



Efflux Transporter ArsK Is Responsible for Bacterial Resistance to Arsenite, Antimonite, Trivalent Roxarsone, and Methylarsenite

Kaixiang Shi,^a Chan Li,^a Christopher Rensing,^{b,c} Xingli Dai,^a Xia Fan,^a Gejiao Wang^a

^aState Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan, China

^bInstitute of Environmental Microbiology, College of Resources and Environment, Fujian Agriculture & Forestry University, Fuzhou, Fujian, China

^cKey Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China

ABSTRACT Arsenic-resistant bacteria have evolved various efflux systems for arsenic resistance. Five arsenic efflux proteins, ArsB, Acr3, ArsP, ArsJ, and MSF1, have been reported. In this study, comprehensive analyses were performed to study the function of a putative major facilitator superfamily gene, *arsK*, and the regulation of *arsK* transcriptional expression in *Agrobacterium tumefaciens* GW4. We found that (i) *arsK* is located on an arsenic gene island in strain GW4. ArsK orthologs are widely distributed in arsenic-resistant bacteria and are phylogenetically divergent from the five reported arsenic efflux proteins, indicating that it may be a novel arsenic efflux transporter. (ii) Reporter gene assays showed that the expression of *arsK* was induced by arsenite [As(III)], antimonite [Sb(III)], trivalent roxarsone [Rox(III)], methylarsenite [MAs(III)], and arsenate [As(V)]. (iii) Heterologous expression of ArsK in an arsenic-hypersensitive *Escherichia coli* strain showed that ArsK was essential for resistance to As(III), Sb(III), Rox(III), and MAs(III) but not to As(V), dimethylarsenite [dimethyl-As(III)], or Cd(II). (iv) ArsK reduced the cellular accumulation of As(III), Sb(III), Rox(III), and MAs(III) but not to As(V) or dimethyl-As(III). (v) A putative arsenic regulator gene *arsR2* was cotranscribed with *arsK*, and (vi) ArsR2 interacted with the *arsR2-arsK* promoter region without metalloids and was derepressed by As(III), Sb(III), Rox(III), and MAs(III), indicating the repression activity of ArsR2 for the transcription of *arsK*. These results demonstrate that ArsK is a novel arsenic efflux protein for As(III), Sb(III), Rox(III), and MAs(III) and is regulated by ArsR2. Bacteria use the *arsR2-arsK* operon for resistance to several trivalent arsenicals or antimonials.

IMPORTANCE The metalloid extrusion systems are very important bacterial resistance mechanisms. Each of the previously reported ArsB, Acr3, ArsP, ArsJ, and MSF1 transport proteins conferred only inorganic or organic arsenic/antimony resistance. In contrast, ArsK confers resistance to several inorganic and organic trivalent arsenicals and antimonials. The identification of the novel efflux transporter ArsK enriches our understanding of bacterial resistance to trivalent arsenite [As(III)], antimonite [Sb(III)], trivalent roxarsone [Rox(III)], and methylarsenite [MAs(III)].

KEYWORDS ArsK, ArsR2, arsenic efflux regulation, bacterial arsenic resistance, efflux transporter

Arsenic is a metalloid that is widely distributed in the environment (1). Arsenic exists in various forms, such as inorganic trivalent arsenite [As(III)], inorganic pentavalent arsenate [As(V)], organic pentavalent roxarsone [Rox(V)], methylarsenate [MAs(V)], and the trivalent forms Rox(III) and methylarsenite [MAs(III)] (2, 3). Microorganisms partici-

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Address correspondence to Gejiao Wang, gejiao@mail.hzau.edu.cn.

pate in the geochemical cycle of arsenic; as a result of continuous exposure to arsenic, most microorganisms have evolved pathways for arsenic resistance and detoxification (4, 5). Arsenic resistance genes are organized in the *ars* operon (6, 7). In general, the minimal *ars* operon contains a repressor encoded by the gene *arsR*, *arsC* encoding an As(V) reductase, and a gene encoding one of the As(III) efflux transporters (8, 9).

ArsR is a well-studied transcriptional repressor that has an autoregulatory function for metals or metalloids (10). When a metal or metalloid is taken up into the cells and binds ArsR, the conformation of ArsR changes, and the ArsR protein disassociates from the DNA, thereby enabling the DNA to be transcribed (10). The regulatory activities of four ArsR proteins, named ArsR1 to ArsR4, have been studied in *Agrobacterium tumefaciens* 5A, revealing a complex regulatory network among these repressors (8). The As(V) reductase ArsC catalyzes the reduction of As(V) to As(III) and is involved in bacterial As(V) resistance (9). In addition, the efflux transporter is the main resistance protein in the *ars* operon; it reduces the intracellular arsenic concentration via its efflux activity (11).

To date, five types of arsenic transporters (ArsB, Acr3, ArsJ, ArsP, and MFS1) have been found in various arsenic-resistant bacteria, and each confers resistance to different types of arsenic compounds (11–15). ArsB and Acr3 are both widespread determinants of As(III) efflux transporters in arsenic-resistant bacteria (16, 17). ArsB is classified in the ion transporter superfamily, and Acr3 is in the bile/arsenite/riboflavin transporter (BART) superfamily (18, 19). ArsB and Acr3 catalyze As(III) export coupled to electrochemical energy, and they can also couple with ArsA (ATPase encoded by *arsA*) to form primary As(III) transporter systems, which are much more efficient in extruding As(III) (11, 16). For resistance to As(V), As(V) is converted to As(III) by ArsC, and As(III) is then excreted by ArsB or Acr3 (9). Recently, a novel As(V) resistance protein, ArsJ, was identified in *Pseudomonas aeruginosa* DK2 (12). ArsJ belongs to the major facilitator superfamily (MFS), which is a ubiquitous transporter superfamily mediating the transport of various substrates (12, 20). Typically, *arsJ* is adjacent to the gene that encodes glyceraldehyde-3-phosphate (G3P) dehydrogenase (GAPDH) (12). When As(V) is taken up by phosphate transporters, GAPDH catalyzes the addition of As(V) to G3P to form 1-arseno-3-phosphoglyceric acid, and then ArsJ extrudes As(V) as the 1-arseno-3-phosphoglyceric acid form (12, 21). The MAs(III) efflux permease ArsP was identified in *Campylobacter jejuni* and shown to confer resistance to organic arsenicals Rox(III) and MAs(III) but not to inorganic arsenicals (22). Additionally, another MFS superfamily gene, *mfs1*, was found upstream of the *mfs2*-GAPDH genes in *Halomonas* sp. strain GFAJ-1 (15). MFS2 is highly homologous to ArsJ in *P. aeruginosa* DK2, and experimental results showed that the *mfs1*-*mfs2*-GAPDH gene operon functions in As(V) resistance but not in As(III) resistance. However, the individual function of *mfs1* is still unclear (15).

Arsenic extrusion appears to be the most ubiquitous resistance pathway for arsenic-resistant bacteria (23), and genes in the *ars* operon are usually related to arsenic resistance. In a previous study, we isolated the highly arsenic-resistant bacterial strain *A. tumefaciens* GW4 from arsenic-enriched groundwater sediment (24). A putative MFS superfamily gene, which we named *arsK*, was found on the arsenic gene island of strain GW4. A phylogenetic tree analysis based on amino acid sequences showed that ArsK is different from the five known transporters (ArsB, Acr3, ArsJ, ArsP, and MFS1) (11, 12, 14, 15, 22). In this study, a novel arsenic efflux transporter, ArsK, was identified, revealing that bacteria can use one transport system for the resistance to several trivalent arsenicals or antimoniols.

RESULTS

ArsK is widely distributed in arsenic-resistant bacteria. Arsenic is widely distributed in the environment, and most bacteria and archaea have *ars* operons (12). A genome analysis identified a putative gene, which we named *arsK*, in the arsenic gene island of *A. tumefaciens* GW4 (Fig. 1). The *arsK* homologs were also closely related to other arsenic-resistant bacteria, such as *A. tumefaciens* 5A, *Rhizobium* sp. strain NT-26, *Ensifer adhaerens*, *Mesorhizobium* sp. strain YR577, *Pseudochochromatium* sp. strain B5,

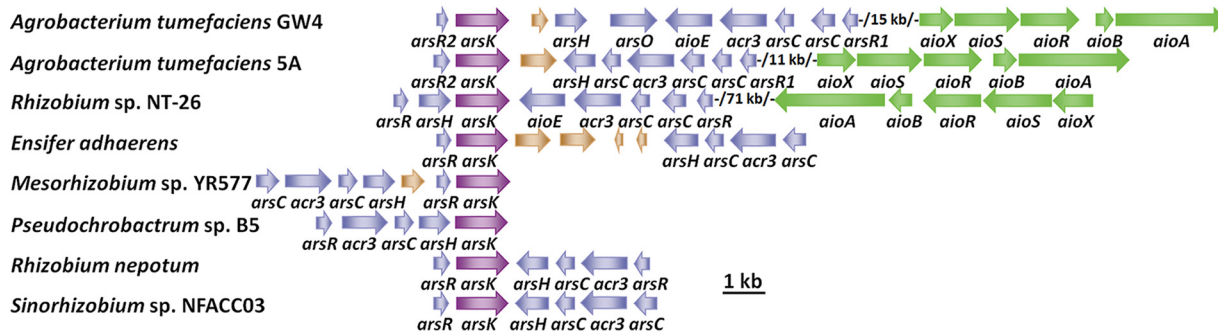


FIG 1 Putative *arsK* genes are conserved in bacterial *ars* operons. Representative *ars* operons containing *arsK* genes (purple) in As(III)-resistant bacteria. The accession numbers of bacterial genomes are [NZ_AWGV000000000](#) (*A. tumefaciens* GW4), [NZ_AGVZ000000000](#) (*A. tumefaciens* 5A), [NZ_FO082821](#) (*Rhizobium* sp. NT-26), [NZ_CP015882](#) (*E. adhaerens*), [NZ_FPBI000000000](#) (*Mesorhizobium* sp. YR577), [NZ_MOYL000000000](#) (*Pseudochrobactrum* sp. B5), [NZ_JWJH000000000](#) (*R. nepotum*), and [NZ_FMXF000000000](#) (*Sinorhizobium* sp. NFACC03).

Rhizobium nepotum, and *Sinorhizobium* sp. strain NFACC03 (Fig. 1). The multiple sequence alignment results showed that three Cys residues (Cys97, Cys183, and Cys318) exist in ArsK from *A. tumefaciens* GW4 and that the Cys183 is conserved (see Fig. S1 in the supplemental material). Besides, the ArsK orthologs were found in bacteria (including *Cyanobacteria*), but not in archaea, fungi, plants, or animals (data not shown), revealing that ArsK may be widely distributed in bacteria only or that the similar function proteins are much less homologous in archaea or eukaryotes.

The *arsK* gene product belongs to the MFS superfamily, and BLAST and conserved domains analyses showed that ArsK has the MFS_1 domain and the predicted arabinose efflux permease domain. MFS is an ancient and ubiquitous transporter superfamily, and members of this family can move substrates across membranes (20). Three major nomenclature and classification systems have been proposed for transporters to predict their substrates: the Pfam protein family database (<http://pfam.xfam.org/clan/MFS>), the HUGO Gene Nomenclature Committee (<http://slc.bioparadigms.org/>), and the Transporter classification database (<http://www.tcdb.org/>) (20). However, the substrate transported by ArsK was not predicted by any of these three databases. These results indicated that ArsK may be involved in a common efflux mechanism in arsenic-resistant bacteria.

In addition, a genome analysis showed that there are four *arsR* genes in the genomes of strains *A. tumefaciens* GW4 and 5A (8), and they have been named *arsR1* to *arsR4* (8). The *arsR2* is present upstream of *arsK* in strains GW4 and 5A (Fig. 1). A genome analysis showed that the ArsR2 ([WP_020810055](#)) from *A. tumefaciens* GW4 is closest to an ArsR of *Agrobacterium* sp. strain D14 ([WP_059754788](#)) and other *Agrobacterium* or *Rhizobium* strains. The ArsR2 amino acid sequence was also aligned with the ArsR orthologues in the NCBI GenBank database (see Fig. S2). Interestingly, the most closely related *arsR2*-like genes are all located adjacent to *arsK*, indicating that ArsR2 may regulate the expression of *arsK*. The multiple sequence alignment results showed that four Cys residues (Cys91, Cys92, Cys108, and Cys109) of ArsR2 are conserved in the related strains (Fig. S2), and the roles of the Cys residues can be investigated in the future.

ArsK is phylogenetically divergent from other arsenic efflux proteins. To associate the distribution of ArsK and the other arsenic efflux proteins with their phylogenetic affiliation, a phylogenetic tree based on their amino acid sequences was constructed using the neighbor-joining method in MEGA7 software (Fig. 2). ArsP sequences formed distinct groups, which was consistent with their different efflux functions related to organic arsenicals. In addition, because they both function in As(III) efflux, ArsB and Acr3 sequences formed distinct groups together, but they were clearly divergent from each other at the subgroup level (Fig. 2). ArsJ and MFS1 were reported to be different MFS superfamily proteins (12, 15), and ArsK also belongs to MFS superfamily according to a bioinformatics analysis. ArsK, ArsJ, and MFS1 sequences also

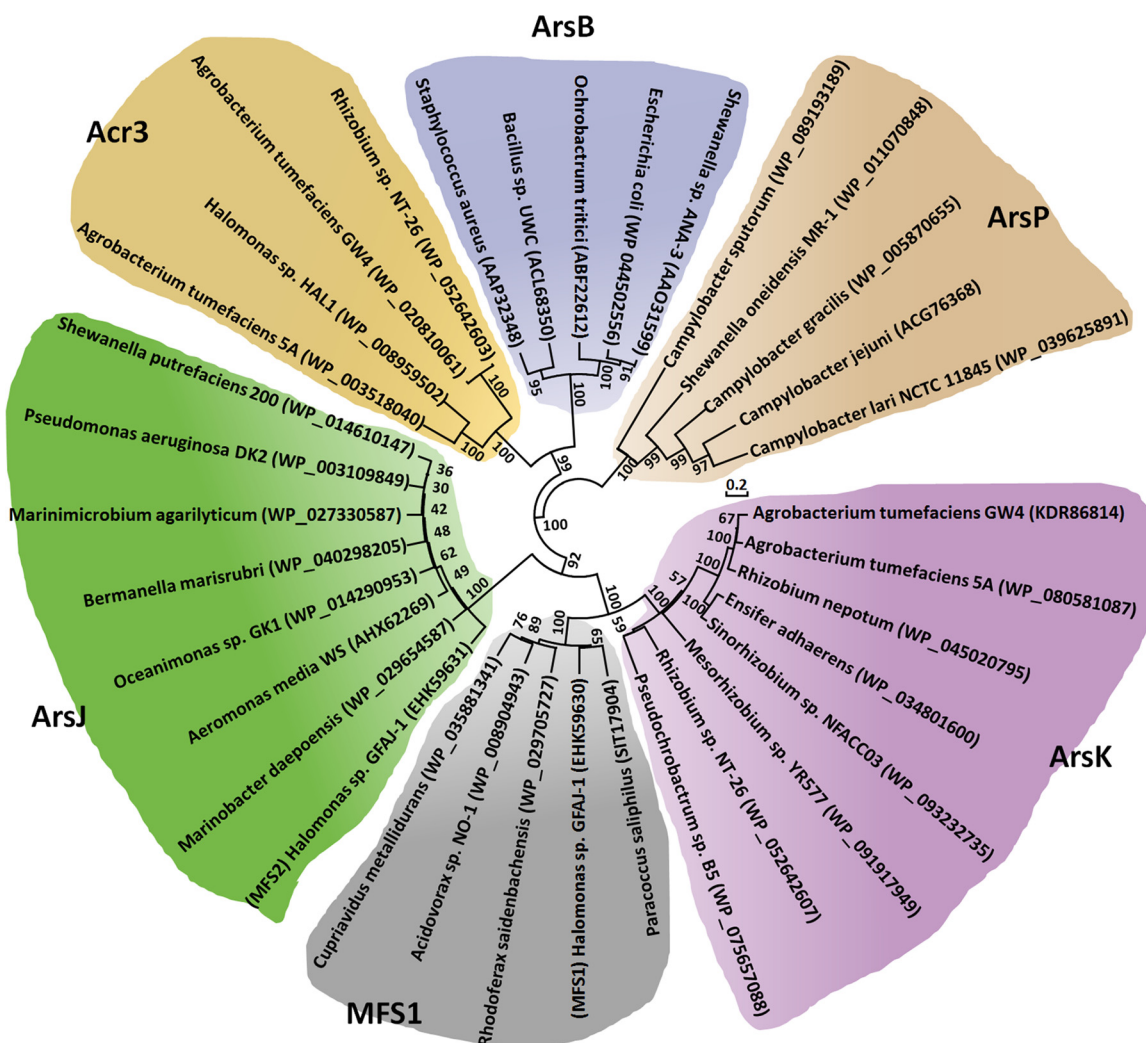


FIG 2 Neighbor-joining phylogenetic tree of ArsK amino acid sequences and other arsenic efflux proteins. The tree was produced by MEGA7. The bacterial names and the protein accession numbers are shown.

formed distinct groups together, but they were clearly divergent from each other in the subgroups (Fig. 2). The multiple sequence alignments and phylogenetic analysis revealed that ArsK may be a novel arsenic efflux protein that is clearly divergent from ArsB, Acr3, ArsP, ArsJ, and MFS1.

ArsK confers the resistance to As(III), Sb(III), Rox(III), and MAs(III). To investigate the function of the *arsK* gene in the *ars* operon, the *arsK* gene from strain GW4 was cloned into pUC19 under the control of the *lac* promoter, creating the plasmid pUC19-ArsK. ArsK was expressed in the arsenic-hypersensitive strain AW3110, and the resistance to arsenicals was assayed. Arsenicals exist in several different oxidation states and species, and the following arsenicals/antimonites were used to testify the function of ArsK: trivalent inorganic arsenical As(III), trivalent inorganic antimonite Sb(III), pentavalent inorganic arsenical As(V), and trivalent organic arsenicals Rox(III), MAs(III), and dimethyl-As(III). Due to the low toxicity of pentavalent inorganic antimonate Sb(V) and pentavalent organic arsenicals Rox(V), MAs(V), and dimethylarsenate [DMAs(V)], strain AW3110 grew well even with high concentrations of these substances (data not shown). Therefore, we did not test bacterial resistance to Sb(V), Rox(V), MAs(V), and DMAs(V).

As shown in Fig. 3A, strain AW3110 cells harboring the plasmid pUC19-ArsK were resistant up to 200 μ M As(III). Strain AW3110(pUC19-ArsK) was resistant up to 100 μ M

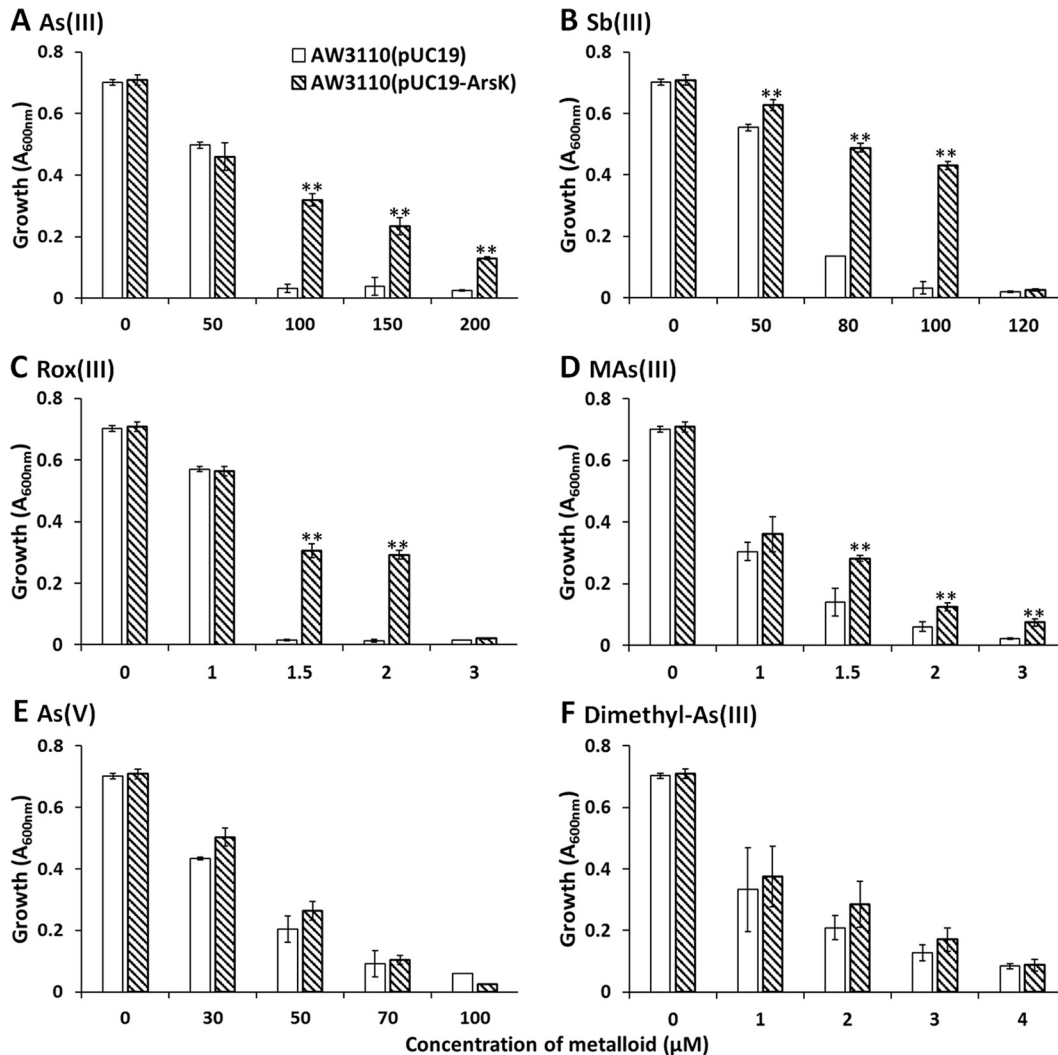


FIG 3 ArsK conferred resistance to As(III), Sb(III), Rox(III), and MAs(III). Growth of *E. coli* strain AW3110 with plasmid pUC19 or pUC19-ArsK was measured with the addition of different amounts of As(III) (A), Sb(III) (B), Rox(III) (C), MAs(III) (D), As(V) (E), and dimethyl-As(III) (F). The data are the means from three replicates. **, $P < 0.01$.

Sb(III) (Fig. 3B). Strain AW3110(pUC19-ArsK) was resistant up to 2 μM Rox(III) (Fig. 3C). Strain AW3110(pUC19-ArsK) was resistant up to 3 μM MAs(III) (Fig. 3D). In contrast, cells expressing *arsK* had the same level of resistance to As(V) and dimethyl-As(III) as cells of the same strain harboring an empty pUC19 vector (Fig. 3). In addition to arsenicals, we also tested bacterial resistance to Cd(II), and ArsK did not confer resistance to Cd(II) (see Fig. S3).

ArsK is essential for reducing cellular As(III), Sb(III), Rox(III), and MAs(III) accumulation. To investigate the efflux function of ArsK, the effect of *arsK* expression on the accumulation of As(III), Sb(III), Rox(III), MAs(III), As(V), and dimethyl-As(III) was examined (Fig. 4). Cells expressing *arsK* accumulated less As(III), Sb(III), Rox(III), and MAs(III) than cells lacking the *arsK* gene (Fig. 4). However, there was no significant difference in either As(V) or dimethyl-As(III) between the cells expressing *arsK* and those with empty vector only (Fig. 4E and F). These results indicated that ArsK can export As(III), Sb(III), Rox(III), and MAs(III) out of cells but has no effect on As(V) or dimethyl-As(III). In addition, compared to that in strain AW3110(pUC19), the intracellular accumulation of As(III) and Sb(III) in strain AW3110(pUC19-ArsK) was reduced by approximately 50% in 15 min, and so we speculated that As(III) and Sb(III) may be the best substrates of ArsK. Next, we performed a mutational analysis by site-directed mutagen-

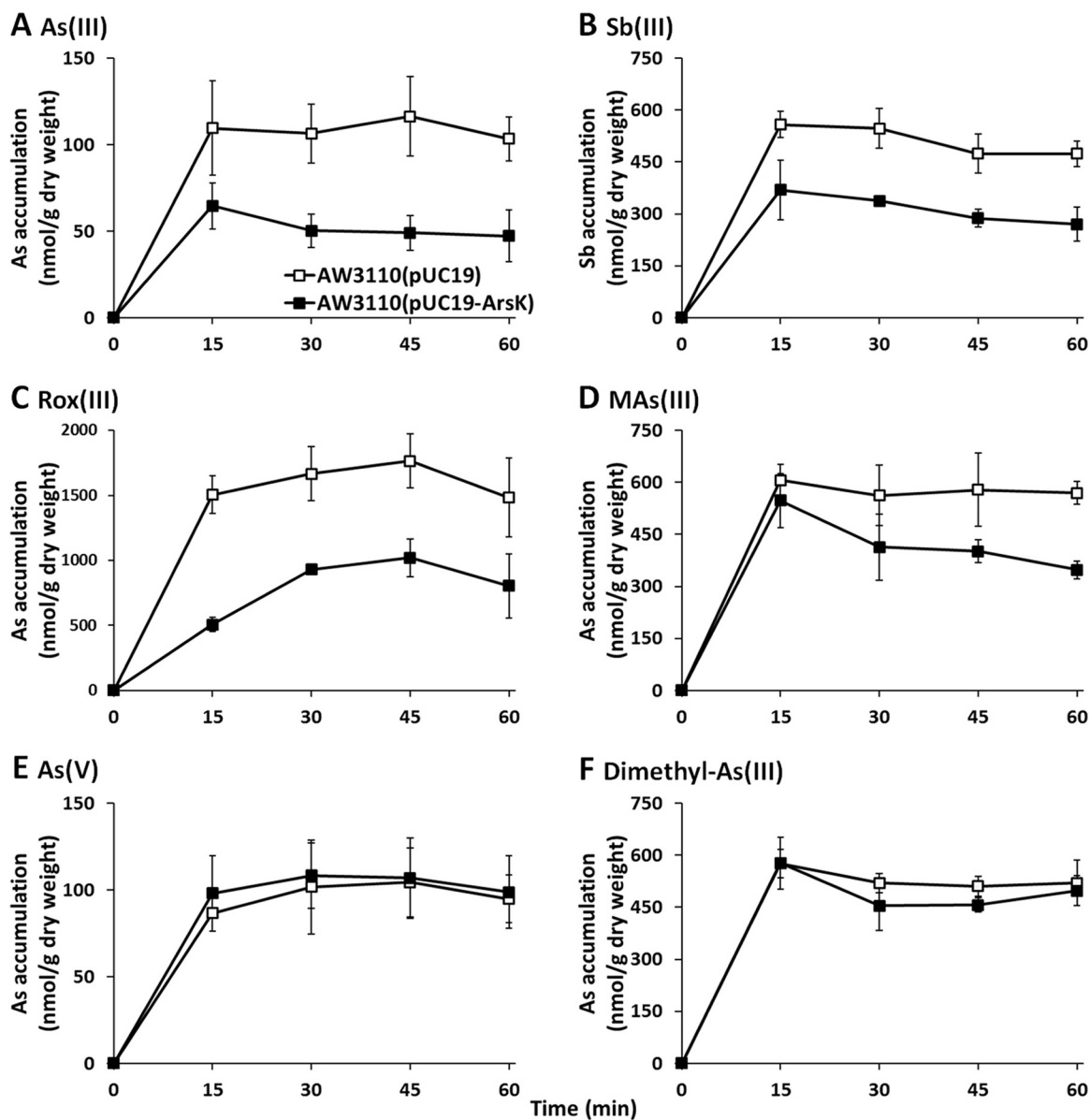


FIG 4 ArsK reduced the cellular accumulation of As(III), Sb(III), Rox(III), and MAs(III). The levels of cellular As(III) (A), Sb(III) (B), Rox(III) (C), MAs(III) (D), As(V) (E), and dimethyl-As(III) (F) in *E. coli* strain AW3110 with plasmid pUC19 or pUC19-ArsK are shown. The data are the means from three replicates.

esis of each of the three residues (Cys97, Cys183, and Cys318) and then examined the metalloid resistance of these mutant strains. However, the mutation of a single cysteine residue did not influence the function of ArsK (see Fig. S4).

As(III), Sb(III), Rox(III), MAs(III), and As(V) induce *arsK* expression. To further investigate the function of the *arsK* gene, *lacZ* reporter gene assays were performed. As expected, *arsK::lacZ* expression was significantly induced by As(III), Sb(III), Rox(III), and MAs(III) in wild-type GW4 (Fig. 5), which was consistent with the resistance and efflux phenotype of ArsK to As(III), Sb(III), Rox(III), and MAs(III) (Fig. 3 and 4). Dimethyl-As(III) did not induce the expression of *arsK::lacZ*, consistent with the null resistance and efflux phenotype of ArsK related to dimethyl-As(III) (Fig. 3F and 4F). However, the expression of *arsK::lacZ* was also induced by As(V) (Fig. 5E), even though ArsK had no effect on the resistance or efflux of As(V) (Fig. 3E and 4E). We speculate that when As(V) was added to the medium, As(V) was reduced to As(III) by ArsC, and then As(III) induced the expression of *arsK::lacZ*. These results indicate that ArsK can be induced by As(III), Sb(III),

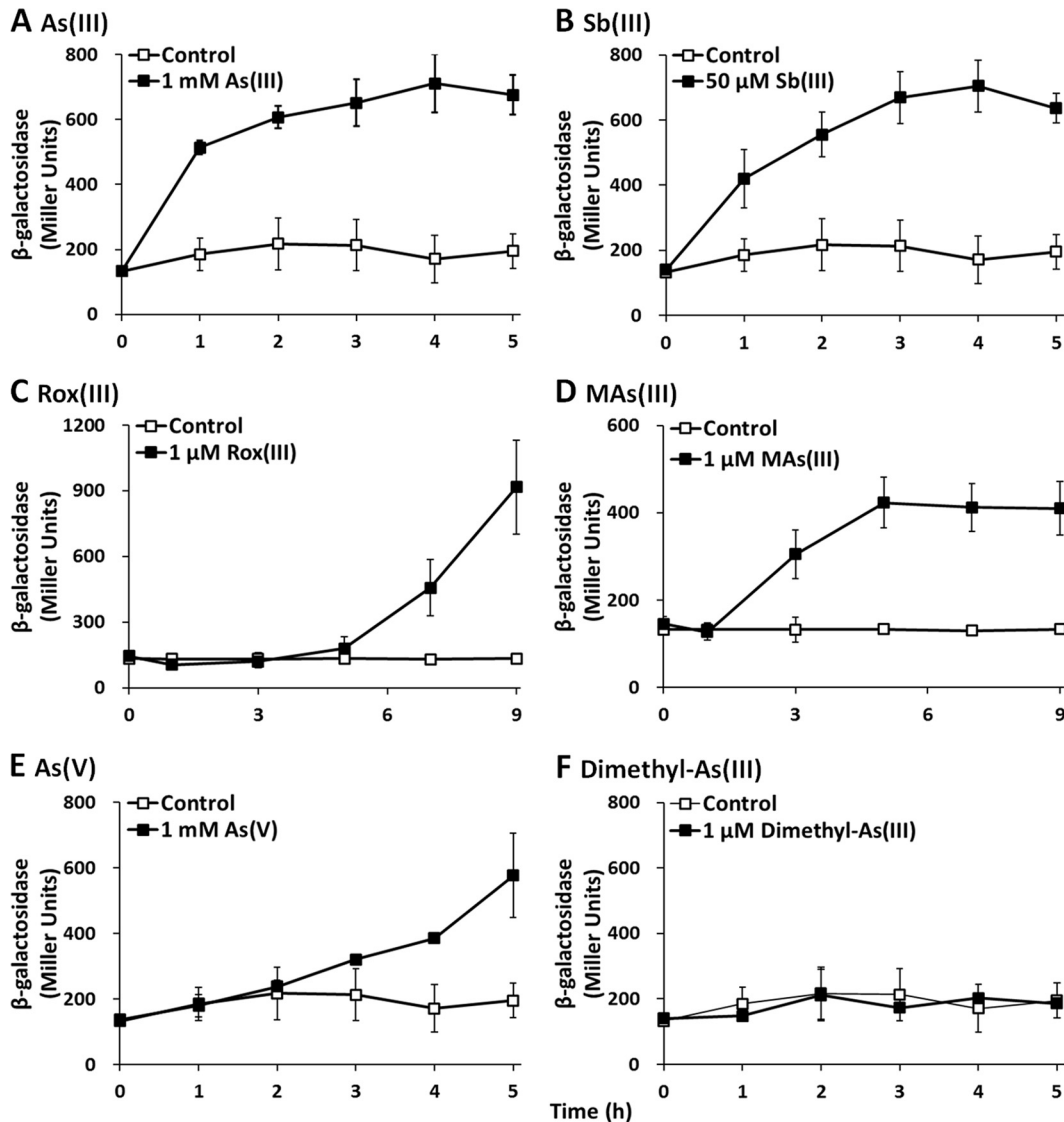


FIG 5 Expression of *arsK* was induced by As(III), Sb(III), Rox(III), MAs(III), and As(V) in strain GW4. Strain GW4 containing the expression plasmid pLSP-*arsR2-arsK* was cultured in MMNH₄ medium with or without the addition of As(III) (A), Sb(III) (B), Rox(III) (C), MAs(III) (D), As(V) (E), or dimethyl-As(III) (F). The data are the means from three replicates.

Rox(III), and MAs(III), which then extrudes As(III), Sb(III), Rox(III), and MAs(III) out of the cells.

As(III) and Sb(III) induced the expression of *arsK* within 1 h, but MAs(III) induced expression after 1 h and Rox(III) induced expression only after 5 h. The delayed time of induction of MAs(III) and Rox(III) may be caused by the slow uptake of MAs(III) and Rox(III) into the cells. Currently, the uptake mechanisms of MAs(III) and Rox(III) remain unknown. In addition, the amounts of As(III), Sb(III), Rox(III), and MAs(III) needed for the induction of *arsK* expression varied from micromoles to millimoles (Fig. 5). The toxicity of Rox(III)/MAs(III), Sb(III), and As(III) is in a decreasing order, which appears to be correlated with the increasing substrate amount for induction (Fig. 5).

ArsR2 negatively regulates the expression of *arsK*. Reverse transcriptase PCRs (RT-PCRs) showed that *arsR2* and *arsK* are cotranscribed (Fig. 6A), revealing that *arsR2* and *arsK* are in the same operon. To investigate the regulation of *ArsR2* and the transcription expression of *arsK*, electrophoretic mobility shift assays (EMSAs) were performed. As shown in Fig. 6B, an increase in *ArsR2* protein in the binding assay resulted in electrophoretic shifts of the 6-carboxyfluorescein (FAM)-labeled *arsR2-arsK*

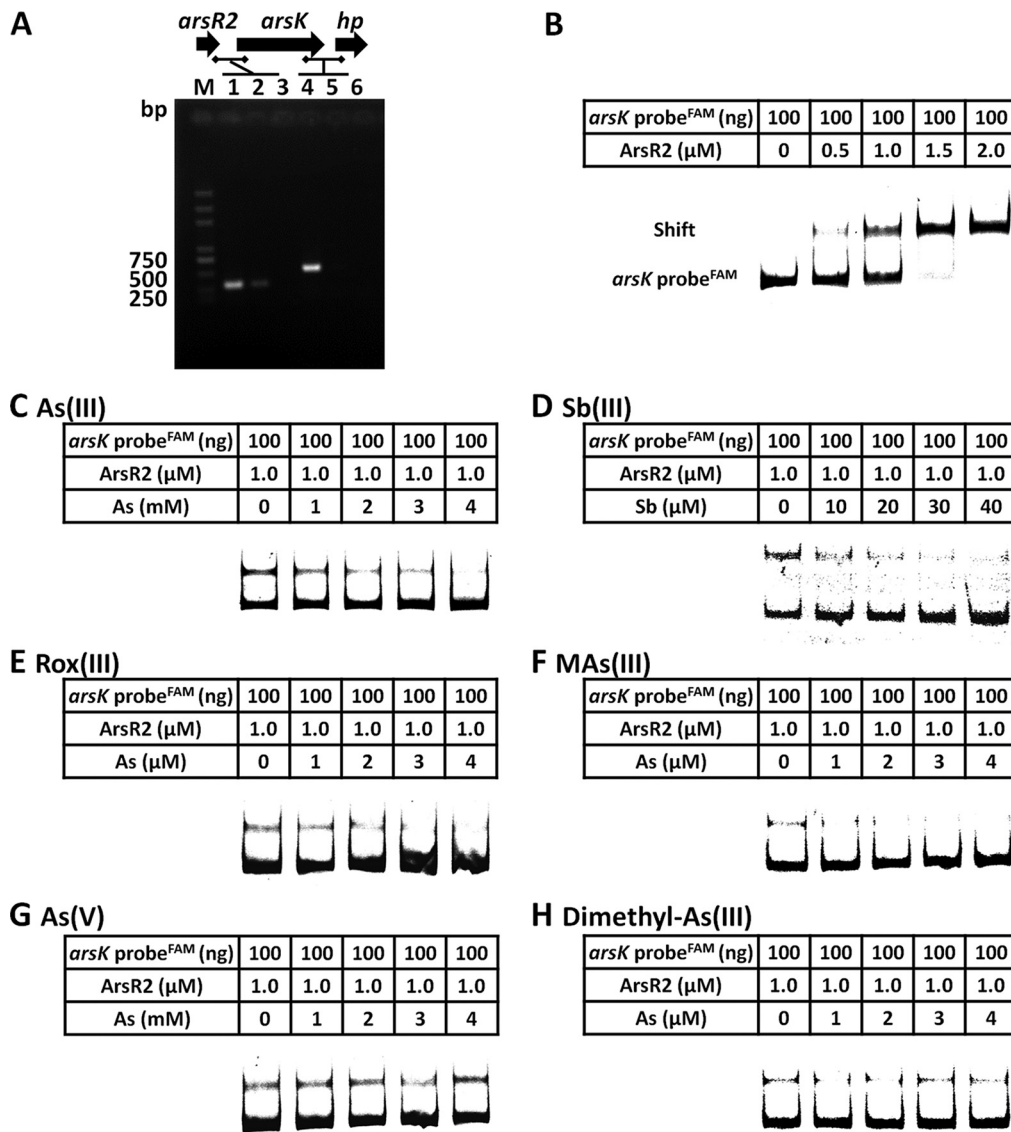


FIG 6 ArsR2 negatively regulates the expression of *arsK*. (A) RT-PCR analysis illustrating the cotranscription of *arsR2* and *arsK*. Total RNA was extracted from strain GW4 grown in MMNH₄ medium with 1 mM As(III). M, molecular weight marker (DL 2000 plus). In lanes 1 and 4, total DNA was used as the template; in lanes 2 and 5, total RNA was used as the template; in lanes 3 and 6, double-distilled water (ddH₂O) was used as the template. The horizontal lines represent the locations of primers. (B) EMSAs. FAM-labeled *arsR2-arsK* probe interacted with ArsR2 protein. The amounts of DNA probes and ArsR2 are shown in the tables above each panel. Competition EMSAs including *arsR2-arsK* probe and ArsR2 protein with increasing amounts of As(III) (C), Sb(III) (D), Rox(III) (E), MAs(III) (F), As(V) (G), or dimethyl-As(III) (H). The amount of DNA probe was 100 ng, the amount of ArsR2 was 1.0 μM, and the amounts of each metalloid are shown in the tables above each panel.

promoter. These results indicated that ArsR2 interacted with the *arsR2-arsK* operon regulatory region. Furthermore, with the addition of increasing amounts of As(III), Sb(III), Rox(III), and MAs(III), the electrophoretic shifts of the FAM-labeled *arsR2-arsK* promoter gradually disappeared (Fig. 6C to F). However, As(V) and dimethyl-As(III) had no effect on the interaction between ArsR2 and DNA (Fig. 6G and H). These results were in accordance with the resistance and efflux function of ArsK for As(III), Sb(III), Rox(III), and MAs(III) but not for As(V) or dimethyl-As(III) (Fig. 3 and 4). These results indicate that As(III), Sb(III), Rox(III), and MAs(III) bind ArsR2, affecting a change in the conformation of ArsR2. ArsR2 then regulates the expression of *arsK*, and the ArsK transporter excretes the relevant substance out of the cells. Besides, the derepression amounts of As(III), Sb(III), and Rox(III)/MAs(III) for EMSAs were in a decreasing order, which was correlated between the metalloid toxicities.

DISCUSSION

The results from this study are consistent with the conclusion that ArsK is a novel membrane transporter mediating resistance to several organic and inorganic arsenicals or antimonials in *A. tumefaciens* GW4. This conclusion is supported by multiple lines of evidence. The expression of *arsK* in the arsenic-sensitive strain AW3110 resulted in increased resistance to As(III), Sb(III), Rox(III), and MAs(III) but had no effect on bacterial resistance to As(V) or dimethyl-As(III). ArsK reduced intracellular As(III), Sb(III), Rox(III), and MAs(III) accumulation in strain AW3110 but did not affect the concentration of As(V) or dimethyl-As(III). In addition, the expression of *arsK* was induced by As(III), Sb(III), Rox(III), and MAs(III). These results indicate that ArsK functions in an efflux mechanism specific for As(III), Sb(III), Rox(III), and MAs(III). A genome analysis showed that the *arsK* gene is widely distributed in various species, indicating that the apparent efflux function mediated by ArsK is a common resistance mechanism in arsenic-resistant bacteria. This finding enriches our knowledge of the various mechanisms of bacterial arsenic resistance.

A previous study analyzed approximately 2,500 genomes and over 700 putative membrane transporters encoded in the identified *ars* operons (23). Distinct clusters of ArsB, Acr3, and ArsP were found, whereas MFS proteins were divided into two sub-clusters (23). A phylogenetic analysis revealed that there are at least two types of MFS superfamily arsenic efflux transporters in arsenic-resistant bacteria. As expected, ArsJ and MFS1 belong to the MFS superfamily (12, 15), and now another MFS superfamily transporter, ArsK, has been identified in this study. Although these three transporters belong to the same MFS superfamily, multiple lines of evidence show conspicuous differences among ArsK, MFS1, and ArsJ. First, the phylogenetic affiliation of ArsK, ArsJ, and MFS1, based on their amino acid sequences, revealed that they formed distinct groups and were clearly divergent. Second, ArsJ and the *mfs1-arsJ-GAPDH* gene operon both confer resistance to As(V) but not to As(III) (12). However, ArsK confers resistance to As(III), Sb(III), Rox(III), and MAs(III) but not to As(V) or dimethyl-As(III). These results clearly reveal that ArsK is a novel arsenic transporter with different functions compared to those of ArsJ and MFS1 (21).

ArsK has very different characteristics compared to the other five types of arsenic efflux transporters, namely, ArsB, Acr3, ArsP, ArsJ, and MFS1. As a toxic metalloid element, arsenic exists in several different oxidation states and species (25, 26). Interestingly, arsenic efflux transporters show high specificity for oxidation states and inorganic/organic species. ArsB, Acr3, and ArsP only confer resistance to trivalent arsenicals, and ArsJ only confers resistance to pentavalent arsenicals (11, 12, 14, 22). ArsB, Acr3, and ArsJ only confer resistance to inorganic arsenicals, and ArsP only confers resistance to organic arsenicals (11, 12, 14, 22). Although ArsK only confers resistance to trivalent arsenicals, it confers resistance to both inorganic and organic forms. Therefore, ArsK is a new arsenic transporter that confers resistance to both inorganic and organic arsenicals. A genome analysis of eight arsenic-resistant bacteria showed that ArsK frequently coexists with Acr3 or ArsB. We speculate that ArsK independently confers organic arsenical resistance and confers inorganic arsenical resistance by cooperating with Acr3 or ArsB.

ArsK confers a novel arsenic resistance mechanism in *A. tumefaciens* GW4, and a model for the mechanism is presented in Fig. 7. ArsR2 is a repressor and interacts with the promoter region of the *arsR2-arsK* operon. When As(III), Sb(III), Rox(III), or MAs(III) is taken up by cells, they can react with protein sulfhydryl groups, thereby causing cellular stress. As(III), Sb(III), Rox(III), or MAs(III) interacts with ArsR2 and changes the protein conformation. Subsequently, the interaction between ArsR2 and the *arsR2-arsK* operon is weakened, which activates the expression of *arsK*. ArsK specifically extrudes As(III), Sb(III), Rox(III), and MAs(III) out of the cells and reduces the intracellular concentrations to avoid toxicity. In summary, an inorganic and organic arsenical resistance mechanism was identified in *A. tumefaciens* GW4. The *arsR2-arsK* operon gene *arsK* encodes a

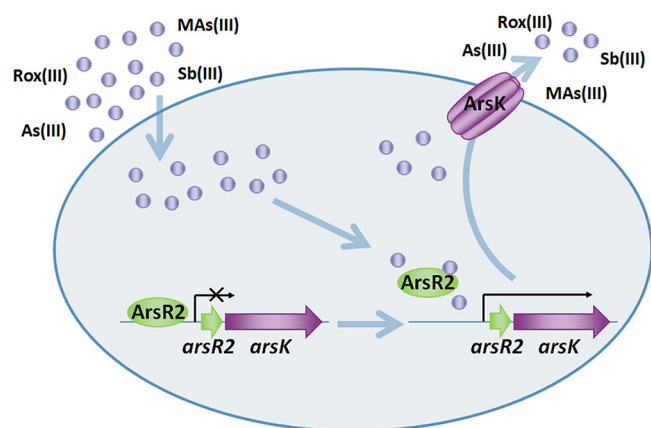


FIG 7 Proposed roles of ArsK and ArsR2 in As(III), Sb(III), Rox(III), and MAs(III) transport and resistance. As(III), Sb(III), Rox(III), or MAs(III) is taken up into the cells and then interacts with ArsR2. The altered conformation of ArsR2 releases its suppression of the *arsR2-arsK* operon, and then the *arsK* gene is expressed. ArsK specifically catalyzes the efflux of As(III), Sb(III), Rox(III), and MAs(III) out of the cells.

trivalent inorganic and organic arsenical efflux transporter, and the gene *arsR2* encodes a regulator involved in the regulation of *arsK* transcriptional expression.

MATERIALS AND METHODS

Strains, media, and reagents. *Escherichia coli* AW3110, which is hypersensitive to As(III), was used (Table 1) (12). For most experiments, *E. coli* cultures bearing the indicated plasmids were grown aerobically in lysogeny broth (LB) or low-phosphate minimal mannitol (MMNH₄) medium at 37°C supplemented with 100 μg/ml ampicillin (Amp) or 50 μg/ml chloramphenicol (Cm), as indicated. MMNH₄ medium contains 10 g of D-mannitol, 1 g of K₂HPO₄, 1 g of KH₂PO₄, 0.25 g Na₂PO₄, 0.01 g of FeCl₃, 0.25 g of MgCl₂, and 0.1 g of CaCl₂ per liter. Bacterial growth was monitored by measuring the absorbance at 600 nm (27). All reagents were obtained from commercial sources. Rox(III), MAs(III), and dimethyl-As(III) were prepared by reduction of the pentavalent forms. Briefly, 0.2 mM arsenical was mixed with 27 mM Na₂S₂O₃, 66 mM Na₂S₂O₅, and 82 mM H₂SO₄, after which the pH was adjusted to 6 with NaOH (28).

Plasmid construction. For the expression of ArsK (accession number KDR86814.1) in *E. coli*, the *arsK* gene was cloned from *A. tumefaciens* GW4 genomic DNA into the plasmid pUC19 under the control of the *lac* promoter, creating plasmid pUC19-ArsK (Table 1) (12). The primers pUC19-ArsK-F and pUC19-ArsK-R were used for cloning *arsK* (Table 2). The PCR fragment was gel purified, digested with the indicated restriction enzymes, and ligated into the vector plasmid pUC19, which had been digested with HindIII and BamHI, generating plasmid pUC19-ArsK (Table 1).

Metalloid resistance assays. For metalloid resistance assays in liquid media, AW3110 competent cells were transformed with the indicated plasmids. Cells were grown overnight with shaking at 37°C in LB with 100 μg/ml Amp and 50 μg/ml Cm. The overnight cultures were diluted 100-fold in MMNH₄ medium with 100 μg/ml Amp and 50 μg/ml Cm containing various concentrations of metal(loid)s plus

TABLE 1 The strains and plasmids used in this research

Strain or plasmid	Description ^a	Reference or source
Strains		
<i>A. tumefaciens</i> GW4	Wild type, As(III)-oxidizing strain	24
<i>E. coli</i> DH5α	<i>supE44 lacU169(φ80lacZΔM15) hrdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Invitrogen
<i>E. coli</i> S17-1	F ⁻ RP4-2-Tc::Mu <i>aphA</i> ::Tn7 <i>recA λpir</i> lysogen; Sm ^r Tp ^r	Invitrogen
<i>E. coli</i> BL21	F ⁻ <i>ompT hsdS_B (r_B⁻ m_B⁻) gal dcm me131 (DE3) pLysS (Cm^r)</i>	Invitrogen
<i>E. coli</i> AW3110	<i>ΔarsRBC::cam F-IN(rrn-rrnE)</i>	12
Plasmids		
pUC19	Amp ^r , clone and expression vector	MiaoLingBio
pUC19-ArsK	pUC19 containing ArsK coding region	This study
pLSP-KT2lacZ	Km ^r , <i>oriV</i> , <i>lacZ</i> -fusion vector used for <i>lacZ</i> fusion constructs	8
pLSP- <i>arsR2-arsK</i>	pLSP-KT2lacZ containing <i>arsR2-arsK</i> promoter	This study
pET-28(a+)	Km ^r , His6 tag expression vector	Novagen
pET-ArsR2	pET-28(a+) containing ArsR2 coding region	This study
pUC19-C97	Cys97 site mutant of ArsK	This study
pUC19-C183	Cys183 site mutant of ArsK	This study
pUC19-C318	Cys318 site mutant of ArsK	This study

^aTc, tetracycline; Sm, streptomycin; Tp, trimethoprim; Cm, chloramphenicol; Km, kanamycin; Amp, ampicillin.

TABLE 2 The primers used in this research

Primer	Description ^a	Use
pUC19-ArsK-F	5'- <u>AAAAAGCTT</u> GATGAGGGTGACGTCGTTG-3'	Cloning for gene expression
pUC19-ArsK-R	5'-AAAGGATCCCGTCTCACTGTCAATGCCTC-3'	
arsR2-arsK-F	5'-GACATCCGACAGTCAGGG-3'	Verification of cotranscription
arsR2-arsK-R	5'-CACGGAGCCGATAGTCA-3'	
arsK-hp-F	5'-GGTGCCATCAGTGTGCG-3'	Verification of cotranscription
arsK-hp-R	5'-CGCTGCAATCAGTCCC-3'	
pLSP-arsK-F	5'-AAAGAATTCCGCTTGACTTCGGTGAC-3'	Cloning for <i>lacZ</i> reporter gene
pLSP-arsK-R	5'-AAAGGATCCCAACGCACGGACAAT-3'	
pET28-arsK-F	5'-AAAGGATCCATGGAAGAACGTCA-3'	Overexpression of ArsK
pET28-arsK-R	5'-AAAAAGCTTAGAGCCGATACGATGC-3'	
arsK-EMSA-F	5'-GTGTCTTGCCGTTTGC-3'	Cloning for EMSA
arsK-EMSA-R	5'-CCCTGACTGTCGGATGT-3'	
C97-F	5'-ACGCTCGCGATTGGCGCTTGGTGC-3'	Site mutant for ArsK
C97-R	5'-GCCAATCGCGAGCGTCAGGGCCGCT-3'	
C183-F	5'-AACCTGTTTCTTGGTATGCCACTTC-3'	Site mutant for ArsK
C183-R	5'-ACCAAGAAACAGGTTTATTCTGCA-3'	
C318-F	5'-GCCTTCGCCATCGGCCTTGGGCTCG-3'	Site mutant for ArsK
C318-R	5'-GCCGATGGCGAAGGCGACCCGACCC-3'	

^aThe underlined sequences denote the restriction enzyme sites.

0.3 mM isopropyl- β -D-thiogalactoside (IPTG) and incubated at 37°C with shaking for another 48 h. The growth was estimated from the absorbance at 600 nm.

Cotranscription assays. Overnight cultures were inoculated into 100 ml MMNH₄ medium with the addition of 1 mM As(III) and incubated at 28°C with 100 rpm shaking. The samples used for RNA isolation were taken after a 16-h cultivation (mid-log phase). Total RNA was extracted with TRIzol (Invitrogen) and incubated with RNase-free DNase I (TaKaRa) at 37°C to remove genomic DNA, and the reaction was then terminated by the addition of 50 mM EDTA at 65°C for 10 min. After determining the concentration of RNA by spectrophotometry (NanoDrop 2000; Thermo), 1,000 ng total RNA was reverse transcribed into cDNA with a RevertAid First Strand cDNA synthesis kit (Thermo). The resulting cDNA was used as the template in the PCR system, and the primers arsR2-arsK-F and arsR2-arsK-R were used for testing the cotranscription of *arsR2* and *arsK* genes (Table 2). The primers arsK-hp-F and arsK-hp-R were used for testing the cotranscription of *arsK* and hypothetical protein (HP) genes (Table 2) (29). The correct sizes of these two PCR products are 321 bp and 534 bp, respectively. The lack of the arsK-HP gene product with RNA indicated that the RT-PCRs were not contaminated with DNA.

Detection of the amount of cellular metalloids. *E. coli* cells expressing ArsK and with the expression vector only were grown to an optical density at 600 nm (OD₆₀₀) of 2 at 37°C in LB. The cells were harvested and suspended in buffer A (75 mM HEPES-KOH [pH 7.5], 0.15 M KCl, and 1 mM MgSO₄) (22). To initiate the transport reaction, 20 μ M each metalloid was added to a 5-ml cell suspension. Aliquots (1 ml) from the cell suspension were withdrawn at the indicated times, washed twice at room temperature with 1 ml buffer A, and lysed using an ultrasonic cell disruptor (Ningbo Xinzhi Instruments). To monitor the metalloid concentration, we used high-performance liquid chromatography with hydride generation-atomic fluorescence spectroscopy (HPLC-HG-AFS) (Beijing Haiguang Instruments) (30).

Site-directed mutagenesis. The site-directed mutagenesis of ArsK was generated by the Fast Mutagenesis system (TransGen Biotech), which was used in our previous study (31). The primers C97-F/C97-R, C183-F/C183-R, and C318-F/C318-R were used for PCR and the construction of the mutational vectors (Table 2).

Reporter gene assays. The reporter gene assays in this study were evaluated based on β -galactosidase activity. The promoter region of *arsR2* was predicted by BPROM (<http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>) and PCR amplified using primers pLSP-arsK-F and pLSP-arsK-R (Table 2). The promoter sequence (Table 2) was inserted into the EcoRI-BamHI sites of pLSP-KT2lacZ, and the resulting plasmids were then introduced into strain GW4 via conjugation. Overnight cultures were inoculated (200 μ l) into 100 ml MMNH₄ with or without metalloids and incubated at 28°C with 100 rpm shaking. During the incubation, β -galactosidase assays were conducted as described previously (31, 32).

Plasmid construction, expression, and purification of ArsR2. The *arsR2* gene was PCR cloned as a BamHI-HindIII fragment using primers pET28-arsK-F and pET28-arsK-R (Table 2) into pET-28a(+), resulting in pET-28a-*arsR2* (Table 1). BL21 cells containing pET-28a-*arsR2* were induced at an OD₆₀₀ of 0.4 by adding 0.3 mM IPTG and cultivated at 20°C for 12 h. They were then harvested by centrifugation (7,000 \times g for 10 min at 4°C) and resuspended in borate saline buffer (pH 8.0) with 20 mM imidazole (33). Unbroken cells and fragments were collected by centrifugation at 7,000 rpm for 10 min. The supernatant was mixed with 2 ml Ni-NTA His-Bind resin (7sea Biotech) and gently agitated at 4°C for 1 h to allow the polyhistidine-tagged protein to bind to the resin. The resin was washed with 10 ml borate saline buffer containing 60 mM imidazole and then eluted with 5 ml borate saline buffer containing 300 mM imidazole. Fractions were collected and analyzed by SDS-PAGE, and protein concentrations were determined spectrophotometrically (NanoDrop 2000; Thermo) (31).

Electrophoretic mobility shift assays. The DNA fragment of the *arsR2* regulatory region was amplified using the primers arsK-EMSA-F and arsK-EMSA-R (Table 2). The forward primer was labeled with

the fluorophore 6-carboxyfluorescein (FAM). All reaction mixtures with or without metalloids were incubated at 28°C for 30 min in binding buffer (20 mM Tris-HCl [pH 7.0], 50 mM NaCl, 1 mM dithiothreitol [DTT], 10 mM MgCl₂, 100 μg/ml bovine serum albumin [BSA]). The binding solution was then loaded onto a 6% native PAGE gel. After 3 h of running at 80 V in 1× Tris-glycine-EDTA (TGE) buffer (120 mM Tris, 950 mM glycine, 5 mM EDTA), the gels were exposed in a phosphor imaging system (Fujifilm FLA-5100) (31).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01842-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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