Novel Pathway for Arsenic Detoxification in the Legume Symbiont *Sinorhizobium meliloti*

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We report a novel pathway for arsenic detoxification in the legume symbiont *Sinorhizobium meliloti*. Although a majority of *ars* operons consist of three genes, *arsR* (transcriptional regulator), *arsB* [$\text{As}(\text{OH})_3/\text{H}^+$ antiporter], and *arsC* (arsenate reductase), the *S. meliloti* *ars* operon includes an aquaglyceroporin (*aqpS*) in place of *arsB*. The presence of AqpS in an arsenic resistance operon is interesting, since aquaglyceroporin channels have previously been shown to adventitiously facilitate uptake of arsenite into cells, rendering them sensitive to arsenite. To understand the role of *aqpS* in arsenic resistance, *S. meliloti* *aqpS* and *arsC* were disrupted individually. Disruption of *aqpS* resulted in increased tolerance to arsenite but not arsenate, while cells with an *arsC* disruption showed selective sensitivity to arsenate. The results of transport experiments in intact cells suggest that AqpS is the only protein of the *S. meliloti* *ars* operon that facilitates transport of arsenite. Coexpression of *S. meliloti* *aqpS* and *arsC* in a strain of *E. coli* lacking the *ars* operon complemented arsenate but not arsenite sensitivity. These results imply that, when *S. meliloti* is exposed to environmental arsenate, arsenate enters the cell through phosphate transport systems and is reduced to arsenite by ArsC. Internally generated arsenite flows out of the cell by downhill movement through AqpS. Thus, AqpS confers arsenate resistance together with ArsC-catalyzed reduction. This is the first report of an aquaglyceroporin with a physiological function in arsenic resistance.

Arsenic compounds are widespread in the biosphere, arising from both natural and anthropomorphic sources. The two biologically relevant oxidation states of inorganic arsenic are arsenite [$\text{As}(\text{III})$] and arsenate [$\text{As}(\text{V})$], the former being more toxic than the latter. The primary mechanism of arsenite toxicity is due to its ability to react with protein sulfhydryl groups, thereby affecting their function. By itself, arsenate has low toxicity as a phosphate analogue, and its main toxicity is the result of its conversion to arsenite.

In response to toxicity, microorganisms have evolved mechanisms for arsenic resistance. Arsenic resistance (*ars*) genes are common in microbes and are localized to *ars* operons on either the chromosome or plasmid (11). Many, if not most, *ars* operons consist of three genes: *arsR*, *arsB*, and *arsC*. ArsR is a *trans*-acting repressor (23, 25) that senses environmental As(III) and controls the expression of ArsB and ArsC. ArsC is a reductase that reduces As(V) to As(III) (14), while ArsB extrudes As(III) out of the cells by functioning as an $\text{As}(\text{OH})_3/\text{H}^+$ antiporter (10). Therefore, expression of both ArsB and ArsC provides resistance to both As(III) and As(V). In addition to the three-gene chromosomal *ars* operon, some *ars* operons such as those carried by *Escherichia coli* plasmids R773 and R46 have five genes, *arsRDABC*, that encode two additional proteins, ArsD and ArsA. ArsD exhibits weak As(III)-responsive transcriptional repressor activity (4), and

ArsA is an ATPase that forms a complex with ArsB to provide higher levels of As(III) resistance than ArsB alone (15).

While conducting a genomics analysis of the *ars* operon in microbial populations, we observed that the chromosome sequence of legume symbiont *Sinorhizobium meliloti* strain Rm1021 (GenBank accession no. AL591786) displays a cluster of four open reading frames (ORFs) that are putative arsenic resistance genes. The first ORF (SMc02647) codes for a hypothetical polypeptide of 137 amino acids that shows sequence similarity to the ArsR subfamily of helix-turn-helix bacterial transcription regulatory proteins. However, the putative *S. meliloti* ArsR does not contain the N-terminal CXCXXC motif, which has been shown to be required for binding of the inducer arsenite in *E. coli* and R773 ArsR (19, 25). Instead, *S. meliloti* ArsR has two pairs of vicinal cysteines located near the C-terminal end of the protein, but the involvement of these thiols in metalloid binding remains to be determined. The second ORF (SMc02648) codes for a putative membrane protein that belongs to the major intrinsic protein or aquaporin superfamily (6) and shows sequence homology with the bacterial glycerol facilitator (GlpF), yeast aquaglyceroporin Fps1p, and mammalian aquaglyceroporin AQP9. This putative 233-residue transmembrane channel will henceforth be referred to as AqpS (aquaglyceroporin from *Sinorhizobium*). The third ORF (SMc02649) codes for a putative 140-amino-acid arsenate reductase, which is homologous to the *E. coli* ArsC sequence. The fourth ORF (SMc02650) encodes a 241-residue hypothetical protein designated ArsH and has conserved domains related to the NADPH-dependent flavin mononucleotide reductase class of proteins.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s) ^a	Reference or source
Strains		
<i>S. meliloti</i>		
Rm1021	SU47; <i>str-21</i>	3
SmK956	In-frame <i>SMc02648</i> (<i>aqpS</i>) deletion, Sm ^r	This study
SmK957	In-frame <i>SMc02649</i> (<i>arsC</i>) deletion, Sm ^r	This study
SmK958	<i>SMc02650</i> (<i>arsH</i>)- <i>gusA</i> transcriptional fusion, Sm ^r Gm ^r	This study
<i>E. coli</i>		
W3110	K12 F ⁻ IN(<i>rrnD-rrnE</i>)	1
AW3110	Δ <i>ars::cam</i> F ⁻ IN(<i>rrnD-rrnE</i>)	2
OSBR1	AW3110 <i>glpF::TnpA</i> , Km ^r	17
TOP10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80/ <i>acZ</i> Δ M15 Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ (<i>araA-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>endA1</i> <i>nupG</i>	Invitrogen
Plasmids		
pBAD/ <i>Myc</i> -HisA	<i>E. coli</i> cloning and expression vector, Ap ^r	Invitrogen
AqpS-pBAD/ <i>Myc</i> -HisA	PCR amplified <i>aqpS</i> gene with an NcoI site at its 5' and HindIII at its 3' end inserted into the NcoI-HindIII site of pBAD/ <i>Myc</i> -HisA, Ap ^r	This study
ArsC-pBAD/ <i>Myc</i> -HisA	PCR-amplified <i>arsC</i> gene with an NcoI site at its 5' and HindIII at its 3' end inserted into the NcoI-HindIII site of pBAD/ <i>Myc</i> -HisA, Ap ^r	This study
AqpS-ArsC-pBAD/ <i>Myc</i> -HisA	PCR-amplified <i>aqpS</i> and <i>arsC</i> from <i>S. meliloti</i> genomic DNA, with an NcoI site at the 5' of <i>aqpS</i> and HindIII at the 3' end of <i>arsC</i> inserted into the NcoI-HindIII site of pBAD/ <i>Myc</i> -HisA, Ap ^r	This study
AqpS-ArsC-ArsH-pBAD/ <i>Myc</i> -HisA	PCR-amplified <i>aqpS</i> , <i>arsC</i> , and <i>arsH</i> from <i>S. meliloti</i> genomic DNA, with an NcoI site at the 5' of <i>aqpS</i> and HindIII at the 3' end of <i>arsH</i> inserted into the NcoI-HindIII site of pBAD/ <i>Myc</i> -HisA, Ap ^r	This study

^a Abbreviations: Ap, ampicillin; Km, kanamycin; Sm, streptomycin; Gm, gentamicin.

The presence of *aqpS* in place of *arsB* in the *S. meliloti* *ars* operon is of considerable interest. GlpF, the glycerol facilitator in *E. coli* and a member of the aquaporin superfamily, was the first to be identified as a trivalent metalloid transporter, responsible for the uptake of antimonite (17) and arsenite (10) and for metalloid sensitivity. Fps1p, the yeast homologue of GlpF, was subsequently shown to be the route of uptake of As(III) in *Saccharomyces cerevisiae* (24). The mammalian aquaglyceroporin AQP9 has recently been shown to transport both As(III) and Sb(III) (8, 9). In *E. coli*, uptake of As(III) by GlpF renders cells sensitive to As(III). How can the AqpS channel, which, by analogy with all other aquaporins, can only equilibrate As(III) on both sides of the membrane, replace the ArsB antiporter, whose function is to actively extrude As(III) against a concentration gradient? This apparent paradox can be resolved if the *S. meliloti* *ars* operon confers resistance to As(V) but not to As(III). In this scenario, once As(V) enters the cell, it is reduced to As(III) by ArsC. This establishes a concentration gradient of As(III) inside the cell relative to the outside, so that As(III) flows out of the cell through the AqpS channel. In this paper, we provide evidence in support of the hypothesis that AqpS and ArsC together confer a novel pathway of As(V) detoxification in *S. meliloti*.

MATERIALS AND METHODS

Reagents. Unless otherwise mentioned, all reagents were from Sigma. As(III), Sb(III), As(V), and Sb(V) were purchased in the form of sodium arsenite, potassium antimonite tartrate, sodium arsenate, and potassium hexahydroxyantimonate, respectively. Restriction enzymes were obtained from Invitrogen. DNA purification kits were obtained from QIAGEN.

Bacterial strains and growth conditions. The strains and plasmids used in this work are listed in Table 1. *S. meliloti* cells were grown either in Luria-Bertani (LB) medium (16) supplemented with 2.5 mM CaCl₂ and 2.5 mM MgSO₄ or in

a low-phosphate medium (12). *E. coli* cells were grown either in LB or low-phosphate medium.

DNA manipulations. Plasmid isolation, DNA restriction endonuclease analysis, ligation, and transformation were performed as described previously (16). Either a Qiaprep Spin Miniprep kit or a Qiaquick gel extraction kit (QIAGEN) was used to prepare plasmid DNA for restriction enzyme digestion, sequencing, and recovering DNA fragments from agarose gels. The sequence of each PCR product was confirmed by DNA sequencing of the entire gene. Sequencing was performed using a CEQ2000 DNA sequencer (Beckman Coulter).

Disruption of *ars* genes in *S. meliloti*. Crossover PCR (21) was used to generate the *S. meliloti* *ars* deletion strains. An internal fragment of either the *aqpS* or *arsC* gene was deleted and replaced with a short synthetic fragment so that the final PCR product maintained the translational reading frame. The PCR fragment was restricted with SstI and PstI and ligated to similarly digested suicide vector pJQ200mp18 (13). Plasmid pJQ200mp18 is based on the p15A origin of replication and incorporates *sacB* from *Bacillus subtilis*, which is inducible by sucrose and is lethal when expressed in *S. meliloti*. Gene replacement was carried out by mobilizing the deletion-insertion construct from *E. coli* into *S. meliloti* Sm1021. Double recombinants were selected on sucrose. The resulting gentamicin-sensitive streptomycin-resistant transconjugants were further analyzed by PCR to verify the expected deletion.

The *aqpS* gene was deleted by removal of the sequence for amino acids 12 to 177 of the 233-residue AqpS. The deleted region was replaced with a 24-bp synthetic fragment (5'-TGGATCCTGCGCTCGACGGCATC-3'), coding for amino acids WILRLDGI. Similarly, insertional inactivation of the 140-residue ArsC resulted in deletion of amino acids 15 to 84, which were replaced with the WILRLDGI sequence. To disrupt *arsH*, an internal fragment of the targeted gene was PCR amplified and cloned upstream of the promoterless reporter gene *gusA* and *tdimer2* in plasmid pTH1469 (J. Cheng and T. M. Finan, unpublished data). This plasmid is unable to replicate in *S. meliloti*. A single homologous recombination created transcriptional reporter gene fusion with simultaneous disruption of *arsH*. All primer sequences that were used for the disruption and expression cloning of *S. meliloti* *ars* genes will be provided upon request.

Cloning of *S. meliloti* genes. *aqpS*, *arsC*, and *arsH* genes were amplified from the genomic DNA of *S. meliloti*, which was purified from an overnight culture of strain Rm1021 by use of the DNeasy tissue kit (QIAGEN). The DNA sequences were amplified by PCR using nucleotide primers complementary to the 5' and 3' regions flanking the open reading frames to produce a PCR fragment with an NcoI site at the 5' end and a HindIII site at the 3' end. A 30-cycle PCR (94°C for 1 min, 55°C

for 1 min, and 72°C for 2 min) was performed with strain Rm1021 genomic DNA. The PCR products were ligated to pGEM-T Easy Vector (Promega). The resulting constructs were digested with NcoI and HindIII, and the NcoI-HindIII fragments containing the complete coding sequence for *ars* genes were ligated to the expression vector pBAD/*Myc*-HisA, which had been similarly digested. The resulting constructs contained the full-length *aqpS*, *arsC*, or *arsH* gene cloned in frame with the sequence for a C-terminal *myc* tag and six-histidine tag followed by a stop codon. The correct reading frame was verified by DNA sequencing.

Transport assays. For uptake assays in *E. coli*, OSBR1 competent cells were transformed with constructs bearing *S. meliloti* arsenic resistance genes and grown in a low-phosphate medium at 37°C supplemented with 125 µg/ml ampicillin. Overnight cultures were diluted 20-fold in low-phosphate medium containing 0.2% arabinose plus 125 µg/ml ampicillin and grown at 37°C to an A_{600} of 1. The cells were harvested, washed, and suspended in a buffer consisting of 75 mM HEPES-KOH (pH 7.5) containing 150 mM KCl and 1 mM MgSO₄ and brought to an A_{600} of 10 at room temperature. To initiate the transport assay, 0.1 ml of the concentrated cell suspension was diluted in 1 ml of the same buffer at room temperature containing 50 µM sodium arsenite or sodium arsenate. Samples (0.1 ml) were withdrawn at the indicated times, filtered through 0.2-µm-pore-diameter nitrocellulose filters (Whatman), and washed with 15 ml of the same buffer, all at room temperature. The filters were digested overnight at room temperature with 0.3 ml of concentrated HNO₃ (EM Science) (69 to 70%). The dissolved filters were incubated for 10 min at 70°C, allowed to cool to room temperature, and diluted with 5.7 ml of high-pressure-liquid-chromatography-grade water (Sigma) to produce a final concentration of HNO₃ of approximately 4%. Standard solutions were made in the range of 0.5 to 150 ppb in 4% HNO₃ by use of an arsenic standard (Ultra Scientific). The total arsenic content was determined with a Perkin-Elmer ELAN 9000 inductively coupled plasma-mass spectrometer. Kinetic data were analyzed using SigmaPlot version 9.0.

Metalloid sensitivity assays. For measurement of the As(III) or Sb(III) resistance phenotype, *S. meliloti* strains were grown for 24 h at 30°C in LB medium supplemented with 2.5 mM CaCl₂, 2.5 mM MgSO₄, and appropriate antibiotics. The cells were diluted 100-fold into fresh, prewarmed medium with the indicated concentrations of As(III) or Sb(III) and incubated at 30°C with shaking for another 24 h. Growth was estimated from the absorbance at 600 nm. For measurement of the As(V) or Sb(V) resistance phenotype, cells were grown in a low-phosphate medium (12) and processed as described above.

For an *E. coli* metalloid resistance assay, competent cells of either AW3110 or OSBR1 were transformed with constructs bearing *S. meliloti* arsenic resistance genes. Assays to determine the As(III) or Sb(III) resistance phenotype were performed in LB medium. Cells were grown overnight at 37°C in LB medium with ampicillin (125 µg/ml). Overnight cultures were diluted 100-fold in fresh LB medium containing various concentrations of As(III) or Sb(III) plus 0.2% arabinose and grown for another 5 h, and the cell density was measured at 600 nm. To determine the As(V) or Sb(V) resistance phenotype, *E. coli* cells were grown in a low-phosphate medium at 37°C supplemented with 125 µg/ml ampicillin. Overnight cultures were diluted 100-fold in low-phosphate medium containing various concentrations of sodium arsenite and 0.2% arabinose. Growth was estimated from the absorbance at 600 nm after 5 h of growth at 37°C.

Purification of *S. meliloti* ArsC. *S. meliloti* ArsC was purified from cultures of *E. coli* strain TOP10 harboring the *S. meliloti* ArsC-pBAD/*Myc*-HisA plasmid. Cells were grown at 37°C in LB medium to an A_{600} of 0.5, at which point 0.02% arabinose was added to induce ArsC expression. The cells were grown for another 4 h before being harvested by centrifugation. ArsC was purified according to the protocol described previously (26). The concentration of ArsC in purified preparations was determined from the absorbance at 280 nm by use of an extinction coefficient of 2,800 M⁻¹ cm⁻¹ (5).

Assay of arsenate reductase activity. Arsenate reductase activity was assayed at 37°C using a coupled assay as described previously (18). The assay buffer contained 50 mM MOPS (morpholinepropanesulfonic acid)-MES (morpholineethanesulfonic acid) (pH 6.5), 0.1 mg/ml bovine serum albumin, 0.4 mM NADPH, 1 mM glutathione, 1 µM yeast glutaredoxin, 50 nM yeast glutathione reductase (Calbiochem), and 5 to 10 µM *S. meliloti* ArsC. Sodium arsenate was added as indicated. Arsenate reductase activity was monitored at 340 nm and expressed as nanomoles of NADPH oxidized per milligram of *S. meliloti* ArsC by using a molar extinction coefficient of 6,200 for NADPH.

RESULTS

The *aqpS*, *arsC*, and *arsH* genes are involved in arsenic resistance in *S. meliloti*. To elucidate the role of *S. meliloti* *aqpS*, *arsC*, and *arsH* genes in arsenic resistance, each was indi-

FIG. 1. Trivalent metalloid sensitivity in *ars* deletion strains of *S. meliloti*. Overnight cultures of *aqpS*-, *arsC*-, and *arsH*-deleted *S. meliloti* were diluted 100-fold into fresh LB medium supplemented with 2.5 mM CaCl₂ and 2.5 mM MgSO₄ and containing the indicated concentrations of sodium arsenite (top panel) and potassium antimonyl tartrate (bottom panel). After 24 h of growth at 30°C, the absorbance at 600 nm was measured. Strains used: (●), *S. meliloti* Rm1021 (wild type); (○), SmK956 ($\Delta aqpS$); (▼), SmK957 ($\Delta arsC$); (△), SmK958 ($\Delta arsH$).

vidually inactivated as described in the Materials and Methods section to generate strains SmK956 ($\Delta aqpS$), SmK957 ($\Delta arsC$), and SmK958 ($\Delta arsH$), respectively. Each of these mutant strains was viable, and their growth rates were similar to that of the wild-type strain in the absence of arsenical salts. Deletion of *aqpS* resulted in increased resistance to either Sb(III) or As(III), the level of resistance being higher for the former than the latter (Fig. 1). These results are in agreement with earlier observations that deletion of GlpF in *E. coli* and Fps1p in *S. cerevisiae* results in increased resistance to trivalent metalloid. These data suggest that AqpS is involved in the uptake of As(III) and Sb(III). In contrast, AqpS-disrupted cells were more sensitive to As(V) than the wild-type cells (Fig. 2, top panel). This is most likely because once As(V) enters the cell through phosphate transport systems, it is reduced to As(III) by ArsC, which then reaches toxic levels due to the absence of the AqpS channel. These results support the hypothesis that AqpS is the primary transport system for As(III) in *S. meliloti* but functions in efflux, not uptake.

FIG. 2. Pentavalent metalloid sensitivity in *ars* deletion strains of *S. meliloti*. Overnight cultures of *aqpS*-, *arsC*-, and *arsH*-deleted *S. meliloti* were diluted 100-fold into fresh low-phosphate medium containing the indicated concentrations of sodium arsenate (top panel) and potassium hexahydroxyantimonate (bottom panel). Metalloid resistance assays were performed as described in Materials and Methods. Strains used: (●), *S. meliloti* Rm1021 (wild type); (○), SmK956 ($\Delta aqpS$); (▼), SmK957 ($\Delta arsC$); (△), SmK958 ($\Delta arsH$).

The ability of AqpS to facilitate bidirectional movement of As(III) was investigated by uptake assays. We have earlier demonstrated that the aquaglyceroporin GlpF in *E. coli* is the major uptake pathway for both As(III) and Sb(III) (10, 17). *E. coli* strain OSBR1, which lacks the chromosomal *arsRBC* operon and also has a *TnphoA* insertion in *glpF*, showed low levels of As(III) uptake (Fig. 3, top panel). OSBR1 cells expressing *aqpS* showed high levels of As(III) accumulation (Fig. 3, top panel). There was no significant difference in metalloid uptake when *S. meliloti* ArsC and ArsH were coexpressed with AqpS (Fig. 3, top panel). This experiment clearly demonstrates that AqpS facilitates transport of As(III) and is probably the only protein of the *S. meliloti* *ars* operon able to do so. When similar transport experiments were performed in the presence of As(V), cells of strain OSBR1, either alone or expressing *S. meliloti* *ars* genes, showed identical levels of arsenate uptake (Fig. 3, bottom panel), demonstrating that AqpS facilitates uptake of trivalent but not pentavalent arsenic.

FIG. 3. Arsenite uptake into cells of *E. coli* OSBR1 ($\Delta ars \Delta glpF$) is facilitated by the *S. meliloti* aquaglyceroporin channel AqpS. Transport of As(III) (top panel) and As(V) (bottom panel) was assayed as described in Materials and Methods. (●), OSBR1; (○), AqpS-pBAD/*Myc*-HisA in OSBR1; (▼), AqpS-ArsC-pBAD/*Myc*-HisA in OSBR1; (△), AqpS-ArsC-ArsH-pBAD/*Myc*-HisA in OSBR1.

Disruption of the *arsC* gene resulted in sensitivity to As(V) (Fig. 2, top panel), but the disrupted strain exhibited levels of resistance to trivalent metalloids similar to those seen with the wild-type strain (Fig. 1), consistent with a role of *S. meliloti* *arsC* in arsenate detoxification. The function of *S. meliloti* ArsC as an arsenate reductase was determined from the catalytic activity of purified ArsC. *S. meliloti* *arsC* was cloned behind the arabinose promoter in plasmid pBAD/*Myc*-HisA with a C-terminal six-histidine tag. The protein was expressed in *E. coli* TOP10 strain and purified by nickel-nitrilotriacetic acid chromatography. Arsenate reductase activity was examined using a coupled assay method (18). The K_m for As(V) was estimated to be 25 mM, while the V_{max} for the arsenate reductase activity was approximately 100 nmol/min/mg of protein. These values are in range with those reported for *E. coli* plasmid R773 ArsC (18). *S. meliloti* ArsC did not display antimonate reductase activity (data not shown), indicating that *S. meliloti* ArsC is selective for As(V) and is not an Sb(V) reductase.

Inactivation of the *arsH* gene resulted in increased sensitivity at low levels of As(III) (Fig. 1, top panel) and, to a lesser degree, As(V) (Fig. 2, top panel). Thus, ArsH also appears to be involved

FIG. 4. *S. meliloti aqpS* and *arsC* genes do not confer arsenite resistance in *E. coli*. Arsenite resistance in *E. coli* strains AW3110 and OSBR1 bearing *S. meliloti ars* genes was assayed as described in Materials and Methods. Expression of the *ars* genes was induced with 0.2% arabinose. (Top panel) (●), W3110 (wild type); (○), AW3110; (▼), AqpS-pBAD/Myc-HisA in AW3110; (△), ArsC-pBAD/Myc-HisA in AW3110; (■), AqpS-ArsC-pBAD/Myc-HisA in AW3110. (Bottom panel) (▲), W3110 (wild type); (▼), OSBR1; (○), AW3110; (△), AqpS-pBAD/Myc-HisA in OSBR1; (■), ArsC-pBAD/Myc-HisA in OSBR1; (□), AqpS-ArsC-pBAD/Myc-HisA in OSBR1.

in arsenic detoxification. Finally, each of the *S. meliloti ars* deletion strains exhibited levels of sensitivity to Sb(V) similar to those seen with the wild-type strain (Fig. 2, bottom panel), indicating that these *ars* genes are not involved in Sb(V) detoxification.

Complementation studies of *S. meliloti ars* genes in *E. coli* strains AW3110 and OSBR1. The *S. meliloti aqpS*, *arsC*, and *arsH* genes were cloned behind the arabinose promoter in plasmid pBAD/Myc-HisA and expressed in *E. coli* strains AW3110, in which the chromosomal *arsRBC* operon was lacking (2), and OSBR1, which was created from AW3110 by inserting *TnphoA* into *glpF* (17). All complementation experiments were done in the presence of 0.2% arabinose as inducer. None of the *S. meliloti ars* genes was expressed in the absence of arabinose, as analyzed by immunoblotting (data not shown). Since OSBR1 has a disrupted *glpF*, it is unable to accumulate As(III) and, consequently, is more resistant to As(III) than AW3110 (Fig. 4, bottom panel). However, upon expression of

FIG. 5. *S. meliloti aqpS* and *arsC* genes confer arsenate resistance in *E. coli*. Arsenate resistance in *E. coli* strain AW3110 and OSBR1 bearing *S. meliloti ars* genes was assayed as described in Materials and Methods. (Top panel) (●), W3110 (wild type); (○), AW3110; (▼), AqpS-pBAD/Myc-HisA in AW3110; (△), ArsC-pBAD/Myc-HisA in AW3110; (■), AqpS-ArsC-pBAD/Myc-HisA in AW3110. (Bottom panel) (●), W3110 (wild type); (○), OSBR1; (▼), AqpS-pBAD/Myc-HisA in OSBR1; (△), ArsC-pBAD/Myc-HisA in OSBR1; (■), AqpS-ArsC-pBAD/Myc-HisA in OSBR1.

AqpS in either OSBR1 or AW3110, both strains showed similar sensitivities to As(III) (Fig. 4). When the experiments were conducted in the presence of As(V), there were no differences in phenotypes between the *aqpS* complemented and parental strains (Fig. 5). These experiments support the hypothesis that AqpS serves as an As(III) channel and does not carry As(V).

Expression of *S. meliloti arsC* in strain AW3110 partially complemented As(V) sensitivity (Fig. 5, top panel) but not As(III) sensitivity (Fig. 4, bottom panel). In contrast, strain OSBR1 cells expressing ArsC are resistant to As(III) (Fig. 4, bottom panel) but sensitive to As(V) (Fig. 5, bottom panel). The complementation result showing As(V) sensitivity in AW3110 (Fig. 5, top panel) but not in OSBR1 (Fig. 5, bottom panel) is consistent with *S. meliloti arsC* encoding an arsenate reductase that reduces As(V) to As(III), followed by downhill transport of As(III) through the GlpF channel of AW3110 cells. OSBR1 cells expressing both AqpS and ArsC showed As(V) resistance (Fig. 5, bottom panel) but not As(III) resis-

FIG. 6. Detoxification of arsenical in *E. coli* K-12 and *S. meliloti* 1021. In both *E. coli* and *S. meliloti* arsenate is brought into cells by the phosphate transporters. The first step of detoxification involves reduction of arsenate to arsenite by either *E. coli* or *S. meliloti* ArsC. Subsequent detoxification steps in *E. coli* involves removal of the trivalent form of the metalloid from the cytosol by active extrusion through the $\text{As}(\text{OH})_3/\text{H}^+$ antiporter ArsB, while in *S. meliloti*, the AqpS channel facilitates downhill transport of As(III). Since arsenite can be taken up directly by cells, using either GlpF in *E. coli* or AqpS in *S. meliloti*, the detoxification mechanism functions when *S. meliloti* cells are exposed to arsenate.

tance (Fig. 4, bottom panel). These experiments support our model that, once As(V) is reduced to As(III) by ArsC, the trivalent metalloid flows out of cell through the AqpS channel. Thus, even in *E. coli*, an aquaglyceroporin can replace ArsB to confer resistance to internally generated As(III).

DISCUSSION

S. meliloti utilizes a unique detoxification pathway wherein reduction of As(V) by ArsC is coupled to downhill transport of As(III) through the AqpS channel (Fig. 6). This is the first report of an aquaglyceroporin with a physiological function of trivalent arsenic detoxification. While aquaglyceroporin channels have been shown to facilitate adventitious uptake of As(III) and Sb(III) into cells (8, 17, 24), most prokaryotes utilize the ubiquitous $\text{As}(\text{OH})_3/\text{H}^+$ antiporter ArsB to extrude As(III) from cells (Fig. 6) (14). The AqpS channel facilitates downhill transport of As(III) (Fig. 6), whereas transport through ArsB is to be coupled to the electrochemical proton gradient (10). Obviously, As(III) efflux via AqpS occurs when extracellular As(III) levels are lower than the intracellular concentration. Consequently, the AqpS-ArsC detoxification pathway is functional when *S. meliloti* cells are exposed to As(V) but not As(III).

An interesting issue is whether AqpS is an arsenic-specific channel, with higher selectivity for As(III) than polyols. AqpS has features that are different from those of other aquaglyceroporins. For example, it lacks the two large extracellular loops between TM4 and TM5 and between TM7 and TM8 that contribute to the binding of glycerol at the lumen of the channel in GlpF. Trp48, Gly199, Phe200, and Arg206, which are known to be involved in forming the selectivity filter in GlpF (20), are not conserved in AqpS. These differences imply that AqpS has a selectivity filter that is quite different from those of other aquaglyceroporins and raises the intriguing possibility that the AqpS channel has evolved metalloid specificity. AqpS shows much closer similarity with aquaglyceroporin

(major intrinsic protein) sequences of *Mesorhizobium loti* (GenBank accession no. BAB49364), *Caulobacter crescentus* (GenBank accession no. NP_420315), *Ralstonia solanacearum* (GenBank accession no. NP_522688), and a number of unknown organisms identified from environmental genome shotgun sequencing of the Sargasso Sea (22). The specificity of AqpS is currently under investigation.

S. meliloti ArsC shows close sequence similarity to R773 ArsC. The catalytic mechanism of the R773 ArsC has been studied in detail, and the *S. meliloti* enzyme almost certainly uses a similar mechanism (7). *S. meliloti* ArsC did not show Sb(V)-reductase activity, and *S. meliloti* *ars* deletion strains showed levels of sensitivity to Sb(V) similar to those seen with the wild-type strain (Fig. 2, bottom panel). Therefore, AqpS-ArsC combination can provide resistance to As(V) but not Sb(V).

Genomic sequence analysis indicated that only a few other organisms (for example, *Mesorhizobium loti*, *Caulobacter crescentus*, and *Ralstonia solanacearum*) exploit this unique AqpS-ArsC detoxification system. However, the presence of this pathway may be more widespread in organisms that encode an arsenate reductase and an aquaglyceroporin and are exposed primarily to As(V).

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