsenate resistance. GstB was previously shown to be involved in bromoacetate resistance via a dehalogenation mechanism in which the conjugation of bromoacetate and glutathione results in the release of a free bromide and formation of glutathione S-acetate.[21] In this study, *in vitro* and *in vivo* biochemical analysis further revealed that, independently of glutaredoxins, GstB can directly facilitate the reduction of arsenate with only GSH as the reductant. While the beta class *E. coli* glutathione S-transferase enzyme (GST), encoded by the gene *gstA*, contains a catalytic cysteine residue essential for its reductive activity,[22] we found that neither of the cysteine residues found in *E. coli* GstB is required for arsenate resistance. Instead, arginine residues near the GSH binding site of GstB are critical for imparting resistance against both arsenate and bromoacetate. Thus, our results define an auxiliary mechanism for arsenate resistance in *E. coli*.

RESULTS AND DISCUSSION

GstB Confers Arsenate Resistance to *E. coli* in an ArsC Null Background. EQ217, an *arsC* null derivative of *E. coli* strain DH10B was created and found to be hypersensitive to arsenate with complete colony growth inhibition observed on MOPS minimal media agar plates containing 0.4 mM sodium arsenate (NaH_2AsO_4). EQ217 was then transformed with the ASKA library[18] and transformants were plated on MOPS minimal media agar plates with 0.4 mM sodium arsenate and 0.1 mM IPTG to induce protein expression from the ASKA plasmids. Several colonies of varied size arose after 2 days of incubation at 37 °C. Sequencing revealed that all large colonies selected from the screen contained ASKA plasmids bearing the *E. coli arsC* gene. The majority of the smaller colonies contained plasmids bearing the *gstB* gene, which encodes for the glutathione S-transferase enzyme GstB. Additionally, we found three resistant clones, which contained plasmids encoding for colonic acid biosynthesis protein, CpsB, and the transcription factors *dksA* and *fruR*. To confirm that arsenate resistance was conferred by the respective ASKA plasmids, plasmid DNA was isolated and retransformed into cells carrying the Δ*arsC* allele in a different genetic background (*E. coli* DHB4 Δ*arsC*, strain EQ279). Resistance phenotypes were again measured on MOPS plates containing sodium arsenate. Among the four genes identified in the screen, multicopy expression of *dksA* showed a mild increase in resistance to sodium arsenate as compared to the parent strain EQ279 (Supporting Information Figure 1). Only multicopy expression of *gstB* could confer growth on MOPS plates containing more than 1 mM sodium arsenate (Figure 1a). Further analysis showed that *E. coli* EQ279 cells transformed with the plasmid pGstB formed good size colonies on plates containing up to 3 mM sodium arsenate and even formed small colonies on plates containing 5 mM sodium arsenate (Figure 1b). Notably, neither ArsC nor GstB expression could mediate growth of the glutathione null strain WP758 (DHB4 Δ*gshA*) in media containing more than 0.1 mM sodium arsenate (data not shown). The inability to rescue growth in WP758 indicates that both arsenate resistance mechanisms are dependent upon the presence of intracellular glutathione.

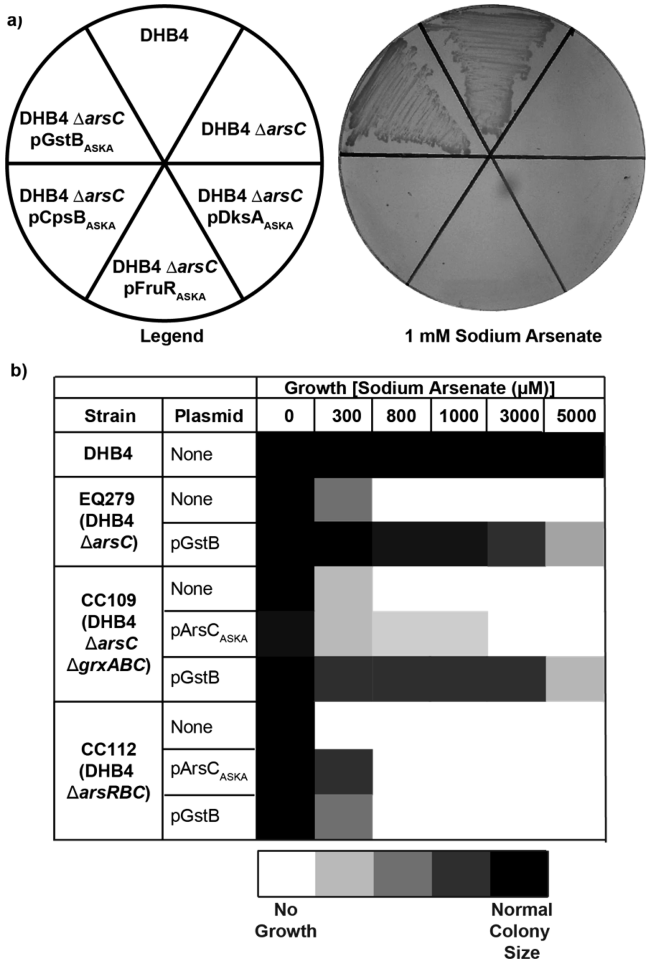

Figure 1. GstB overexpression confers arsenate resistance to *E. coli* Δ*arsC* cells. (a) Arsenate resistance of DHB4 Δ*arsC* cells expressing *gstB*, *cpsB*, *dksA*, and *fruR* selected from the genetic screen. Strains are plated on MOPS agar plates containing 1 mM sodium arsenate. (b) Growth of varied selected mutant strains expressing either the *gstB* construct (pGstB) or the ASKA *arsC* construct (pArsC$_{ASKA}$). Mutants were grown for 24 h on MOPS plates containing between 0 and 5 mM sodium arsenate. DHB4 Δ*arsC*Δ*grxABC* represents mutants lacking the *arsC* gene and the glutaredoxin genes *grxA*, *grxB*, and *grxC*; Δ*arsRBC* represents mutants lacking the *arsRBC* operon.

The catalytic cycle of ArsC is known to be dependent on glutaredoxins for reduction.[10] At concentrations above 0.1 mM sodium arsenate, the Δ*arsC* *E. coli* mutant, CC109, carrying three additional deletions of glutaredoxins A, B, and C, showed increased sensitivity to arsenate relative to the parental strain EQ279 (DHB4 Δ*arsC*) (Figure 1b). Transformation of mutant strain CC109 with a plasmid encoding ArsC could not restore healthy growth on plates containing low millimolar concentrations of arsenate. In contrast, CC109 cells transformed with a plasmid encoding GstB could grow in the presence of media containing of up to 3 mM sodium arsenate. Thus, unlike arsenate reductase, resistance to arsenate by GstB is not dependent on the expression of the *E. coli* glutaredoxins.

ArsC and ArsB together with the trans-acting repressor ArsR comprise the arsenate resistance operon *arsRBC*. ArsC reduces

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arsenate to arsenite, which is then exported from the cell by the ArsB transporter.[4] A DHB4 derived strain containing a complete deletion of the *arsRBC* operon, CC112, could not grow on plates containing more than 0.3 mM sodium arsenate. Moreover, arsenate sensitivity of CC112 could not be rescued by multicopy expression of ArsC or by GstB (Figure 1b). Considering that GstB can rescue bacterial growth of a Δ*arsC* mutant and that ArsR is a transcription factor responsible for regulating ArsC and ArsB transcription, the data from Figure 1b indicates that ArsB expression from the *arsRBC* operon is critical for arsenate detoxification by GstB.

GstB Residues Involved in Arsenate Reduction. A common feature in redox regulation is the presence of a CXXC motif in the catalytic site of redox sensitive proteins such as *E. coli* glutaredoxins and thioredoxins. The N-terminal and C-terminal cysteine residues within this motif reversibly form disulfide bonds and, depending upon the amino acids between the cysteine pair, are involved in a range of redox reactions.[23,24] *E. coli* GstB also contains the canonical thioredoxin-like CXXC motif comprising cysteine residues C134 and C137. To delineate a role of the cysteine residues in arsenate resistance, we constructed a variant of the *E. coli* GstB enzyme in which both Cys residues in the CXXC motif had been mutated to alanine. Cells expressing $GstB_{C134A/C137A}$ from the multicopy plasmid were fully resistant to arsenate and grew in the presence of 1 mM sodium arsenate with a doubling time of 200 ± 10 min compared to 205 ± 10 min for cells expressing wild type GstB (Figure 2 and Supporting Information Figure 2). The observed CXXC motif is not conserved across GstB homologues. *Salmonella enterica* encode for a GstB homologue, YliJ, which displays 83% amino acid identity with *E. coli* GstB but does not contain the N-terminal cysteine residue, C134. Moreover, the crystal structure of *S. enterica* YliJ (PDB 4KH7)[25] suggests that the conserved cysteine residue, C137, is too distant from both the GSH binding site and the putative electrophilic binding site (20−25 Å) to be directly involved in a catalytic mechanism involving glutathione (Figure 2a).

The YliJ crystal structure also indicated that three arginine (Arg) residues common to both GstB and YliJ form a patch of positive charge in close proximity (7 Å) to the glutathione binding site. Closer inspection of the crystal structure suggested that R7 may be directly involved in glutathione binding. Therefore, we chose to focus on the role of arginine residues, R111 and R119, which might be important for positioning anions such as arsenate in close proximity to the nucleophilic thiol of GSH. Substitution of either R111 or R119 for a glutamine residue in the *E. coli* GstB amino acid sequence impaired GstB conferred arsenate resistance. Notably, cells expressing the latter variant, $GstB_{R119Q}$, were most sensitive to sodium arsenate and could not grow in media containing more than 2 mM sodium arsenate (Supporting Information Figure 3). Substitution of both Arg residues from GstB, $GstB_{R111Q/R119Q}$, completely abolished the ability of GstB to confer arsenate resistance to *E. coli* EQ301 (DHB4 Δ*arsC*Δ*gstB*) cells (Figure 2 and Supporting Information Figure 2). To rule out the possibility that the R111Q/R119Q substitutions might have affected the expression or stability of the enzyme rather than having a direct effect on catalysis, we expressed and purified GstB and $GstB_{R111Q/R119Q}$ to near homogeneity. GstB and $GstB_{R111Q/R119Q}$ displayed identical elution on size exclusion chromatography demonstrating that these two mutations did not result in protein aggregation or denaturation (Supporting Information Figure 4). Differential

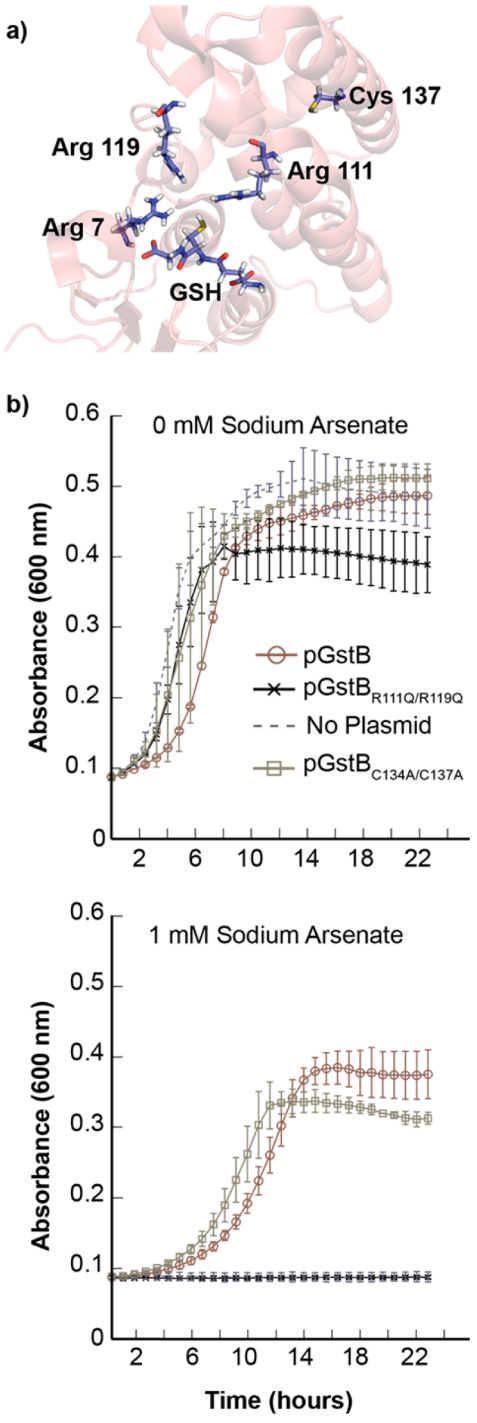

Figure 2. Mutational analysis of GstB residues involved in arsenate resistance. (a) Crystal structure of the *E. coli* GstB homologue, YliJ, from *Salmonella enterica* (PDB 4KH7). Three arginine residues (blue stick models), R7, R111, and R119, form the putative electrophilic binding site near glutathione (GSH). (b) Growth curves of *E. coli* EQ301 (DHB4 Δ*arsC*Δ*gstB*) expressing selected GstB variants from the multicopy pCA24N construct in liquid minimal MOPS media containing either 0 mM sodium arsenate (top panel) or 1 mM sodium arsenate (bottom panel). Growth curves are plotted as the average absorbance values from three independent growth studies. Error bars represent the standard deviation in absorbance at each time point.

scanning fluorometry also revealed that the $GstB_{R111Q/R119Q}$ mutant exhibits a slightly increased stability to thermal unfolding (47 ± 0.2 °C) relative to the wild type enzyme

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(44 ± 0.4 °C) (Supporting Information Figure 5). Substitution of residues R111 and R119 was shown not only to abolish the enzyme's ability to mediate resistance to arsenate but also its activity toward other electrophilic substrates. Earlier studies established that GstB can dehalogenate bromoacetate.[21] Unlike the wild type GstB, $GstB_{R111Q/R119Q}$ showed no activity with respect to glutathione S-acetate formation (Supporting Information Figure 6). This loss of activity indicates that the two Arg residues are involved in the reactivity of GstB toward electrophilic substrates.

GstB Catalyzes the Reduction of Arsenate to Arsenite. The finding that GstB does not confer arsenate resistance in *E. coli* cells lacking the ArsB arsenite transporter, suggests that GstB overexpression may facilitate arsenate reduction to arsenite. To further investigate this hypothesis, we used a two-step assay to detect the accumulation of arsenite in biological mixtures such as culture supernatants or cell lysates. First, arsenite is separated from arsenate in solution by anion exchange chromatography on a silica based resin (Phenomenex Strata SAX columns) at a pH above 3. Arsenate, which is negatively charged at pH > 2.2, stays bound to the column whereas neutral arsenite, not retained by the resin, elutes in the flow through. Arsenite collected from the flow through can be detected using a colorimetric assay in which all arsenic species are first reduced into arsine gas within an enclosed compartment. The formation of arsine gas reacts with mercuric bromide adsorbed onto strip paper and results in a mixed arsenic–mercury halogenide that discolors the test strip proportionally to the concentration of total arsenic in solution.[26] We first established that by using the combination of ion exchange chromatography together with colorimetric detection of arsenic eluted from the column flow-through, we could semi-quantitatively measure between 0.05 and 0.5 mM arsenite in 3–5 mL of growth media containing 50 mM sodium arsenate (Supporting Information Figure 7).

We used the method described above to detect secreted arsenite that had accumulated in the culture supernatants of cells incubated with 1 mM sodium arsenate. For the *in vivo* assay, sodium arsenate was added to the media 1 h after enzyme induction by 0.1 mM IPTG. The degree of arsenite accumulation in the supernatant was determined after a subsequent hour of incubation with arsenate (Figure 3). No arsenite was detected in the *E. coli* Δ*gshA* strain, WP758, which is incapable of synthesizing the reductant GSH. A very low level of arsenite was detected in the supernatant of *E. coli* Δ*arsC* (strain EQ279). The formation of arsenite in EQ279 cells, as opposed to the Δ*gshA* mutant strain, WP758, is most likely due to nonenzymatic reduction of arsenate by intracellular GSH.[27] This observation is consistent with the fact that EQ279 can grow on plates containing low concentrations of sodium arsenate (<0.4 mM) whereas the growth of Δ*gshA* cells (*E. coli* WP758) was inhibited even in the presence of 0.1 mM sodium arsenate.

While DHB4 cells accumulated low levels of arsenite in the culture media, induced expression of ArsC prior to the addition of sodium arsenate resulted in a much higher level of arsenite (Figure 3, Panel 4). This result suggests that the catalytic activity of arsenate reductase expressed by the chromosomal *arsC* gene is rate-limiting. Cells expressing GstB from the pGstB construct also accumulated high levels of arsenite indicating that enzyme plays a direct role in the reduction of arsenate (Figure 3, Panel 6). We note that overexpression of the better characterized *E. coli* glutathione S-transferase, Gst,

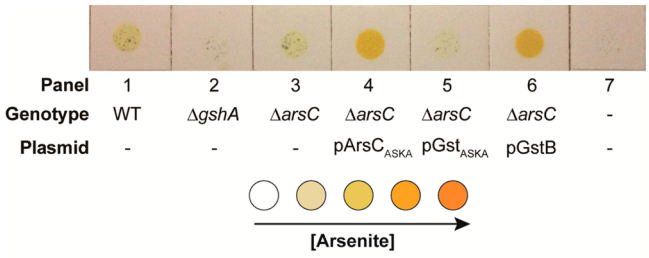

Figure 3. GstB overexpression results in the accumulation of arsenite in culture supernatant. Levels of arsenite accumulated in the supernatant of bacteria cultured in MOPS minimal media containing 1 mM sodium arsenate were measured using a two-step colorimetric assay. Exported arsenite was isolated from arsenate in the media using anion exchange. Separated arsenite was subsequently detected using a colorimetric assay with mercuric bromide. Panels 1–6 illustrate the levels of arsenite produced by selected *E. coli* mutant strains containing either no plasmid (−), the ASKA *arsC* construct ($pArsC_{ASKA}$), the *gstB* construct (pGstB), or the ASKA *gstA* construct ($pGst_{ASKA}$). Panel 7 represents the detection of arsenic in uninoculated growth media containing only 1 mM sodium arsenate.

did not result in significant arsenite accumulation or increased resistance of *E. coli* EQ279 to arsenate (Figure 3, Panel 5).

We next sought to evaluate whether GstB directly catalyzes the reduction of arsenate *in vitro*. First, purified GstB was incubated with 1 mM GSH and 50 mM sodium arsenate for either 30 or 60 min. The accumulation of arsenite was directly measured by the assay described above. As expected, incubation with wild type GstB resulted in a significant increase in the level of arsenite accumulation over the background rate due to the direct reaction of arsenate with GSH (Figure 4a). In addition, the rate of arsenate reduction by GstB was monitored by measuring the oxidation of NADPH in a redox coupled assay containing NADPH, glutathione reductase (GOR), GSH, and sodium arsenate.[11] With respect to wild type arsenate resistance, glutaredoxins are required to reduce the intermediate ArsC–arsenate–glutathione complex formed and thereby complete the redox cycle.[11] In contrast to the ArsC catalyzed mechanism, addition of purified Glutaredoxin 2 (Grx2) in the reaction above, did not appreciably increase *in vitro* GstB activity by a significant degree (Supporting Information Figure 8). Maintaining the concentration of sodium arsenate at 50 mM while varying the concentration of GSH from 0.1 to 5 mM showed that the rate of GSH oxidation increased linearly at a rate of 0.02 $(\text{min}*[\text{GSH mM}])^{-1}$. Similarly, varying the concentration of sodium arsenate from 20 to 100 mM in a reaction containing 1 mM GSH showed that NADPH oxidation increased linearly at a rate of 5.6×10^{-4} (min*[sodium arsenate mM]) (Supporting Information Figure 9). We found that, presumably due to high salt concentration, the reaction rate of arsenate reduction decreased when GstB was incubated with more than 100 mM sodium arsenate and 1 mM GSH. The maximum observed rate was 0.11 $(\text{min}*[\text{GSH mM}])^{-1}$ at 5 mM GSH and 50 mM arsenate with 0.12 mM GstB. Finally, the inactive variant $GstB_{R111Q/R119Q}$ showed no appreciable increase in arsenite concentration or NADPH oxidation as compared to the noncatalyzed reactions (Figure 4a and b).

DISCUSSION

Using a genetic selection to identify suppressors of arsenate sensitivity in *E. coli* Δ*arsC* cells, we identified a glutathione S-

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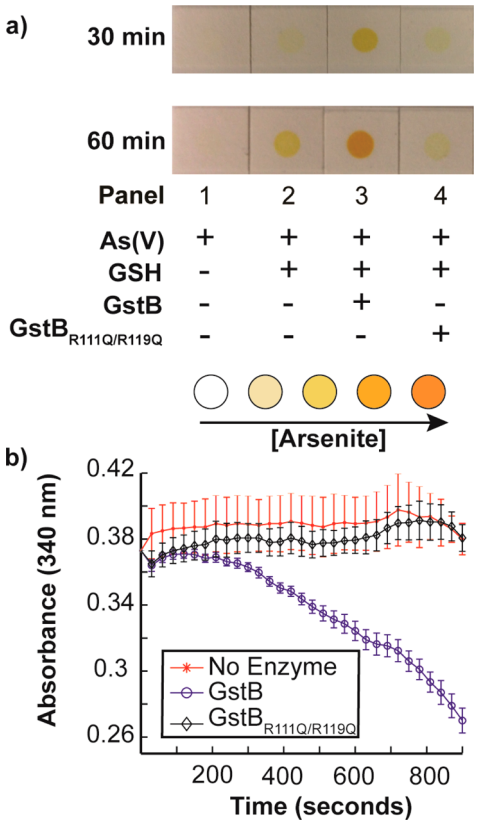

Figure 4. Reduction of arsenate to arsenite by GstB *in vitro*. GstB directed reduction of arsenate via GSH was measured using two separate *in vitro* assays. (a) Levels of arsenite were measured using the two-step colorimetric assay after a 30 or 60 min reaction. Reactions were performed using 50 mM sodium arsenate and 1 mM GSH. When indicated, active GstB or inactive $GstB_{R111Q/R119Q}$ was added to the reaction at a final concentration of 0.12 mM (Panels 3 and 4, respectively). (b) NADPH redox-coupled oxidation assays monitor the oxidation of NADPH at an absorbance of 340 nm. The plot shows the rate of NADPH oxidation in the presence of GOR, 50 mM sodium arsenate and 1 mM GSH. When indicated, active GstB and inactive $GstB_{R111Q/R119Q}$ were added to the reaction at a concentration of 0.12 mM. Error bars represent the standard deviation in absorbance across three replicate samples.

transferase, GstB, that confers high levels of arsenate resistance when overexpressed. Similar to the canonical pathway of arsenate resistance via the arsenate reductase, ArsC, we find that GstB conferred arsenate resistance is dependent upon the presence of the ArsB transporter, and that GstB overexpression facilitates the *in vivo* reduction of arsenate into arsenite. *In vitro*, our experiments provide direct evidence that GstB accelerates arsenate reduction in the presence of the electron donor, glutathione (GSH). However, in contrast to the enzymatic mechanism utilized by ArsC, GstB does not contain a catalytic cysteine residue and is not dependent on glutaredoxins for arsenate reduction. In nature, glutathione S-transferase enzymes, which catalyze the conjugation of glutathione to a broad range of electrophilic substrates, play an essential role in the detoxification of xenobiotics.[28] Recognition of a particular electrophilic substrate is highly dependent upon the residues found within its substrate binding domain (H-site).[29] In this study, we show that two arginine residues, R111 and R119, within the H-site are essential for arsenate detoxification.

Sequence homology analysis revealed that at least 30% of all genera from the *Enterobacteriaceae* family include species encoding for enzymes which share more than 50% sequence identity with GstB. Most importantly, the arginine residue, R119, is conserved in each homologue. To our knowledge GstB is the first bacterial GST found to confer resistance to high concentrations of arsenate in solution. In higher organisms, enzymes containing GST activity have been reported to also confer resistance to select arsenic species via reductive mechanisms distinct from that of GstB. Specifically, hGSTO1 or MMAV reductase, the human reductase responsible for monomethylarsenate (MMAV) detoxification, is a member of a unique class of glutathione S-transferases termed the omega class GST.[30,31] The omega class of GST enzymes displays close structural and phylogenetic relationships with glutaredoxins, a class of enzymes that perform a broad range of reductive functions. Specifically, hGSTO1 has been found to be highly similar in both function and tertiary structure to *E. coli* Grx2, a glutaredoxin found to be the most active with respect to catalyzing arsenate reduction in the ArsC mediated mechanism.[11,32] In addition to hGSTO1, the protozoan, *Leishmania major*, expresses a unique arsenate and monomethylarsenate reductase enzyme, TDR1, that contains two distinct glutathione S-transferase subdomains bearing high similarity to omega class GSTs.[33,34]

Comparison of the crystal structures of hGSTO1, TDR1, and the GstB homologue, YliJ, (PDB 1EEM, 4AGS, and 4KH7, respectively) reveal similarities in the substrate binding site (H-site) of the transferase domains. Specifically, the H-site of hGSTO1 was found to be relatively polar as compared to most GST enzymes.[31] The crystal structure of hGSTO1 (PDB 1EEM) indicates that two arginine residues R183 and R132 are within 10 Å of the cysteine thiol from bound glutathione. In addition to hGSTO1, both glutathione S-transferase subdomains of TDR1 contain two arginine residues and one histidine residue within 10 Å of the glutathione thiol. Similar to the two essential arginine residues within 7 Å of bound glutathione in the GstB homologue, YliJ, these basic residues may support a binding pocket for negatively charged species such as arsenate and monomethylarsenate. In contrast, analysis of the crystal structure of the beta class *E. coli* Gst (PDB 1NT2A),[35] which displays reductase activity but does not support arsenate reduction, reveals that the H-site contains only one basic residue, His 106. This residue is not responsible for substrate binding but instead activates the glutathione thiol via hydrogen bonding.[22]

One striking difference with respect to GstB is that both hGSTO1 and TDR1 contain a cysteine residue within the glutathione binding site (G-site).[31,34,36] This catalytic cysteine residue is capable of forming a disulfide bond with glutathione and is essential for conferring arsenate reductase activity in hGSTO1 and TDR1.[30,33] An overlay of the eukaryotic crystal structures with YliJ indicates that the GstB homologue contains a serine residue (Ser 10) in place of the cysteine residues found in hGSTO1 and TDR1. Serine residues have been shown to be the catalytic residue for transferase activity in both θ and ζ class GSTs.[36] In contrast to the eukaryotic GST enzymes, GstB appears to confer arsenate resistance by accelerating the rate of arsenate reduction via a ligand exchange reaction.

We propose two possible mechanisms that may account for the GSH-dependent reduction of arsenate by GstB (Scheme 1). In one schema, GstB could be serving to accelerate a rate-limiting step in which reduced glutathione is first conjugated to

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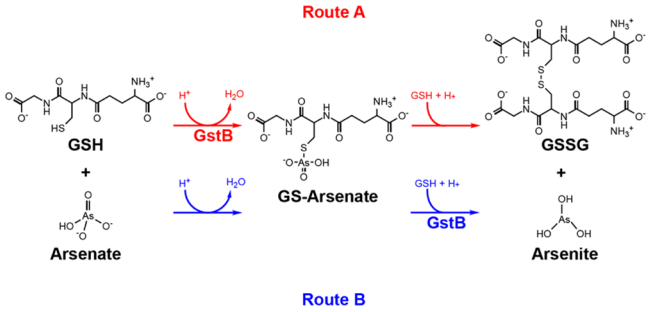
Scheme 1. Proposed Mechanisms of Arsenate Reduction by GstB[a]



[a]Route A. GstB directly enhances the rate of GS-arsenate conjugation. Once formed, the intermediate undergoes a spontaneous reaction with a second equivalent of GSH, which results in reduction to arsenite and oxidized GSH (GSSG). Route B. Conjugation of one reduced GSH molecule to arsenate occurs spontaneously in solution. The presence of GstB then catalyzes the conjugation of a second GSH molecule to arsenate and subsequent reduction of $(GS)_2$–arsenate intermediate into oxidized GSSG and arsenite.

arsenate to form an arsenate–glutathione intermediate that is then reduced by a second GSH molecule in close proximity or in solution. An alternate mechanism assumes that first, GSH spontaneously conjugates to arsenate in solution resulting in a GSH–arsenate complex. Then, GstB catalyzes the rate-limiting step whereby a second glutathione molecule is conjugated to the intermediate complex. We believe that the former mechanism is more plausible for two reasons: first, steric restrictions around the putative GSH binding site of GstB would hinder the ability of a preformed GSH–arsenate complex to interact with a second glutathione equivalent bound to GstB; second, a nonenzymatic arsenate–GSH complex could not be detected *in vitro* by NMR.[27] The inability to detect this intermediate implies that the formation of an arsenate–GSH complex is rate-limiting and that the reduction of arsenate following an interaction with a second GSH equivalent would be rapid. Therefore, it seems more plausible that GstB catalyzes the formation of an initial arsenate–GSH conjugate, which then encounters a second equivalent of GSH, resulting in the reduction of arsenate to arsenite. Collectively our results provide evidence for a mechanistically novel pathway for arsenate detoxification in bacteria involving the glutathione S-transferase enzyme, GstB.

METHODS

Reagents. Sodium arsenate hepta-hydrate (S9663-50G), sodium arsenite (S225I-100G), MOPS salts (M1254-250G), reduced nicotinamide adenine dinucleotide phosphate (NADPH N7505-25MG), and glutathione reductase (GOR) from Baker's Yeast (060M7405) were purchased from Sigma-Aldrich. Disposable Strata-SAX anion-exchange columns (8B-S008-HCH) were purchased from Phenomenex. Isopropyl β-D-1-thiogalactopyranoside (206-703-0), sodium chloride (S640-500), and imidazole (AC39674-5000) were purchased from Fisher Scientific. All reagents for arsenic detection (EZ Arsenic Test Kit Catalog #280000) including arsenic test strips, zinc sulfate, and phosphate were purchased from Hach Chemicals.

Bacterial Strains, Plasmids, and Media. Cells challenged for arsenate resistance were grown in MOPS minimal growth media, which contains minimal levels of phosphate.[37] MOPS growth media consisted of MOPS salts, 0.2% casein amino acids, 0.2% glucose, and 1.32 mM KH_2PO_4. When necessary, chloramphenicol and/or kanamycin were added to the growth media at a concentration of 33 μg mL^{-1} and 25 μg mL^{-1}, respectively. To induce gene expression from the pCA24N constructs (ASKA plasmids), IPTG was added to growth media at a concentration of 0.1 mM. For protein purification, cell strains were grown in 2xYT rich media containing 33 μg mL^{-1} of chloramphenicol. The details for all *E. coli* strains are described in Supporting Information Table 1. Briefly, the *arsC* gene from the *E. coli* DH10B derivative strain, Jude 1, and the *E. coli* DHB4 strain was replaced with a kanamycin resistance marker derived from plasmid pKD4 using the Wanner gene knockout method.[38] For each subsequent gene knockout, the kanamycin resistance marker was first removed using FRT recombinase encoded on the pCP20 plasmid.[38] The *E. coli* gene *gstB* was also knocked out of strain EQ279 yielding the mutant strain EQ301 (Δ*arsC*Δ*gstB*). Finally, DHB4 mutants containing knockouts of the individual glutaredoxin genes (*grxA*, *grxB*, and *grxC*) were constructed using P1 transduction[39] of the mutated alleles from donor strains obtained from the Keio Collection.[40] The *arsC* gene was also removed from the mutant strain DHB4Δ*grxA*Δ*grxB*Δ*grxC* (strain CC105), which yielded mutant strain CC109. The *arsRBC* operon was removed from *E. coli* DHB4 using the Wanner method (strain CC112).

The details for all *E. coli* constructs are described in Supporting Information Table 2. Genetic selection was performed using the library of ASKA clones where the GFP tag had been previously removed, leaving behind a C-terminal peptide scar sequence of GLCGR. After the *gstB* gene, encoded by the pCA24N ASKA construct ($pGstB_{ASKA}$), was identified from the genetic screen, the scar sequence was removed from the C-terminal end using quick change PCR (construct pGstB). Select point mutations to the CXXC motif in pGstB (C134A, C137A, and C134A/C137A) and to the putative electrophilic binding pocket (R111Q, R119Q, R111Q/R119Q) were also made using the quick change mutagenesis protocol. A g-block of the GstB homologue gene, *yliJ*, was ordered from Integrated DNA Technologies and was subsequently cloned into pCA24N using the Gibson cloning method (construct $pGstB_{YliJ\text{-}S.enterica}$).

Genetic Selection. The electrocompetent *E. coli* Δ*arsC* mutant strain, EQ217, was transformed with the ASKA library. Transformed cells were recovered in LB media containing 0.1 mM IPTG for 2 h. After recovery, cells were plated on MOPS agar plates containing chloramphenicol, kanamycin, 0.4 mM sodium arsenate, and 0.1 mM IPTG. Plates containing colonies were incubated for 2 days at 37 °C. After 2 days, colonies were selected and their corresponding pCA24N constructs from the ASKA library were isolated and sequenced.

Measurement of Arsenite in Solution. Solutions of arsenite were separated from arsenate using disposable Strata SAX anion exchange columns from Phenomenex. Arsenate, which is negatively charged at a pH > 2.2, binds to the positively charged column, whereas arsenite, which is uncharged at pH < 9.2, will not bind to the column. Therefore, when a solution containing a mixture of arsenate and arsenite is passed through the column, purified arsenite can be collected in the flow through. The following steps were performed to separate arsenite from arsenate using anion exchange. First, the SAX anion exchange columns were pretreated with 6 mL of methanol followed by 6 mL of 25 mM Tris-OAc buffer (pH 8). Next, 3–6 mL of sample was applied to the column and the flow through was collected. The levels of arsenite in the flow through can then be semiquantitated using the EZ arsenic detection kit provided by Hach chemicals. This kit reportedly detects levels of arsenic as low as 10 ppb in 50 mL water or 35 ppb in 10 mL water. Total arsenic is detected by the zinc catalyzed reduction of arsenite to arsine gas, which is then captured by mercuric bromide strips causing a color change. Strips that appear dark orange/black indicate levels of arsenic above 500 ppb in 50 mL water or 4000 ppb in 10 mL water.

Measurement of Arsenite Accumulation *In Vivo*. Cell strains grown overnight were subcultured at a dilution of 1:100 at 37 °C in MOPS media supplemented with appropriate antibiotics for strains containing kanamycin and/or chromosomal resistance markers and plasmids. When cell cultures reached an OD_{600} of 0.3, IPTG was added to the growth media to a final concentration of 0.1 mM and allowed to express protein until attaining an OD_{600} of 0.6–0.8 (approximately 1 h). After induction, 1 mM sodium arsenate (final concentration) was added to each strain and growth was continued for

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1 h. After 1 h of growth, all cell strains were normalized to the same OD, and the supernatant was taken from 5 mL of pelleted cell cultures. As described above, arsenite present in the 5 mL solution was separated from arsenate using the disposable anion exchange columns; the flow through was diluted to 10 mL using water and the amount of arsenite present in each sample was measured using mercuric bromide strips.

***In Vitro* Arsenate Reduction.** The reaction buffer for *in vitro* arsenate reduction was 100 mM HEPES at neutral pH 7. In the noncatalyzed reaction, 1 mM of GSH was incubated with 50 mM sodium arsenate. For the enzyme catalyzed reaction, purified enzyme was added to the mixture at a final concentration of 0.12 mM. The total reaction volume was 500 μL. After 30 min of incubation, arsenite production was measured using half of the reaction mixture; after 60 min, arsenite production was measured using the remaining solution. At each time point, the pH of the reaction mixture was lowered to 4, diluted to 3 mL, and subsequently separated using the anion exchange columns. The reaction mixture was lowered to pH 4 to separate any arsenite−GSH conjugates known to freely form in solution. The column flow through was diluted to a volume of 10 mL using water before arsenic detection. Arsenite in each reaction mixture was semiquantitated using the arsenite detection assay described above.

■ ASSOCIATED CONTENT

Supporting Information

E. coli strains and plasmids used in this study. Survival in the presence of sodium arsenate of *E. coli* EQ279 mutants expressing plasmids isolated from the genetic screen; doubling time of *E. coli* EQ301 mutant strain expressing selected GstB variants; growth in liquid media of *E. coli* EQ301 mutants expressing GstB variants containing point mutations in either the Arg111 and/or Arg119 residue; size exclusion analysis of purified GstB enzymes; differential scanning fluorimetry analysis of purified GstB enzymes; analysis of GstB activity with respect to the conversion of bromoacetate into glutathione S-acetate; two-step semi-quantitative assay for measuring arsenite in solution; comparison of *in vitro* GstB activity with respect to arsenate reduction with and without the addition of purified Glutaredoxin 2; *in vitro* assay for GstB catalyzed arsenate reduction using NADPH redox-coupled assays; supplemental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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