Identification of the Biosynthetic Gene Cluster for the Organoarsenical Antibiotic Arsinothricin

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ABSTRACT The soil bacterium Burkholderia gladioli GSRB05 produces the natural compound arsinothricin [2-amino-4-(hydroxymethylarsinoyl) butanoate] (AST), which has been demonstrated to be a broad-spectrum antibiotic. To identify the genes responsible for AST biosynthesis, a draft genome sequence of B. gladioli GSRB05 was constructed. Three genes, arsQML, in an arsenic resistance operon were found to be a biosynthetic gene cluster responsible for synthesis of AST and its precursor, hydroxyarsinothricin [2-amino-4-(dihydroxyarsinoyl) butanoate] (AST-OH). The arsL gene product is a noncanonical radical S-adenosylmethionine (SAM) enzyme that is predicted to transfer the 3-amino-3-carboxypropyl (ACP) group from SAM to the arsenic atom in inorganic arsenite, forming AST-OH, which is methylated by the arsM gene product, a SAM methyltransferase, to produce AST. Finally, the arsQ gene product is an efflux permease that extrudes AST from the cells, a common final step in antibiotic-producing bacteria. Elucidation of the biosynthetic gene cluster for this novel arsenic-containing antibiotic adds an important new tool for continuation of the antibiotic era.

IMPORTANCE Antimicrobial resistance is an emerging global public health crisis, calling for urgent development of novel potent antibiotics. We propose that arsinothricin and related arsenic-containing compounds may be the progenitors of a new class of antibiotics to extend our antibiotic era. Here, we report identification of the biosynthetic gene cluster for arsinothricin and demonstrate that only three genes, two of which are novel, are required for the biosynthesis and transport of arsinothricin, in contrast to the phosphonate counterpart, phosphinothricin, which requires over 20 genes. Our discoveries will provide insight for the development of more effective organoarsenical antibiotics and illustrate the previously unknown complexity of the arsenic biogeochemical cycle, as well as bring new perspective to environmental arsenic biochemistry.

KEYWORDS arsinothricin, organoarsenical antibiotic, biosynthetic gene cluster, Burkholderia gladioli GSRB05

Arsenic is one of the most ubiquitous environmental toxic substances. Bacteria have taken advantage of its prevalence to evolve mechanisms that give them competitive advantages over bacterial competitors. These include pathways for arsenic respiration for energy generation (1–3), methylation of inorganic arsenic to increase its potency as an antimicrobial (4, 5), and even incorporation into arsenolipids for sparing phosphate under nutrient-limiting conditions (6, 7). A newly recognized adaptation is the synthesis of a novel arsenic-containing antibiotic.

Antibiotic resistance is a global health challenge, and new antibiotics are urgently needed. Recently the rice rhizosphere bacterium Burkholderia gladioli GSRB05 was shown to produce the broad-spectrum arsenic-containing antibiotic arsinothricin (2-amino-4-hydroxymethylarsinoyl) butanoate (AST) (8). AST effectively inhibits growth of the World Health Organization (WHO) priority pathogens such as carbapenem-resistant...
Enterobacter cloacae (CRE) but has low cytotoxicity in human monocytes (9). It also inhibits growth of Mycobacterium bovis BCG, which causes tuberculosis (TB) in animals and humans and is closely related to Mycobacterium tuberculosis, the major causative agent of human TB. TB is classified by the WHO as a global health emergency (10), and the WHO has called for the development of new and innovative antibiotics against M. tuberculosis (https://www.who.int/activities/tackling-the-drug-resistant-tb-crisis). AST is a pentavalent organoarsenical and a nonproteinogenic amino acid analog of glutamate. It inhibits glutamine synthetase (GS), most likely because of its chemical similarity to the acyl-phosphate intermediate γ-glutamyl phosphate, in the GS catalytic cycle (9). GS is essential for nitrogen metabolism in M. tuberculosis, and inhibitors of M. tuberculosis GS are actively sought after as drugs against TB (11).

B. gladioli GSRB05 is the only known AST producer that has been shown to produce both pentavalent hydroxyarsinothricin [2-amino-4-(dihydroxyarsinoyl) butanoate] (AST-OH) and AST from As(III), with a possible precursor-product relationship (8). Here, we identified the biosynthetic gene cluster (BGC) for AST production from the genome of B. gladioli GSRB05. Three genes, arsQ, arsM, and arsL, are organized in an arsenic resistance (ars) operon. By way of comparison, the phosphonate analog of AST is the Streptomyces antibiotic phosphinothricin, which is used commercially as the herbicide glufosinate and has a very complicated biosynthetic pathway (12). The BGC from Streptomyces viridochromogenes consists of 24 genes (accession no. X65195) (13), so a three-gene BGC for arsinothricin production is surprisingly small.

Identification of the AST BGC makes substantial contributions in two important areas—treatment of infectious diseases and radical S-adenosylmethionine (SAM) chemistry. First, the BGC is an uncomplicated pathway with only three steps required for the synthesis of AST. This arsenic-containing compound may be the founding member of a new class of antibiotics, adding to our arsenal of weapons against multidrug-resistant pathogens. Second, the radical SAM enzyme BgArsL (i.e., B. gladioli ArsL) catalyzes the key enzymatic reaction in AST biosynthesis. Radical SAM enzymes form the largest enzyme superfamily (14). Most members catalyze the transfer of a 5′-deoxyadenosyl radical to the substrate or function as methyltransferases using a methylene fragment from SAM (15). In contrast, BgArsL is a noncanonical radical SAM enzyme that transfers the 3-amino-3-carboxypropyl moiety to As(III), forming AST-OH, a unique radical SAM reaction.

**RESULTS**

**Identification of the AST biosynthetic gene cluster.** In this study, a time course of AST biosynthesis by B. gladioli GSRB05 was conducted in cells grown in ST 10⁻¹ medium (Fig. 1). After a lag period of approximately 10 h, both trivalent and pentavalent AST-OH and pentavalent AST were produced. After approximately 15 h, all of the As(III) was consumed, the trivalent AST-OH peak decreased, and both the AST and the pentavalent AST-OH peaks increased correspondingly. We interpret this result as As(III) conversion into trivalent AST-OH in the first step, which is then either methylated to AST in a second step or oxidized to pentavalent AST-OH. After 24 h, both the pentavalent and trivalent AST-OH peaks decreased, and the AST peak increased reciprocally, suggesting that the strain is able to reduce pentavalent AST-OH to the trivalent form to produce more AST. It is not clear if the product is trivalent or pentavalent AST because in our hands trivalent AST oxidizes in air too rapidly to be isolated. Trivalent AST-OH also oxidizes in air, but less rapidly than AST.

To identify the AST BGC knowing that methylation is involved, we made the assumption that a B. gladioli GSRB05 enzyme would be related to other known methylating enzymes. Arsenic methylation catalyzed by the ArsM As(III) S-adenosylmethionine methyltransferase is a common reaction in arsenic metabolism (16). Bacterial and algal ArsM enzymes methylate inorganic As(III) up to three times to produce methylarsenite [MAs(III)], dimethylarsenite [DMAs(III)], and trimethylarsenite [TMAs(III)]. These are...
rapidly oxidized nonenzymatically in air to the pentavalent species, which are neither substrates nor products of the enzyme-catalyzed reaction. We predicted that a *B. gladioli* GSRB05 ArsM ortholog is involved in the methylation of trivalent AST-OH and that its gene would be in the AST BGC. For that reason, a draft sequence of the *B. gladioli* GSRB05 genome was constructed (see Fig. S2 in the supplemental material). As anticipated, an *arsM* sequence was identified next to a cluster of genes related to arsenic metabolism but divergently oriented (Fig. 2A). These genes located on the opposite DNA strand are *arsR* (17), *pitA* (18), *aioAB* (2), encoding a putative ArsR As(III)-responsive transcriptional repressor, PitA, a low-affinity inorganic phosphate/arsenate transporter, and the AioAB arsenite oxidase that oxidizes As(III) to As(V), respectively. The predicted products of the genes upstream and downstream of *arsM* were searched against the basic local alignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov) using the BLASTP program. The gene immediately upstream of *arsM* is termed *arsQ* and encodes a putative membrane transporter annotated as related to the GntP family of gluconate permeases (19). The gene immediately downstream of *arsM* encodes a putative radical SAM protein and is termed *arsL*. The *arsQML* cluster is found in the genomes of other proteobacterial genomes but is not widespread in the bacterial kingdom (Fig. 2B). Their association suggests that these three genes have a related function. The next four genes were termed *orf1–4* and are annotated as encoding two class I SAM-dependent methyltransferases, a cytochrome P450-like protein, and an α/β-hydrolase. The four genes are not adjacent to *arsQML* in other bacterial genomes, and as described below, these four genes appear to be unrelated to AST biosynthesis.

The *B. gladioli* *arsM* gene product (BgArsM) is a 378-amino-acid-residue protein with a predicted mass of 41.71 kDa (accession no. MBW5287222). Most ArsM and related animal AS3MT enzymes have four conserved cysteine residues that are required...
for catalysis (20, 21). Multiple-sequence alignment of BgArsM and orthologs shows that BgArsM has four cysteine residues at positions 30, 54, 181, and 233 (see Fig. S3 in the supplemental material). The N terminus of BgArsM does not align well with those of other ArsMs, but Cys30 is in approximately the position expected for the first conserved cysteine, and the other three align well with the remaining three conserved cysteine residues. The *arsL* gene product (BgArsL) is a 428-amino-acid-residue protein with a predicted mass of 47.5 kDa (accession no. MBW5287221). Multiple-sequence alignment with other putative ArsL orthologs shows that BgArsL has three conserved cysteine residues at positions 194, 198, and 201 (see Fig. S4 in the supplemental material). It is annotated as a radical SAM enzyme, with these three cysteine residues forming a CX$_3$CX$_2$C motif that is found in more than 90% of members of the radical SAM superfamily. In the cyanobacterium *Synechocystis* sp. strain PCC 6803, a radical SAM enzyme, SsArsS, has been shown to function with SsArsM to catalyze the initial steps in arsenosugar biosynthesis (22). It was therefore reasonable to consider that BgArsM and BgArsL might function together in AST biosynthesis. The *arsQML* genes plus the four downstream *orf* genes were cloned and transformed into *Escherichia coli* Top10. However, the transformants grew poorly. Considering that the heterologous expression of membrane proteins, in general, might be toxic for the host cells, *arsQ* was eliminated from the construct, leaving *arsML* and the four *orf* genes. Cells of *E. coli* Top10 with the remaining six genes grew well, indicating that *arsQ* was responsible for growth retardation. With the addition of 1 $\mu$M As(III), cells

**FIG 2** The *B. gladioli* GSRB05 AST biosynthetic gene cluster. (A) The *B. gladioli* GSRB05 genome (accession no. JAGSIB000000000) contains a cluster of *ars* genes, including *arsQML*. (B) Phylogenetic distribution of the genes of the AST BGC. GenBank accession numbers are given in Materials and Methods.

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This document is a continuation of the previous text, discussing the biosynthesis of arsenosugars in *B. gladioli* GSRB05. It highlights the roles of the proteins BgArsM and BgArsL in this process, focusing on their cysteine residues and their alignment with orthologs. The study includes the cloning and expression of the *ars* genes in *Escherichia coli* Top10, leading to an understanding of the growth properties of the transformants in the presence of As(III). The figure illustrates the gene cluster and its phylogenetic distribution among various bacteria, providing insights into the evolutionary context of this biosynthetic pathway.
expressing the six genes produced AST and smaller amounts of AST-OH (Fig. 3A). Cells expressing an \textit{arsL}–orf1–4 construct lacking \textit{arsM} produced only AST-OH and not AST. These results indicate that one or more of the five genes downstream of \textit{arsM} are involved in AST-OH biosynthesis, followed by methylation to AST by \textit{BgArsM}.

**BgArsM and \textit{BgArsL} are both required for AST biosynthesis.** To determine which gene or genes are required for AST-OH production, a mutation producing a stop codon was introduced into \textit{arsL} and each of the other four genes in the \textit{arsL–orf1–4} construct. Cells expressing each mutant were incubated with As(III), and the culture medium was analyzed by high-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS). Stop codons in the downstream \textit{orf1–4} genes had no effect, and only the strain with the point mutation in the \textit{arsL} gene lost the ability to produce AST-OH (Fig. 3B). Thus, only \textit{arsL} is required for AST-OH production. These results strongly suggest that the biosynthetic pathway of AST is composed of only two reactions catalyzed by BgArsM and BgArsL. To confirm this hypothesis, we expressed \textit{arsL} alone and the \textit{arsML} genes together in the pETDuet-1 system (Fig. 4). Cells of \textit{E. coli} Top10 expressing the \textit{arsML–orf1–4} construct produced AST-OH and smaller amounts of AST (Fig. 3A).
coli BL21 expressing *arsL* alone produced AST-OH but not AST. In contrast, AST production was observed when *arsL* and *arsM* were coexpressed.

The *arsQML* genes comprise an As(III)-inducible *ars* operon. To examine whether the *arsQ*, *arsL*, and *arsM* genes comprise an As(III)-responsive *ars* operon, total RNA was obtained from cells of *B. gladioli* GSRB05 with or without As(III), and cDNA was subsequently synthesized by reverse transcription-PCR (RT-PCR). From the amount of RNA detected in quantitative real-time PCR (RT-qPCR) analysis, expression of *arsQ*, *arsM*, and *arsL* increased approximately 8.5-, 7.2-, and 11.9-fold, respectively, following induction with 3 μM As(III) compared with RNA from uninduced cells (Fig. 5). The results demonstrate that the *arsQML* genes comprise an *ars* operon. As(III) responsiveness suggests that the operon is controlled by an ArsR repressor, likely the product of the upstream *arsR* gene. When the reverse primers were designed to anneal a cDNA region including either *orf1* or *orf2*, almost no amplification was observed, indicating that the downstream four *orf* genes are not part of the *arsQML* operon.

**ArsQ is an AST efflux permease.** Antibiotic BGCs frequently have genes for efflux of the antibiotic. This serves dual purposes of removing the active antibiotic from the cytosol, thus protecting the producing organism, and exporting the compound into the medium, where it can exert its antibiotic action against other bacteria. The *arsQ* gene encodes a putative membrane protein of 408 amino acid residues with a predicted mass of 42.9 kDa (accession no. MBW5287223). ArsQ orthologs are found primarily in *Proteobacteria* and are annotated as gluconate permeases. AST and gluconate are similar in size and charge, so a reasonable inference is that the BgArsQ functions as an AST efflux permease. A multiple-sequence alignment with the 100 most closely related proteins shows variation in the N-terminal 90 residues, but the remaining 318 residues are highly conserved (see Fig. S5 in the supplemental material). The *arsQ* gene was cloned into vector pTrcHisA and transformed into the As(III)-hypersensitive strain *E. coli* AW3110 (23).

Addition of the inducer IPTG (isopropyl-β-D-thiogalactopyranoside) produced growth inhibition, so the cells were grown without inducer, and the effect of leaky expression of *arsQ* on AST inhibition was assayed (Fig. 6A). Cells expressing *arsQ* were clearly more resistant to AST than those with vector only. The resistance was not dramatic—probably due to low levels of expression of BgArsQ. Next, the effect of *arsQ* expression on accumulation of AST was examined. Cells expressing BgArsQ accumulated significantly less AST than the control (Fig. 6B). Uptake into everted vesicles...
reflects efflux from cells (24, 25), so everted membrane vesicles were prepared from those cells, and energy-driven uptake of AST into those vesicles was assayed. Cells expressing BgAST took up significantly more AST than the control (Fig. 6C). These results demonstrate that expression of arsQ increases resistance by active efflux of AST.

**DISCUSSION**

Identification of the biosynthetic gene cluster for arsinothricin is an essential step in elucidation of the antimicrobial action of this novel arsenic-containing antibiotic. With only three genes, the BGC is surprisingly small, especially compared with the BGC for the phosphonate counterpart, phosphinothricin, which includes 24 genes. The AST BGC has a rather narrow phylogenetic distribution. The arsQML gene cluster is found primarily in members of the *Proteobacteria* phylum, such as the classes *Alphaproteobacteria* (*Rhodobacter*), *Betaproteobacteria* (*Burkholderia*), and *Gammaproteobacteria* (*Pseudomonas*). Most BgArsQ orthologs are found in *Proteobacteria*, while orthologs of BgArsM and BgArsL are more widely distributed. However, it is not clear if those are involved in AST biosynthesis. Near other arsL genes are also genes for other putative permeases that are unrelated to ArsQ. It is not known if their substrates include AST, but alternate AST transporters would not be surprising. This is reminiscent of the existence of multiple permeases for inorganic and organic arsenicals, such as ArsB, Acr3, ArsP, ArsK, and ArsJ (26). We propose that AST is synthesized in a three-step pathway: (i) AST-OH synthesis by BgArsL, (ii) methylation of AST-OH to AST by BgArsM, and (iii) export of AST by BgArsQ (Fig. 7).

BgArsL can be predicted to be a radical SAM enzyme, a member of the largest enzyme superfamily. It has three cysteine residues (Cys194, Cys198, and Cys201) that form a [4Fe-4S] cluster for reductive
cleavage of SAM. Since BgArsL has only three conserved cysteine residues, it likely has a single $[4\text{Fe}-4\text{S}]^1$ cluster. The majority of radical SAM enzymes produce a 5'-deoxyadenosyl radical that performs a wide variety of organic chemical biotransformations (15). AST-OH is an amino acid with arsenate replacing the γ-carboxyl group of glutamic acid, so it is reasonable to propose that BgArsL catalyzes addition of the 3-amino-3-carboxypropyl (ACP) radical that creates a C-As bond with As(III), producing trivalent AST-OH. This is different from most radical SAM enzymes that form 5'-deoxyadenosyl radical by reductive cleavage of SAM at the 5' position of the adenosine moiety. In the second step, BgArsM uses a second molecule of SAM to methylate trivalent AST-OH, generating trivalent AST, which spontaneously oxidizes nonenzymatically to the antibiotic pentavalent AST.

FIG 6 BgArsQ is an AST efflux permease. (A) Expression of arsQ confers resistance to AST. Overnight cultures of E. coli AW3110 bearing either pTrcHisArsQ (shaded bars) or vector plasmid pTrcHisA (black bars) were diluted 100-fold into fresh M9 medium containing the indicated concentrations of AST. $A_{600}$ was measured after 24 h of growth at 37°C. (B) Uptake of AST (40 μM) was assayed in cells of E. coli AW3110 bearing either pTrcHisArsQ (●) or vector plasmid pTrcHisA (○). (C) Uptake of AST (20 μM) was assayed in everted vesicles prepared from cells of E. coli AW3110 expressing pTrcHisArsQ (●) or vector plasmid pTrcHisA (○) with 5 mM NADH as an energy source, as described in Materials and Methods. Data are the mean ± SE (n = 3).

FIG 7 Proposed pathway of AST biosynthesis. AST biosynthesis by B. gladioli GSRB05 is composed of two steps. In the first step, the noncanonical radical SAM enzyme BgArsL cleaves the Cγ bond of SAM, forming a 3-amino-3-carboxypropyl (ACP) radical that creates a C-As bond with As(III), producing trivalent AST-OH. This is different from most radical SAM enzymes that form 5'-deoxyadenosyl radical by reductive cleavage of SAM at the 5' position of the adenosine moiety. In the second step, BgArsM uses a second molecule of SAM to methylate trivalent AST-OH, generating trivalent AST, which spontaneously oxidizes nonenzymatically to the antibiotic pentavalent AST.
bohydrates and 0.2% glucose. The culture was incubated until As(III) was completely transformed into AST. AST was

A mobile phase of 3 mM malonic acid, 5% methanol, and tetrabutylammonium hydroxide to reach a pH of 5.9 was used to elute a C18 reverse-phase column at a flow rate of 1.0 ml min⁻¹.

The substrate specificity of BgArsM was determined by using a C18 reversed-phase column. The elution conditions were as follows: a mobile phase of 3 mM malonic acid, 5% methanol, and tetrabutylammonium hydroxide to reach a pH of 5.9. The flow rate was 1.0 ml min⁻¹.

The results of transport assays with cells of E. coli heterologously expressing the arsQ gene transform As(III) into AST-OH, and cells expressing both arsM and arsQ genes produce ATS. Thus, we propose that in the second step, trivalent AST-OH is methylated by BgArsM to form the reduced form of AST, which would rapidly oxidize, yielding the pentavalent antibiotic AST. Future tests of this proposal will involve purification of BgArsM for enzymatic assays and development of analytical methods to detect the trivalent form of AST.

Antibiotic producers frequently have transporters that transport the active antibiotic from the cells, both for self-protection and to inhibit growth of competitors (29). In the third and final step, AST is exported from the cells by the efflux permease BgArsQ. The results of transport assays with cells of E. coli heterologously expressing arsQ clearly demonstrate that BgArsQ reduces the intracellular concentration of AST, and in complementary assays with everted membrane vesicles, there is energy-dependent uptake of AST. It is not known whether BgArsQ substrates are trivalent, pentavalent, or both, whether AST-OH and AST are both substrates, or even if gluconate is a substrate, due to apparent toxicity caused by arsQ expression. Future experiments will be designed to produce sufficient BgArsQ for biochemical characterization.

**MATERIALS AND METHODS**

**Strains, plasmid, media, and growth conditions.** Strains and plasmids used in this study are described in Table S1 in the supplemental material. B. gladioli GSRB05 and E. coli cultures were grown aerobically overnight with shaking in lysogeny broth (LB) medium (30) at 30 or 37°C, respectively. M9 medium (30) was supplemented with 0.2% glucose, 0.1 mM CaCl₂, and 1 mM MgSO₄. For resistance assays, antibiotics were supplemented at the following final concentrations, as indicated: 50 μg/ml streptomycin (Sm), 25 μg/ml chloramphenicol (Cm), and 100 μg/ml ampicillin (Ap).

**AST production.** The protocol for AST production was modified from the original method (8). A single colony of B. gladioli GSRB05 was inoculated into 10 ml of LB medium and grown overnight. The culture was 100-fold diluted into 1 liter of fresh LB and grown again to an A₆₀₀ of 1. Next, the cells were harvested and transferred into the same volume of ST 10⁻¹ medium (31) supplemented with 20 μM As(III) and 0.2% glucose. The culture was incubated until As(III) was completely transformed into AST. AST was chromatographically purified as described previously (8) with a yield of approximately 5 mg AST/liter of culture medium and was free of AST-OH.

**Analysis of arsenic species.** Arsenic species in the supernatant were analyzed by high-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS). For sample preparation, 0.5 ml of culture was collected and centrifuged at 13,000 rpm for 2 min at 4°C. Supernatants were filtered with 3-kDa Amicon Ultra Centrifugal filters (MilliporeSigma, St. Louis, MO, USA) for 10 min. A mobile phase of 3 mM malonic acid, 5% methanol, and tetrabutylammonium hydroxide to reach a pH of 5.9 was used to elute a C18 reverse-phase column at a flow rate of 1.0 ml min⁻¹.

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Construction of the draft genome sequence of *B. gladioli* GSRB05. Genome sequencing of *B. gladioli* GSRB05 was performed using the Illumina NextSeq platform at Center for Genomics Technology, University of Miami, Miller School of Medicine (Miami, FL, USA). Sequence data were comprised of 7.8 million paired-end (2 × 150) reads. Quality trimming and filtering were performed using the TrimGalore (version 0.6.4) ([https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/](https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)) tool to remove adapter sequences, base pairs with a quality score of <30, and reads shorter than 50 bp. Quality-trimmed reads were assembled using SPAdes (version 3.13.0) (32) and ABySS (version 2.1.5) (33) at different kmers, and optimal assembly was selected as described previously (34). Genome annotation was accomplished using the NCBI Prokaryotic Genome Annotation Pipeline (35), and predictions of *arsM* orthologous genes were performed via BLAST (36) and OrthoMCL (37) sequence analysis against the predicted proteins.

Cloning and expression. Genomic DNA was extracted from 3 ml of a fresh overnight culture of *B. gladioli* GSRB05 using the E.Z.N.A. Bacterial DNA kit (Omega Bio-tek, Inc., Norcross, GA, USA). The gene cluster was sequentially amplified by PCR with *Pfu* Turbo high-fidelity DNA polymerase (Agilent Technologies, Inc., Santa Clara, CA, USA), and the entire cluster or groups of genes were cloned into plasmid pUC118 between the KpnI and XbaI restriction sites. To construct pUCarsL–orf1–4, the cloning was carried out in two steps, while for pUCarsML–orf1–4, an additional step was necessary (see Fig. S1 in the supplemental material) to amplify fragments of no more than 3.2 kb to avoiding incorrect base insertion. Primers were designed with unique restriction sites to serially construct the final plasmids. The amplified products were gel purified, digested with the appropriate restriction enzymes, and inserted into vector plasmid pUC118 with the first gene in frame with the lacZa gene of the vector. The *arsL* and *arsML* genes were ordered from GenScript (GenScript, Piscataway, NJ, USA) and cloned into pETDuet-1 (Millipore Sigma) and *arsQ* in pTrcHisA (Thermo Fisher Scientific, Inc., Waltham, MA, USA) to construct pETDuet-arsL, pETDuet-arsML, and pTcarsQ, respectively. Each step of cloning was verified by sequencing the fragments. The complete list of the oligonucleotides used in this study is given in Table S1.

Site-directed mutagenesis. The primers for site-directed mutagenesis (see Table S2 in the supplemental material) were designed using the online QuickChange Primer Design program ([https://www.agilent.com/store/primerDesignProgram.jsp](https://www.agilent.com/store/primerDesignProgram.jsp)). A stop codon was individually inserted into each of the five genes in plasmid pUCarsL–orf1–4 by substitution of one nucleotide at the beginning of the sequence. Unmutated plasmid pUCarsL–orf1–4 was removed from the reaction mixture by digesting the methylated DNA with restriction enzyme DpnI (New England Biolabs, Ipswich, MA, USA). Each mutated plasmid was transformed into competent cells of *E. coli* TOP10 and purified, and the presence of the mutation was verified by sequencing.

mRNA extraction, reverse transcription and quantitative real-time PCR. An RNasey minikit (Qiagen, Valencia, CA, USA) was used to isolate total RNA from a 3-ml culture of *B. gladioli* GSRB05 that had been cultured with or without exposure to 3 μM As(III) in ST 10^-1 medium for 13 h. The purity and concentrations of RNA were determined from the absorbance at 260 nm using a Synergy H4 Hybrid microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). RNA integrity was verified by electrophoresis (data not shown). Reverse transcription-PCR (RT-PCR) was performed to synthesize complementary DNA (cDNA) using a Verso cDNA synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions. Primer sets for real-time qPCR of target genes are listed in Table S2. One microliter of each of the purified RT-PCR products corresponding to 50 ng of total RNA was amplified in a 10-μl reaction mixture containing 0.5 μM each primer set and 5 μl of iQSYBR green supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Real-time qPCR assays were carried out using a Realplex2 PCR instrument (Eppendorf, Hamburg, Germany) with the following cycle steps: initial denaturation for 2 min at 94°C, followed by 40 cycles of 15 s at 94°C for denaturation, and then 30 s at 50°C for annealing, and 1 min 30 s at 72°C for extension of *arsQ*, *arsM*, and *arsL*. For fragments containing sequences from *arsM* to *orf1* (*arsM–orf1*) or from *arsL* to *orf2* (*arsL–orf2*), the reaction condition was changed to 30 s at 58°C for annealing and 3 min 10 s at 72°C for extension. All data were normalized to the amount of 16S rRNA. The threshold cycle (2^-ΔCt) was calculated to compare the expression level of each gene. Assays of *arsQ* function. To examine whether the *arsQ* gene could confer AST resistance, cells of *E. coli* AW3110 harboring plasmid pTrcHis2ArsQ were grown overnight in LB medium and washed and suspended in 0.9% NaCl. The washed cells were then inoculated into M9 medium at an A_600nm of 0.03 and incubated for 24 h in the presence of the indicated concentrations of AST.

To determine the function of ArsQ in AST transport, the arsQ gene was cloned into vector plasmid pTrcHisA. Cells of *E. coli* AW3110 expressing plasmid pTrcHisArsQ vector plasmid pTrcHisA only were grown to an A_600nm of 2 at 37°C with aeration in LB medium. The cells were harvested and suspended in buffer A (75 mM HEPES-KOH [pH 7.5], 0.15 M KCl, and 1 mM MgSO4) at an A_600nr of 4. To initiate the transport reaction, 20 μM AST was added to 1 ml of cell suspension. Portions (0.1 ml) from the cell suspension were withdrawn at the indicated times, filtered through nitrocellulose filters (0.2-μm pore diameter; EMD Millipore, Billerica, MA), and washed twice at room temperature with 5 ml of buffer A. The filters were digested with 0.3 μl of concentrated HNO3 (68 to 70%) overnight at room temperature. The dissolved filters were incubated for 10 min at 70°C, allowed to cool to room temperature, and diluted with HPLC-grade water (MilliporeSigma) to produce a final HNO3 concentration of 2%. Arsenic was quantified by ICP-MS. Standard solutions were made in the range of 1 to 50 ppb in 2% nitric acid using arsenic standards (Ultra Scientific, North Kingstown, RI, USA). Protein content was determined using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc.).

Everted membrane vesicles and transport assays were performed as described previously (38). Transport assays were performed in buffer A containing 0.25 M sucrose. The reaction mixture contained 1 mg/ml membrane proteins, 40 μM AST, and 5 mM NADH in a final volume of 0.6 ml of the same buffer.

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Portions (0.1 ml) were withdrawn at the indicated times, filtered through 0.2-μm-pore-size nitrocellulose filters, and washed twice with 5 ml of the same buffer. Arsenic content was determined by ICP-MS.

AST BGC distribution. The prevalence of AST BGC was analyzed in representative organisms. GenBank accession numbers of the following bacterial genomes are given in parentheses: B. gladioli GSRB05 (JAGSB000000000000) is compared with putative orthologs from Burkholderia oklahomensis (NZ_UFUH010000001), Burkholderia cepacia (NZ_CADEU01000000007), Pseudomonas aeruginosa (NZ_CACPETO100000007), Pseudomonas fluorescens (NZ_LVEJO100000018), Pseudomonas amygdali (NZ_LGLI010000031), Actibacterium sp. (NZ_JAFUEL010000009), and Rhodobacter sp. (NVUP010000011). Multiple alignments of the sequences of ArsM (Fig. 53), ArsR (Fig. 54), and ArsQ (Fig. 55) orthologs were performed using T-Coffee (39) and the BoxShade version 3.21 server (https://embnet.vital-it.ch/software/BOX_form.html).

Supplemental Material. The draft genome sequence for B. gladioli GSRB05 has been deposited in NCBI under accession no. JAGSB000000000000. Raw sequence reads have been deposited in NCBI under BioProject accession no. PRJNA722678.

Supplemental Material

Supplemental material is available online only.

Supplemental File 1, PDF file, 0.5 MB.

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We declare no conflicts of interest.

References

1. Krafft T, Macy JM. 1998. Purification and characterization of the respiratory arsenate reductase of Chrysiogenes arenatitis. Eur J Biochem 255:647–653. https://doi.org/10.1046/j.1432-1327.1998.2550647.x
2. Ellis PJ, Conrads T, Hille R, Kuhn P. 2001. Crystal structure of the 100 kDa arsenite oxidase from Alcaligenes faecalis in two crystal forms at 1.64 Å and 2.03 Å. Structure 9:125–132. https://doi.org/10.1016/S0969-2126(01)00566-4.
3. Zargar K, Hoefl S, Oremland R, Saltikow CW. 2010. Identification of a novel arsenate oxidase gene, arxA, in the haloalkaliphilic, arsenite-oxidizing bacterium Alkalilimnicola ehrlichii strain MLHE-1. J Bacteriol 192:3755–3762. https://doi.org/10.1128/JB.00244-10.
4. Qin J, Rosen BP, Zhang Y, Wang G, Franke S, Rensing C. 2006. Arsenic detoxification and evolution of trimethylarsine gas by a microbial arsenite S-adenosylmethionine methyltransferase. Proc Natl Acad Sci U S A 103:2075–2080. https://doi.org/10.1073/pnas.0506831103.
5. Chen J, Yoshinaga M, Rosen BP. 2019. The antibiotic action of methylarsenite is an emergent property of microbial communities. Mol Microbiol 111:487–494. https://doi.org/10.1111/mmi.14169.
6. Devalla S, Feldmann J. 2003. Determination of lipid-soluble arsenic species in seaweed-eating sheep from Orkney. Appl Organometal Chem 17:906–912. https://doi.org/10.1002/aoc.550.
7. Amayo KO, Petursdottir A, Newcombe C, Gunnlaugsdottir H, Raab A, Krupp EM, Feldmann JR. 2011. Identification and quantification of arseno-lipids using reversed-phase HPLC coupled simultaneously to high-resolution ICPIMS and high-resolution electrospray MS without species-specific standards. Anal Chem 83:3589–3595. https://doi.org/10.1021/ac2005873.
8. Kuramata M, Sakakibara F, Kataoka R, Yamazaki K, Baba K, Ishizaka M, Hiradate S, Kamo T, Ishikawa S. 2016. Arsinothricin, a novel organoarsenic species produced by a rice rhizosphere bacterium. Environ Chem 13:723–731. https://doi.org/10.1071/EN14247.
9. Nadar VS, Chen J, Dheeman DS, Galvan AE, Yoshinaga-Sakurai K, Kandavelu P, Sankaran B, Kuramata M, Ishikawa S, Rosen BP, Yoshinaga M. 2019. Arsinothricin, an arsenic-containing non-proteinogenic amino acid analog of glutamyl-tRNA synthetase, is a broad-spectrum antibiotic. Commun Biol 2:131. https://doi.org/10.1038/s42003-019-0365-y.
10. Gygli SM, Borrell S, Trauner A, Gagneux S. 2017. Antimicrobial resistance in Mycobacterium tuberculosis: mechanistic and evolutionary perspectives. FEMS Microbiol Rev 41:354–373. https://doi.org/10.1093/femsre/fux011.
11. Berlinski L, Objojska A, Forlani G, Kafarski P. 2005. Design, synthesis, and activity of analogues of phosphinothricin as inhibitors of glutamine synthetase. J Med Chem 48:6340–6349. https://doi.org/10.1021/jm050474e.
12. Schinko E, Schad K, Eys S, Keller U, Wohlfleben W. 2009. Phosphinothricin-tripeptide biosynthesis: an original version of bacterial secondary metabolism? Phytochemistry 70:1787–1800. https://doi.org/10.1016/j.phytochem.2009.09.002.
13. Blodgett JA, Zhang JK, Yu X, Metcalf WW. 2016. Conserved biosynthetic pathways for phosholacine, bialaphos and newly discovered phosphonic acid natural products. J Antibiot (Tokyo) 69:15–25. https://doi.org/10.1038/ja.2015.77.
14. Wang SC, Frey PA. 2007. S-adenosylmethionine as an oxidant: the radical SAM superfamily. Trends Biochem Sci 32:101–110. https://doi.org/10.1016/j.tibs.2007.01.002.
15. Roach PL. 2011. Radicals from S-adenosylmethionine and their application to biosynthesis. Curr Opin Chem Biol 15:267–275. https://doi.org/10.1016/j.cbpa.2011.08.009.
16. Chen J, Rosen BP. 2020. The arsenic methylcation cycle: how microbial communities adapted methylarsenicals for use as weapons in the continuing war for dominance. Front Environ Sci 8. https://doi.org/10.3389/fenvs.2020.00043.
17. Wu J, Rosen BP. 1991. The ArsR protein is a trans-acting regulatory protein. Mol Microbiol 5:1331–1336. https://doi.org/10.1111/j.1365-2958.1991.tb00779.x.
18. Willsky GR, Malamy MH. 1980. Characterization of two genetically separable inorganic phosphate transport systems in Escherichia coli. J Bacteriol 144:356–365. https://doi.org/10.1128/JB.144.1.356-365.1980.
19. Klemm P, Tong S, Nielsen H, Conway T. 1996. The gntP gene of Escherichia coli involved in gluconate uptake. J Bacteriol 178:61–67. https://doi.org/10.1128/jb.178.1.61-67.1996.
20. Dheeman DS, Packianathan C, Pillai JK, Rosen BP. 2014. Pathway of human ASM3T arsenic methylolation. Chem Res Toxicol 27:1979–1989. https://doi.org/10.1021/tx500313k.
21. Marapakala K, Packianathan C, Ajees AA, Dheenan DS, Sankaran B, Kandavelu P, Rosen BP. 2015. A disulfide-bond cascade mechanism for As (III) S-adenosylmethionine methyltransferase. Acta Crystallogr D Biol Crys-
tallogr 71:505–515. https://doi.org/10.1107/S1399004714027552.
22. Xue YM, Ye J, Raber G, Rosen BP, Francesconi K, Xiong C, Zhu Z, Rensing C, Zhu YG. 2019. Identification of steps in the pathway of arsenosugar biosynthesis. Environ Sci Technol 53:634–641. https://doi.org/10.1021/acs.est.8b04389.
23. Carlin A, Shi W, Dey S, Rosen BP. 1995. The ars operon of Escherichia coli confers arsenical and antimonal resistance. J Bacteriol 177:981–986. https://doi.org/10.1128/jb.177.4.981-986.1995.
24. Rosen BP, Tsuchiya T. 1979. Preparation of everted membrane vesicles from Escherichia coli for the measurement of calcium transport. Methods Enzymol 56:233–241. https://doi.org/10.1016/0076-6879(79)56026-1.
25. Chen J, Madegowda M, Bhattacharjee H, Rosen BP. 2015. ArsP: a methylarsenate efflux permease. Mol Microbiol 98:625–635. https://doi.org/10.1111/mmi.13145.

26. Garbinski LD, Rosen BP, Chen J. 2019. Pathways of arsenic uptake and efflux. Environ Int 126:585–597. https://doi.org/10.1016/j.envint.2019.02.058.

27. Zhang Y, Zhu X, Torelli AT, Lee M, Dzikovski B, Koralewski RM, Wang E, Freed J, Krebs C, Ellick SE, Lin H. 2010. Diphthamide biosynthesis requires an organic radical generated by an iron-sulphur enzyme. Nature 465:891–896. https://doi.org/10.1038/nature09138.

28. Broderick JB, Duffus BR, Duschene KS, Shepard EM. 2014. Radical S-adenosylmethionine enzymes. Chem Rev 114:4229–4317. https://doi.org/10.1021/cr4004709.

29. Li X-Z, Plésiat P, Nikaido H. 2015. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. Clin Microbiol Rev 28:337–418. https://doi.org/10.1128/CMR.00117-14.

30. Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning, a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

31. Maki T, Hasegawa H, Watarai H, Ueda K. 2004. Classification for dimethyldimethylarsenate-decomposing bacteria using a restrict fragment length polymorphism analysis of 16S rRNA genes. Anal Sci 20:61–68. https://doi.org/10.2116/analsci.20.61.

32. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021.

33. Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJM, Birol I. 2009. ABYSS: a parallel assembler for short read sequence data. Genome Res 19:1117–1123. https://doi.org/10.1101/gr.089532.108.

34. Utturkar SM, Klingeman DM, Land ML, Schadt CW, Doktycz MJ, Pelletier DA, Brown SD. 2014. Evaluation and validation of de novo and hybrid assembly techniques to derive high-quality genome sequences. Bioinformatics 30:2709–2716. https://doi.org/10.1093/bioinformatics/btu391.

35. Tatusova T, DiCuccio M, Badetdin A, Chetverin V, Nawrocki EP, Zaslavsky L, Lomsadze A, Pruitt KD, Borodovsky M, Ostell J. 2016. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res 44:6614–6624. https://doi.org/10.1093/nar/gkw569.

36. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL. 2009. BLAST+: architecture and applications. BMC Bioinformatics 10:421. https://doi.org/10.1186/1471-2105-10-421.

37. Li L, Stoeckert CJ, Roos DS. 2003. OrthoMCL: identification of orthologous groups for eukaryotic genomes. Genome Res 13:2178–2189. https://doi.org/10.1101/gr.1224503.

38. Villadangos AF, Fu HL, Gil JA, Messens J, Rosen BP, Mateos LM. 2012. Efflux permease CgAcr3-1 of Corynebacterium glutamicum is an arsenite-specific antiporter. J Biol Chem 287:723–735. https://doi.org/10.1074/jbc.M111.263335.

39. Notredame C, Higgins DG, Heringa J. 2000. T-Coffee: a novel method for fast and accurate multiple sequence alignment. J Mol Biol 302:205–217. https://doi.org/10.1006/jmbi.2000.4042.