



# Arsenic Detoxification by *Geobacter* Species

Yan Dang,<sup>a,c</sup> David J. F. Walker,<sup>a</sup> Kaitlin E. Vautour,<sup>a</sup> Steven Dixon,<sup>b</sup> Dawn E. Holmes<sup>a,b</sup>

Department of Microbiology, University of Massachusetts—Amherst, Amherst, Massachusetts, USA<sup>a</sup>; Department of Physical and Biological Sciences, Western New England University, Springfield, Massachusetts, USA<sup>b</sup>; Beijing Key Laboratory for Source Control Technology of Water Pollution, College of Environmental Science & Engineering, Beijing Forestry University, Beijing, China<sup>c</sup>

**ABSTRACT** Insight into the mechanisms for arsenic detoxification by *Geobacter* species is expected to improve the understanding of global cycling of arsenic in iron-rich subsurface sedimentary environments. Analysis of 14 different *Geobacter* genomes showed that all of these species have genes coding for an arsenic detoxification system (*ars* operon), and several have genes required for arsenic respiration (*arr* operon) and methylation (*arsM*). Genes encoding four arsenic repressor-like proteins were detected in the genome of *G. sulfurreducens*; however, only one (ArsR1) regulated transcription of the *ars* operon. Elimination of *arsR1* from the *G. sulfurreducens* chromosome resulted in enhanced transcription of genes coding for the arsenic efflux pump (Acr3) and arsenate reductase (ArsC). When the gene coding for Acr3 was deleted, cells were not able to grow in the presence of either the oxidized or reduced form of arsenic, while *arsC* deletion mutants could grow in the presence of arsenite but not arsenate. These studies shed light on how *Geobacter* influences arsenic mobility in anoxic sediments and may help us develop methods to remediate arsenic contamination in the subsurface.

**IMPORTANCE** This study examines arsenic transformation mechanisms utilized by *Geobacter*, a genus of iron-reducing bacteria that are predominant in many anoxic iron-rich subsurface environments. *Geobacter* species play a major role in microbially mediated arsenic release from metal hydroxides in the subsurface. This release raises arsenic concentrations in drinking water to levels that are high enough to cause major health problems. Therefore, information obtained from studies of *Geobacter* should shed light on arsenic cycling in iron-rich subsurface sedimentary environments, which may help reduce arsenic-associated illnesses. These studies should also help in the development of biosensors that can be used to detect arsenic contaminants in anoxic subsurface environments. We examined 14 different *Geobacter* genomes and found that all of these species possess genes coding for an arsenic detoxification system (*ars* operon), and some also have genes required for arsenic respiration (*arr* operon) and arsenic methylation (*arsM*).

**KEYWORDS** *ars* operon, *Geobacter*, iron reduction, detoxification, genetics, transcriptomics, arsenic

Arsenic is a naturally occurring metalloid that is found in many minerals, usually in conjunction with such metals as iron, lead, nickel, copper, and cobalt (1). Both inorganic and organic arsenic forms are found in soils, sediments, water, and living organisms, with inorganic forms being most abundant (2, 3). In the environment, the oxidized form of inorganic arsenic [arsenate; As(V)] is relatively nonmobile as it strongly adsorbs to such minerals as ferrihydrite and alumina. The reduced form, arsenite [As(III)], on the other hand, does not adsorb well to many minerals, making it highly

Received 22 September 2016 Accepted 2 December 2016

Accepted manuscript posted online 9 December 2016

**Citation** Dang Y, Walker DJF, Vautour KE, Dixon S, Holmes DE. 2017. Arsenic detoxification by *Geobacter* species. *Appl Environ Microbiol* 83:e02689-16. <https://doi.org/10.1128/AEM.02689-16>.

**Editor** Shuang-Jiang Liu, Chinese Academy of Sciences

**Copyright** © 2017 American Society for Microbiology. All Rights Reserved.

Address correspondence to Dawn E. Holmes, [dholmes@wne.edu](mailto:dholmes@wne.edu).

Y.D. and D.J.F.W. contributed equally to this article.

mobile (4). Arsenite is also 25 to 60 times more toxic than its nonmobile counterpart (5) and poses a greater environmental threat.

The toxic nature and prevalence of arsenic in the environment have led to the evolution of several different arsenic transformation systems within bacteria (3). Some bacteria are able to methylate arsenic with *S*-adenosyl-L-methionine-dependent methyltransferase (ArsM), forming volatile methylarsines, which removes arsenic from the immediate environment by converting it into a gaseous compound (6). Other prokaryotes utilize *arr* and/or *ars* arsenic transformation systems. While both systems involve reduction of arsenate, only the dissimilatory arsenate reductase system (*arr*) allows an organism to gain energy from coupling the oxidation of an electron donor with the reduction of arsenate.

Arsenic is frequently coprecipitated with iron hydroxides and sulfides in sedimentary rocks (7). In fact, arsenopyrite, a compound of iron, arsenic, and sulfur, is the most common arsenic mineral found in nature (8). Therefore, it does not seem too surprising that studies have shown that Fe(III)-respiring bacteria such as *Geobacter* are frequently involved in microbially mediated arsenic release from flooded soils and anoxic sediments (9–17).

Analysis of available *Geobacter* genomes has revealed that all *Geobacter* species have genes coding for an arsenic detoxification system (*ars* genes), and a few of them have the machinery required for arsenate respiration (*arr* genes). The presence of *ars* genes in *Geobacter* is not unusual as arsenic resistance (*ars*) genes have been found in genomes from a diversity of cultured and uncultivated bacteria (18–26); however, the functionality of Ars proteins has only been determined in one other genetically tractable strictly anaerobic dissimilatory metal-reducing subsurface bacterium, *Desulfovibrio desulfuricans* (27). Although genetic studies have been carried out on homologous Ars proteins from facultative anaerobes (28–31), it is premature to assume that Ars proteins function in a similar manner in strict anaerobes until more information is available. Therefore, bioinformatic, transcriptomic, and genetic studies were done to determine the role that the various Ars proteins play in arsenic resistance by *G. sulfurreducens*, another environmentally significant anaerobic bacterium.

## RESULTS AND DISCUSSION

**Arsenic detoxification machinery in *Geobacter*.** All of the *Geobacter* genomes available to date have the arsenic detoxification machinery (*ars* genes), whereas only a few *Geobacter* species have genes required for arsenite methylation (*arsM*) and arsenate respiration (*arr* genes) (Table 1). *G. lovleyi*, *G. uraniireducens*, and *Geobacter* sp. strain OR-1 are the only species or strains whose genomes carry genes from the *arr* operon, and all three of these organisms are capable of arsenate respiration (9, 10, 32). It also appears that three of the species within the genus *Geobacter* (*G. metallireducens*, *G. uraniireducens*, and *G. lovleyi*) have genes that could potentially code for arsenite *S*-adenosylmethyltransferase (ArsM) proteins. These proteins are homologous to the characterized ArsM protein from *Rhodospseudomonas palustris* (33): the Glov\_0896, Gura\_2444, and Gmet\_2791 gene products are 51%, 56%, and 55% similar, respectively.

Similar to the case in other arsenic-resistant bacteria (34), *Geobacter ars* genes are arranged in clusters, and many of them are cotranscribed (see Fig. S1 in the supplemental material). ArsR is a *trans*-acting repressor involved in the regulation of the *ars* operon, ArsC is an arsenate reductase that reduces arsenate to arsenite prior to efflux, and Acr3 is a transmembrane antiporter (35–37) that pumps arsenite out of the cell.

The gene coding for ArsC was found in all of the *Geobacter* genomes except *G. uraniireducens* and *G. daltonii* (9, 32). However, the *G. uraniireducens* genome has a gene coding for dissimilatory arsenate reductase (ArrA), which can also reduce As(V) to As(III) for extrusion from the cell.

It was interesting to find that *Geobacter* ArsC proteins are homologous to thioredoxin-activating ArsC proteins, which use thioredoxin as a source of reducing power. These proteins have three highly conserved cysteine residues located at amino acid positions 12, 82, and 89, are primarily associated with low-G+C Gram-positive

**TABLE 1** Arsenic detoxification and respiratory genes found in the genomes of the *Geobacter* species and strains used in this study

Annotation	Gene name	Gene found in:					
		<i>G. sulfurreducens</i>	<i>Geobacter</i> sp. strain M21	<i>Geobacter</i> sp. strain M18	<i>G. bemidjensis</i>	<i>G. uraniireducens</i>	<i>G. metallireducens</i>
ABC transporter, permease protein		GSU2950			Gbem_0431		Gmet_0524
ABC transporter, ATP-binding protein	<i>tauB</i>	GSU2951			Gbem_0430		Gmet_0523
DNA-binding transcriptional repressor (helix turn helix)	<i>arsR</i>	GSU2952	GM21_1872	GM18_1516	Gbem_2350	Gura_0468	Gmet_0522
		GSU2625	GM21_1776	GM18_2299	Gbem_1845	Gura_0439	Gmet_0846
		GSU0399	GM21_4069	GM18_2397	Gbem_2441	Gura_1710	
		GSU2149	GM21_0522	GM18_1826	Gbem_1345	Gura_4223	
			GM18_1204		Gura_3309		
			GM18_3417		Gura_2445		
Arsenate reductase (thioredoxin activating)	<i>arsC</i>	GSU2953	GM21_1874	GM18_1515	Gbem_2348		Gmet_0521
Integral membrane efflux pump	<i>acr3</i>	GSU2954	GM21_1873	GM18_1514	Gbem_2349	Gura_0467	Gmet_0520
Permease DUF318 family	<i>arsP</i>	GSU2955	GM21_1875			Gura_0438	Gmet_0519
Redox active disulfide protein	<i>trx3</i>	GSU2956	GM21_1877	GM18_2400		Gura_0436	Gmet_0518
Thioredoxin family protein	<i>trxA</i>	GSU2957	GM21_1878	GM18_2401	Gbem_0404	Gura_0435	Gmet_0517
		GSU3281	GM21_0404	GM18_3976		Gura_0363	Gmet_3230
Thiol-disulfide interchange protein	<i>dsbD</i>	GSU2958	GM21_1879	GM18_2402		Gura_0434	Gmet_0516
Co/Zn/Cd cation transporter-like protein	<i>cdf</i>	GSU2959	GM21_1876	GM18_0478	Gbem_2347	Gura_3416	Gmet_0515
Arsenical resistance operon <i>trans</i> -acting repressor ArsD	<i>arsD</i>					Gura_1382	
Molybdopterin-containing oxidoreductase (PMO) family	<i>arrA</i>					Gura_0470	
4Fe-4S ferredoxin iron-sulfur binding domain protein	<i>arrB</i>					Gura_0471	
S-Adenosyl-L-methionine:methylarsonite As-methyltransferase	<i>arsM</i>					Gura_2444	Gmet_2791

bacteria, and prior to this study had only been found in two other Gram-negative bacteria (*Pseudomonas aeruginosa* and *Acidithiobacillus ferrooxidans*) (2, 38–40). Other characterized ArsC proteins from Gram-negative bacteria have six conserved amino acid residues (His8, Cys12, Ser15, Arg60, Arg94, and Arg107) and use glutaredoxin as an electron donor.

*Geobacter* and other deltaproteobacterial species are Gram-negative; however, their ArsC proteins do not cluster within the glutaredoxin clade. Rather, they all have three cysteine residues at positions 12, 82, and 89 (see Fig. S2A in the supplemental material). Phylogenetic trees made from ArsC protein alignments also showed that *Geobacter* and other deltaproteobacterial ArsC proteins fall within the thioredoxin binding clade, while most other Gram-negative bacteria cluster within the glutaredoxin clade (Fig. S2B).

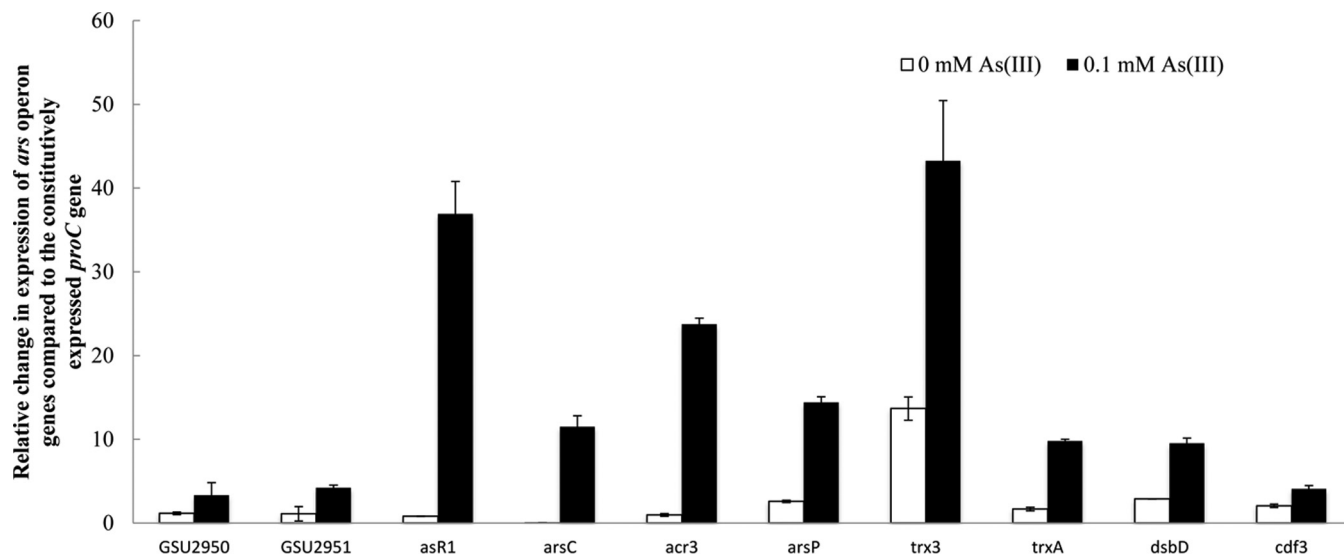
Further analysis of *Geobacter* and deltaproteobacterial ArsC sequences indicated that these genes are unlikely to have been acquired recently via horizontal gene transfer as no plasmid, transposon, or phage-related genes were located in the vicinity of *arsC* and the percentage of G+C content of all of the *arsC* genes was within 7% of

TABLE 1 (Continued)

Gene found in:							
<i>G. lovleyi</i>	<i>G. bremsensis</i>	<i>G. daltonii</i>	<i>Geobacter</i> sp. strain OR-1	<i>G. pickeringii</i>	<i>G. argillaceus</i>	<i>G. soli</i>	<i>G. anodireducens</i>
	K419DRAFT_03027			Ga0069501_11275		Ga0077628_112470	Ga0133348_113083
	K419DRAFT_03028			Ga0069501_11276		Ga0077628_112471	Ga0133348_113084
Glov_1154	K419DRAFT_00586	Geob_2213	Ga0098289_102202	Ga0069501_11744	Ga0052872_03142	Ga0077628_112472	Ga0133348_113085
Glov_1189	K419DRAFT_00674	Geob_3457	Ga0098289_10561		Ga0052872_00732	Ga0077628_11451	Ga0133348_11965
Glov_1665	K419DRAFT_00388	Geob_2249	Ga0098289_1176		Ga0052872_01025	Ga0077628_113344	Ga0133348_11381
Glov_0357	K419DRAFT_04127	Geob_0483	Ga0098289_101332		Ga0052872_02405		
		Geob_0528	Ga0098289_102250				
		Geob_2068	Ga0098289_107231				
Glov_0597	K419DRAFT_00584		Ga0098289_102203	Ga0069007_111441	Ga0052872_03143	Ga0077628_112473	Ga0133348_113086
Glov_1155	K419DRAFT_00585	Geob_2214	Ga0098289_102204 Ga0098289_10562	Ga0069007_111442	Ga0052872_03144	Ga0077628_112474	Ga0133348_113087
Glov_1178		Geob_3501 Geob_2903	Ga0098289_102252 Ga0098289_12258				
Glov_1184		Geob_3502	Ga0098289_102262 Ga0098289_10573		Ga0052872_00157		
Glov_1185	K419DRAFT_03989	Geob_3503	Ga0098289_10574	Ga0069501_113062	Ga0052872_01678	Ga0077628_112757	Ga0133348_113405
Glov_0505		Geob_1303	Ga0098289_102264 Ga0098289_102214 Ga0098289_103279				
Glov_1186		Geob_3504	Ga0098289_102265 Ga0098289_10575				
Glov_1190	K419DRAFT_00583	Geob_0736	Ga0098289_102253	Ga0069501_11586	Ga0052872_02403	Ga0077628_112476	Ga0133348_113089
Glov_1191			Ga0098289_106193				
			Ga0098289_10564				
			Ga0098289_102209 Ga0098289_102212				
Glov_1149			Ga0098289_10560				
Glov_1148			Ga0098289_10559				
Glov_0896							

their overall genomes. Therefore, if these genes were acquired via horizontal gene transfer, it would have occurred in the distant evolutionary past. However, it is interesting to note that many deltaproteobacteria form syntrophic partnerships with low-G+C Gram-positive bacteria, and this close proximity would have made it easy to acquire these genes at some point in the past (41). Although lateral gene transfer of the *arsC* gene between deltaproteobacteria and low-G+C Gram-positive bacteria is not apparent, other studies have found that these two groups of organisms have exchanged other genes such as *dsrAB* (dissimilatory sulfite reductase) and *aps* (adenosine 5-phosphosulfate reductase) (42–44).

Consistent with *Geobacter* ArsC protein structure, several of the *Geobacter ars* operons have genes (*trxA*) coding for a thioredoxin domain protein (Table 1). While all of the *Geobacter* species have at least one copy of a thioredoxin domain protein gene (*trxA*) in their genome, *trxA* clusters with the *arsC* gene in only 3 of the 14 *Geobacter* genomes examined (*G. sulfurreducens*, *Geobacter* sp. strain M21, and *G. metallireducens*). This gene and another thioredoxin-like gene (*trx3*) are more highly transcribed in *G.*



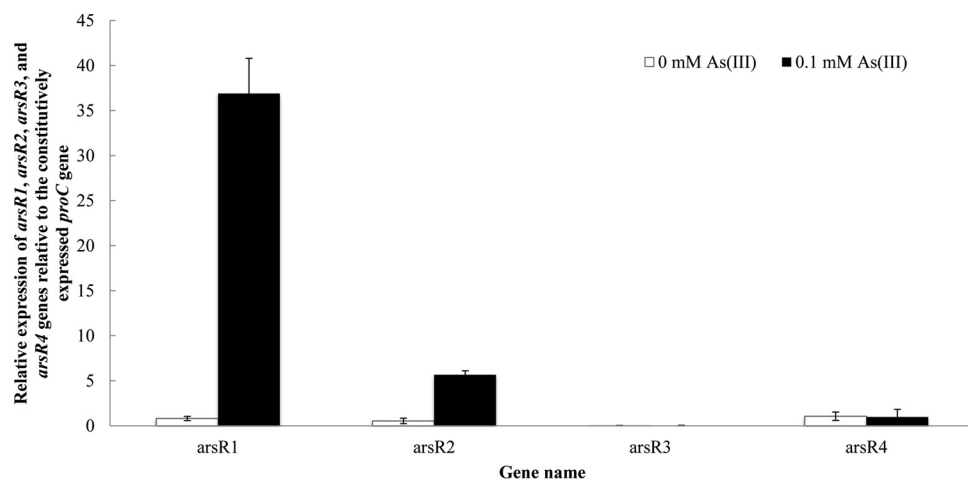
**FIG 1** Relative changes in expression of *ars* operon genes compared to expression of the constitutively expressed *proC* gene in *G. sulfurreducens* cells grown in the presence or absence of 0.1 mM arsenite. Acetate (10 mM) was provided as the electron donor, and fumarate (50 mM) was provided as the electron acceptor for all conditions. All conditions were tested in triplicate.

*sulfurreducens* cells grown in the presence of 0.1 mM arsenite (Fig. 1), indicating that thioredoxin might play a role in electron transfer by *G. sulfurreducens* to ArsC. However, *trxA*, *trx3*, and *trxA trxA* deletion mutant strains did not have these phenotypes when grown in the presence of either arsenite (0.1 mM) or arsenate (5 mM) (see Fig. S3 in the supplemental material). One would also expect that if a thioredoxin protein were required for function of ArsC, its gene would be cotranscribed with the *arsC* gene more consistently across the genus.

Both the arsenic respiratory and detoxification systems are regulated by the ArsR repressor protein, which is induced by arsenite. A copy of the gene coding for ArsR is found in all of the *Geobacter ars* operons examined in this study. In addition, all of the *Geobacter* species have other genes coding for ArsR-SmtB family proteins scattered throughout their genomes (Table 1). For example, *G. sulfurreducens*, *G. bemiidjensis*, *G. lovleyi*, *G. argillaceus*, and *G. bremensis* all have 4 *arsR*-like homologues, *G. uraniireducens*, *G. daltonii*, and *Geobacter* sp. strains M18 and OR-1 have 6 homologues, *Geobacter* sp. strain M21 has 5, *G. soli* and *G. anodireducens* have 3, and *G. metallireducens* has two *arsR* homologues.

**The *ars* detoxification system in *G. sulfurreducens*.** Bioinformatic and gap reverse transcription-PCR (RT-PCR) analyses showed that the *ars* operon in *G. sulfurreducens* is composed of 10 genes (GSU2950 to -2959) (Fig. S1), and rapid amplification of cDNA ends (RACE) showed that the 5' untranslated region (UTR) includes a region 97 bp upstream from GSU2950. Genes found in the *ars* operon include a gene coding for an ABC transporter permease and ATP-binding protein (GSU2950-GSU2951), *arsR* (GSU2952), *arsC* (GSU2953), *acr3* (GSU2954), *arsP* (GSU2955), *trx3* (GSU2956), *trxA* (GSU2957), *dsbD* (GSU2958), and a gene coding for a cation efflux protein (GSU2959) from the cation diffusion facilitator (CDF) family of transporters. All 10 of these genes were upregulated in *G. sulfurreducens* cells grown in the presence of 0.1 mM As(III) compared to cells grown without exposure to arsenic (Fig. 1).

**Regulation of the *ars* operon.** The *G. sulfurreducens* genome has four different genes coding for putative ArsR-Smt family regulatory proteins scattered throughout the genome: GSU2952 (*arsR1*), GSU2149 (*arsR2*), GSU0399 (*arsR3*), and GSU2625 (*arsR4*). All four of these genes' proteins have the helix-turn-helix (HTH) motif that is characteristic of DNA binding transcriptional regulators (see Fig. S4A in the supplemental material), and all of them are found near transport proteins. The *arsR1* gene is part of the *ars* operon, *arsR2* is located 529 bp upstream from a gene coding for heavy



**FIG 2** Relative changes in expression of the *arsR1*, *arsR2*, *arsR3*, and *arsR4* genes relative to the constitutively expressed *proC* gene in *G. sulfurreducens* cells grown in the presence or absence of 0.1 mM arsenite. Acetate (10 mM) was provided as the electron donor, and fumarate (50 mM) was provided as the electron acceptor for all conditions. All conditions were tested in triplicate.

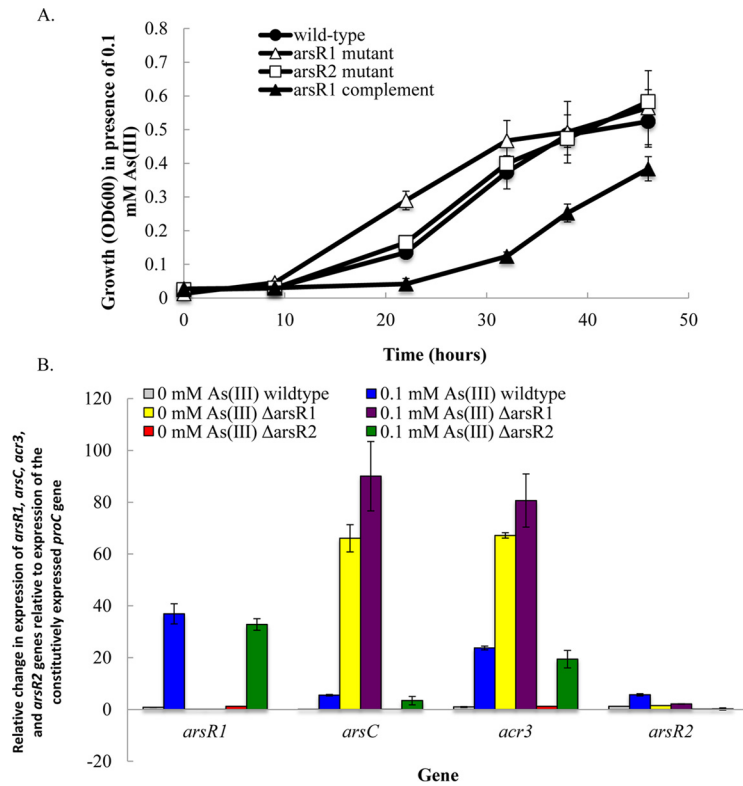
metal-translocating P-type ATPase protein (GSU2147; *cadA*), *arsR3* is located just downstream from an operon that contains genes coding for subunits of a multidrug efflux transporter (GSU0391 to -0394), and *arsR4* is found downstream from genes coding for an ABC-type oligopeptide transport system (GSU2623-GSU2624). However, only ArsR1 and ArsR2 have the metal-binding motif (ELCVCDL) that allows metals to bind to ArsR and the vicinal cysteine pair (Cys-32 and Cys-34) that is part of the inducer (AsIII) binding domain of ArsR in *Escherichia coli* and *Shewanella* (Fig. S4) (29, 45).

In addition to ArsR1 and ArsR2 being the only HTH proteins with conserved metal binding sites, their associated genes (*arsR1* and *arsR2*) were the only *arsR*-like genes differentially transcribed in the presence of 0.1 mM arsenite (Fig. 2). However, the number of *arsR2* mRNA transcripts only slightly increased in the presence of arsenite, while a significant increase in transcription of *arsR1* was observed. Further comparison of ArsR2 amino acid sequences with other regulatory proteins from the ArsR-Smt family suggests that ArsR2 is more closely related to CadC regulatory proteins (Fig. S4A and S4B), which makes sense because it is located upstream from a gene coding for a putative CadA protein.

In order to determine which of these arsenic repressor proteins is involved in regulation of the *ars* operon, *arsR1*, *arsR2*, *arsR3*, and *arsR4* deletion mutant strains were constructed in *G. sulfurreducens*. The only repressor mutant strain that exhibited a phenotype was the  $\Delta$ *arsR1* mutant (Fig. 3). The lag period for growth in the presence of 0.1 mM As(III) was shorter in the *arsR1* deletion mutant than in the wild type, and the mutant's doubling time in the presence of As(III) was slightly faster than that of the wild type (8 versus 9 h) (Fig. 3A). This shortened lag period was not observed in the *arsR2* mutant or the *arsR1* complement. The finding that the *arsR1* complement strain grew slower than the wild-type strain can most likely be explained by the fact that *arsR1* was expressed from a plasmid carrying a second antibiotic resistance marker. Previous studies have shown that plasmids and multiple antibiotic cassettes can interfere with the normal proliferation of an organism, thereby slowing down growth rates and reducing final cell densities (46). Growth rates of *Geobacter* complement strains are almost always slower than that of the wild type using this genetic approach (47). Growth of the other two complement strains constructed in this study (*acr3* and *arsC* complements) was also slightly slower than that in the wild-type strain.

Transcription of *arsC* and *acr3* genes also notably increased in  $\Delta$ *arsR1* cells grown in the presence and absence of As(III) (Fig. 3B). The number of *arsC* mRNA transcripts was ~100-fold higher in *arsR1* mutant strains than in the wild type in the presence and absence of As(III), and *acr3* transcript levels were 67- and 4-fold higher in  $\Delta$ *arsR1* cells

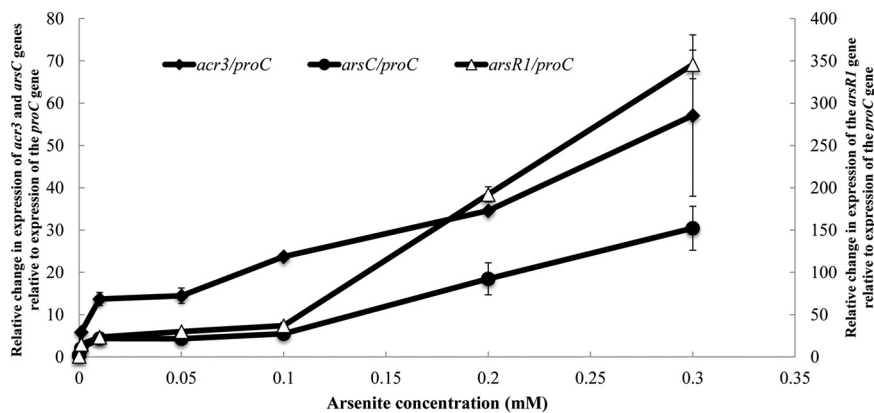




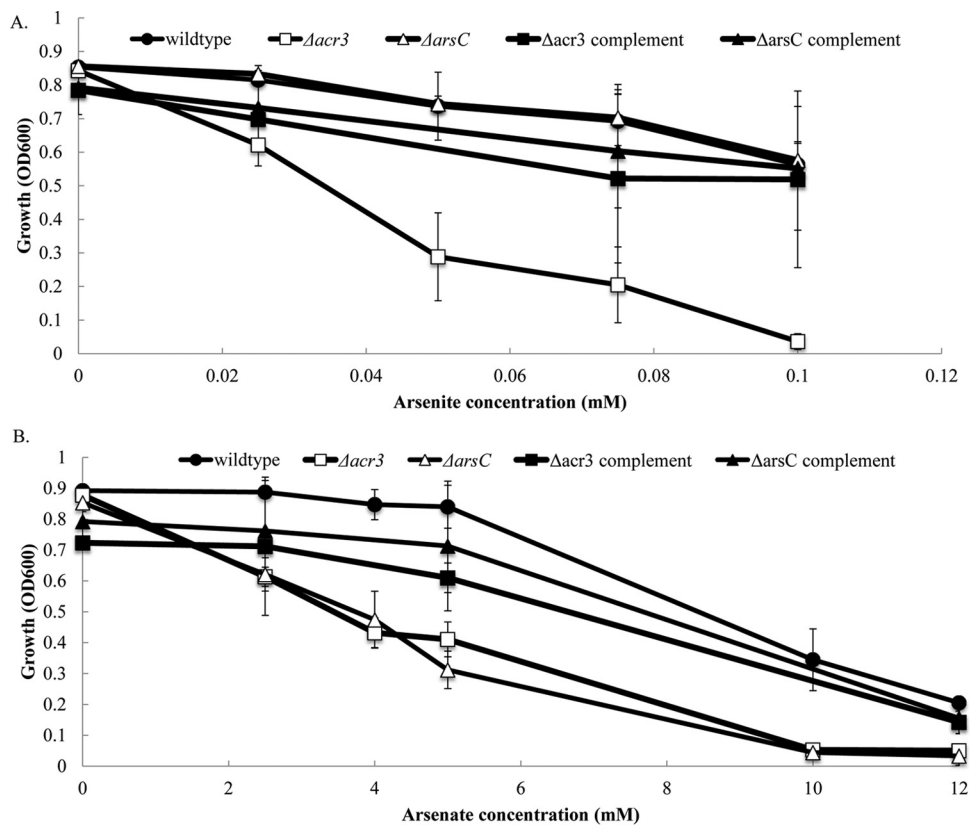
**FIG 3** (A) Growth of the wild-type, *arsR1* deletion mutant, *arsR2* deletion mutant, and *arsR1*-complemented strains in the presence of 0.1 mM As(III). (B) Relative changes in expression of the *acr3*, *arsC*, *arsR1*, and *arsR2* genes compared to expression of the constitutively expressed *proC* gene in the wild-type and *arsR1* and *arsR2* deletion mutant strains grown in the presence or absence of 0.1 mM As(III). Total RNA was extracted from cells after 20 h of incubation. All conditions were conducted in triplicate.

than wild-type cells in the presence or absence of As(III), respectively. Transcription of *acr3* and *arsC*, on the other hand, was not impacted by deletion of the *arsR2* gene. These results suggest that the *ars* operon is regulated by *arsR1*.

**Characterization of the *ars* operon in *G. sulfurreducens*.** In order to confirm that expression of *ars* genes is involved in arsenic detoxification in *G. sulfurreducens*, transcription of *arsR1*, *acr3*, and *arsC* under various concentrations of arsenite was quantified by quantitative RT-PCR (qRT-PCR) (Fig. 4). The numbers of *acr3*, *arsC*, and



**FIG 4** Relative changes in expression of the *arsR1*, *acr3*, and *arsC* genes compared to expression of the constitutively expressed *proC* gene in *G. sulfurreducens* cells grown in the presence of various concentrations of arsenite. All points represent the average from triplicate samples.



**FIG 5** Maximum OD<sub>600</sub> after 45 h of incubation for the wild-type and deletion mutant *acr3* and *arsC* strains of *G. sulfurreducens* in the presence of increasing levels of (A) arsenite [As(III)] or (B) arsenate [As(V)]. Cells were grown with acetate (10 mM) as the electron donor and fumarate (50 mM) as the electron acceptor. All points represent the average from triplicate samples.

*arsR1* mRNA transcripts were 59, 83, and 429 times more abundant in cells grown in the presence of 0.3 mM As(III) than in cells grown without exposure to arsenic.

Deletion of *ars* genes from *G. sulfurreducens* cells further supported their involvement in arsenic detoxification. When the gene coding for Acr3 was deleted from the chromosome, growth on both As(III) and As(V) was impaired (Fig. 5). While wild-type strains were able to grow in the presence of 0.3 mM As(III) or 12.5 mM As(V), growth of the *acr3* deletion mutant strain was impaired at As(III) concentrations as low as 0.025 mM As(III) and 2.5 mM As(V). The phenotype of the *arsC* deletion mutant was similar to that of the wild type when grown in the presence of As(III), as would be expected since the efflux pump (*acr3*) was fully functional in this strain. However, growth of the *arsC* mutant strain was diminished in the presence of As(V), and it had a similar phenotype on As(V) to the *acr3* mutant (Fig. 5B). Complementation of both of these genes restored function and allowed growth in the presence of As(III) and As(V) similar to that of the wild type.

**Implications.** The prevalence and redundancy of many of these arsenic transformation genes within the genus *Geobacter* show how frequently these organisms are likely to encounter this metalloid in the environment. Studies have shown that *Geobacter* species are major players in microbially mediated arsenic release from metal hydroxides, which is a common cause of elevated arsenic concentrations (those exceeding 10  $\mu$ g/liter) in drinking water. In South and Southeast Asia, tens of millions of people are exposed to chronic arsenic poisoning annually by drinking groundwater (48) in which bound arsenic is released from sediments by dissimilatory metal-respiring bacteria such as *Geobacter* (11, 12, 14, 49). Mobilization of arsenic is then further enhanced by species that can transform arsenic to its more mobile form (arsenite), which readily leaches into drinking water supplies. Because *Geobacter* species are



responsible for much of this microbially mediated arsenic release, a better understanding of the mechanisms that they use for arsenic transformation should help us develop methods that can reduce the problem of arsenic-contaminated drinking water in many environments.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *G. sulfurreducens* strain DL1 (ATCC 51573) was obtained from our laboratory culture collection. Cells were grown under anaerobic conditions in a previously described bicarbonate-buffered, defined medium (50) supplemented with acetate (10 mM) as the electron donor and fumarate (50 mM) as the electron acceptor. Cells were incubated at 30°C in the dark under an N<sub>2</sub>-CO<sub>2</sub> (80:20) atmosphere. Arsenic was added to cultures from anoxic stocks of sodium (meta)arsenite (NaAsO<sub>2</sub>; 10 mM) and sodium arsenate (Na<sub>2</sub>HAsO<sub>4</sub>; 10 mM).

**Analytical techniques.** Acetate concentrations were determined with an HP series 1100 high-pressure liquid chromatograph (Hewlett Packard, Palo Alto, CA) with a Bio-Rad fast acid analysis column (Bio-Rad Laboratories, Hercules, CA), with an eluent of 8 mM H<sub>2</sub>SO<sub>4</sub> and UV absorbance detection at 210 nm as previously described (51).

*G. sulfurreducens* cells were counted with acridine orange staining and epifluorescence microscopy as previously described (52). Cells were diluted 100-fold, fixed with a 10% glutaraldehyde solution, and stained with acridine orange (0.01% final concentration). The stained sample (1 ml) was then concentrated with a vacuum pump (pressure of ca. 150 mm Hg) onto a 0.2- $\mu$ m-pore-size polycarbonate membrane filter (Millipore) and visualized by fluorescence microscopy on a Nikon Eclipse E600 microscope.

**Extraction of RNA from samples.** RNA was prepared from triplicate cultures for all growth conditions, and all solutions used during the RNA extraction process were prepared with diethylpyrocarbonate (DEPC)-treated water (Ambion, Foster City, CA). Batch cultures (50 ml) were grown with acetate (10 mM) as an electron donor and fumarate (50 mM) as an electron acceptor in the presence of various concentrations of arsenite (0, 0.05, 0.1, 0.2, and 0.3 mM) or arsenate (0, 2.5, 4, 5, and 10 mM) and harvested during exponential growth. Cells were split into 50-ml conical tubes (BD Biosciences, San Jose, CA) and pelleted by centrifugation at 3,000  $\times$  *g* for 15 min. RNA was then extracted with the RNEasy Plus minikit (Qiagen) and treated with DNA-free DNase (Ambion) according to the manufacturer's instructions. All RNA samples were checked for integrity by agarose gel electrophoresis and had A<sub>260</sub>/A<sub>280</sub> ratios of 1.8 to 2.0, indicating that they were of high purity (53). In order to ensure that RNA samples were not contaminated with DNA, PCR amplification with primers targeting the 16S rRNA gene was conducted on RNA samples that had not undergone reverse transcription.

**Analysis of the *ars* operon in *G. sulfurreducens*.** In order to determine the size of the *ars* operon in *G. sulfurreducens*, primers were designed to link consecutive genes within the putative operon (see Table S1A in the supplemental material). cDNA was generated from RNA (1  $\mu$ g) extracted from *Geobacter* cells grown in the presence of 0.1 mM arsenite with the Durascript enhanced avian reverse transcriptase single-strand synthesis kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions and used as a template for the various PCRs. The 5'/3' RACE (rapid amplification of cDNA ends) kit (Roche, Mannheim, Germany) was used to perform rapid amplification of 5' cDNA ends (5' RACE) to find the start of the *G. sulfurreducens* *ars* operon. For 5' RACE, single-stranded cDNAs were first synthesized with sp1 (Table S1A) and transcriptase reverse transcriptase. After purification of the single-stranded cDNA with the High Pure PCR purification kit (Roche), terminal transferase was used to add a poly(A) tail at the 5' end. Two rounds of PCR were then conducted with sp2 and the oligo(dT) anchor primer and sp3 and the PCR anchor primer.

**qRT-PCR.** Genome sequence data obtained from the DOE Joint Genome Institute (JGI) website ([www.jgi.doe.gov](http://www.jgi.doe.gov)) were used to design quantitative RT-PCR (qRT-PCR) primers. All qRT-PCR primers (Table S1B) were designed according to the manufacturer's specifications (amplicon sizes of 100 to 200 bp), and representative products from each of these primer sets were verified by sequencing.

The Superscript III first-strand synthesis SuperMix for qRT-PCR (Invitrogen) was used to generate cDNA from 1  $\mu$ g total RNA. Once the appropriate cDNA fragments were generated by RT-PCR, qRT-PCR amplification and detection were performed with the 7500 real-time PCR system (Applied Biosystems). Optimal qRT-PCR conditions were determined using the manufacturer's guidelines. Each PCR mixture consisted of a total volume of 25  $\mu$ l and contained 1.5  $\mu$ l of the appropriate primers (stock concentrations, 15  $\mu$ M) and 12.5  $\mu$ l Power SYBR green PCR master mix (Applied Biosystems). Standard curves covering 8 orders of magnitude were constructed with serial dilutions of known amounts of purified cDNA quantified with a NanoDrop ND-1000 spectrophotometer at an absorbance of 260 nm.

**Construction of deletion mutants and complements.** The sequences of all primer pairs used for construction of the deletion mutants used in this study are listed in Table S1C. All mutants were constructed by replacement of the gene of interest with a gentamicin resistance cassette as previously described (54). Restriction digestions were carried out according to the manufacturer's instructions, and JumpStart AccuTaq LA DNA polymerase (Sigma-Aldrich) was used for all PCRs.

Primer pairs were designed to amplify regions approximately 500 bp downstream and upstream of the target genes and to add AvrII (CCTAGG) restriction sites to the PCR products. These products were digested with AvrII restriction endonuclease (NEB, Beverly, MA), ethanol precipitated, and ligated with T4 DNA ligase (NEB). The ligation reaction mixture was then loaded onto an agarose gel, and a 1-kb band was purified with the Qiaquick gel extraction kit (Qiagen) and ligated into the pCR2.1 TOPO cloning vector, resulting in the formation of PCR2.1up5'+3'dn. Sanger sequencing was done to verify the

sequence of the cloned product. A gentamicin resistance cassette was amplified with flanking AvrII restriction sites by PCR using the pBSL141 plasmid as a template. Both the gentamicin cassette and the PCR2.1up5'+3'dn plasmid were digested with AvrII. The gentamicin cassette was ligated into the PCR2.1up5'+3'dn plasmid to complete construction of the mutant allele.

Plasmids carrying mutant alleles were linearized by digestion with KpnI (GGTACC) (NEB) and concentrated by ethanol precipitation. These linearized plasmids were electroporated into *G. sulfurreducens* strain DL1 as previously described (47). Isolated colonies were grown on agar plates supplemented with fumarate (50 mM), acetate (10 mM), and gentamicin (20 µg/ml). Replacement of wild-type alleles by mutant alleles in the *G. sulfurreducens* chromosome was verified by PCR and sequencing.

Deletion mutants made in this study were complemented by amplifying the respective genes with their native ribosome binding site (RBS) and ligating them into a plasmid with a constitutive *lac* promoter (pCM66) (55). Primers (Table S1C) were designed to introduce XbaI (TCTAGA) and BamHI (GGATCC) restriction sites into the PCR product. After digestion of pCM66 and the PCR product with XbaI and BamHI, the gene of interest was ligated downstream from the *lac* promoter with T4 DNA ligase. After complementary plasmids were constructed, they were introduced into *ars* mutant cells by electroporation as previously described (47).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02689-16>.

**TEXT S1**, PDF file, 1.3 MB.

## ACKNOWLEDGMENTS

The authors have no conflicts of interest to declare.

This research was supported by the Office of Science (BER), U.S. Department of Energy, award no. DE-SC0006790.

## REFERENCES

- Nriagu JO. 2002. Arsenic poisoning through the ages, p 1–26. In Frankenberger WT (ed), *Environmental chemistry of arsenic*. CRC Press, Dekker, New York, NY.
- Mukhopadhyay R, Rosen BP, Phung LT, Silver S. 2002. Microbial arsenic: from geocycles to genes and enzymes. *FEMS Microbiol Rev* 26:311–325. <https://doi.org/10.1111/j.1574-6976.2002.tb00617.x>.
- Oremland RS, Stolz JF. 2003. The ecology of arsenic. *Science* 300:939–944. <https://doi.org/10.1126/science.1081903>.
- Smedley PL, Kinniburgh DG. 2002. A review of the source, behaviour and distribution of arsenic in natural waters. *Appl Geochem* 17:517–568. [https://doi.org/10.1016/S0883-2927\(02\)00018-5](https://doi.org/10.1016/S0883-2927(02)00018-5).
- Korte N, Fernando Q. 1991. A review of arsenic(III) in groundwater. *Crit Rev Environ Control* 21:1–39.
- Bentley R, Chasteen TG. 2002. Microbial methylation of metalloids: arsenic, antimony, and bismuth. *Microbiol Mol Biol Rev* 66:250–271. <https://doi.org/10.1128/MMBR.66.2.250-271.2002>.
- Mandal BK, Suzuki KT. 2002. Arsenic round the world: a review. *Talanta* 58:201–235. [https://doi.org/10.1016/S0039-9140\(02\)00268-0](https://doi.org/10.1016/S0039-9140(02)00268-0).
- Henke KR (ed). 2009. *Arsenic: environmental chemistry, health threats and waste treatment*, p 69–235. John Wiley & Sons, Ltd, Hoboken, NJ.
- Giloteaux L, Holmes DE, Williams KH, Wrighton KC, Wilkins MJ, Montgomery AP, Smith JA, Orellana R, Thompson CA, Roper TJ, Long PE, Lovley DR. 2013. Characterization and transcription of arsenic respiration and resistance genes during in situ uranium bioremediation. *ISME J* 7:370–383. <https://doi.org/10.1038/ismej.2012.109>.
- Ohtsuka T, Yamaguchi N, Makino T, Sakurai K, Kimura K, Kudo K, Homma E, Dong DT, Amachi S. 2013. Arsenic dissolution from Japanese paddy soil by a dissimilatory arsenate-reducing bacterium *Geobacter* sp. OR-1. *Environ Sci Technol* 47:6263–6271. <https://doi.org/10.1021/es400231x>.
- Islam FS, Gault AG, Boothman C, Polya DA, Charnock JM, Chatterjee D, Lloyd JR. 2004. Role of metal-reducing bacteria in arsenic release from Bengal delta sediments. *Nature* 430:68–71. <https://doi.org/10.1038/nature02638>.
- Hery M, van Dongen BE, Gill F, Mondal D, Vaughan DJ, Pancost RD, Polya DA, Lloyd JR. 2010. Arsenic release and attenuation in low organic carbon aquifer sediments from West Bengal. *Geobiology* 8:155–168. <https://doi.org/10.1111/j.1472-4669.2010.00233.x>.
- Lear G, Song B, Gault AG, Polya DA, Lloyd JR. 2007. Molecular analysis of arsenate-reducing bacteria within Cambodian sediments following amendment with acetate. *Appl Environ Microbiol* 73:1041–1048. <https://doi.org/10.1128/AEM.01654-06>.
- Hery M, Rizoulis A, Sanguin H, Cooke DA, Pancost RD, Polya DA, Lloyd JR. 2015. Microbial ecology of arsenic-mobilizing Cambodian sediments: lithological controls uncovered by stable-isotope probing. *Environ Microbiol* 17:1857–1869. <https://doi.org/10.1111/1462-2920.12412>.
- Ying SC, Damashek J, Fendorf S, Francis CA. 2015. Indigenous arsenic(V)-reducing microbial communities in redox-fluctuating near-surface sediments of the Mekong Delta. *Geobiology* 13:581–587. <https://doi.org/10.1111/gbi.12152>.
- Hassan Z, Sultana M, Westerhoff H, Khan S, Roling W. 3 November 2015. Iron cycling potentials of arsenic contaminated groundwater in Bangladesh as revealed by enrichment cultivation. *Geomicrobiol J* <https://doi.org/10.1080/01490451.2015.1111471>.
- Osborne TH, McArthur JM, Sikdar PK, Santini JM. 2015. Isolation of an arsenate-respiring bacterium from a redox front in an arsenic-polluted aquifer in West Bengal, Bengal Basin. *Environ Sci Technol* 49:4193–4199. <https://doi.org/10.1021/es504707x>.
- Silver S, Phung LT. 2005. Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. *Appl Environ Microbiol* 71:599–608. <https://doi.org/10.1128/AEM.71.2.599-608.2005>.
- Zhao CG, Zhang Y, Chan ZH, Chen SC, Yang SP. 2015. Insights into arsenic multi-operons expression and resistance mechanisms in *Rhodospseudomonas palustris* CGA009. *Front Microbiol* 6:986. <https://doi.org/10.3389/fmicb.2015.00986>.
- Lv C, Zhao C, Yang S, Qu Y. 2012. Arsenic metabolism in purple nonsulfur bacteria. *Wei Sheng Wu Xue Bao* 52:1497–1507. (In Chinese.)
- Butcher BG, Rawlings DE. 2002. The divergent chromosomal *ars* operon of *Acidithiobacillus ferrooxidans* is regulated by an atypical *ArsR* protein. *Microbiology* 148:3983–3992. <https://doi.org/10.1099/00221287-148-12-3983>.
- Branco R, Chung AP, Morais PV. 2008. Sequencing and expression of two arsenic resistance operons with different functions in the highly arsenic-resistant strain *Ochrobactrum tritici* SCII24T. *BMC Microbiol* 8:95. <https://doi.org/10.1186/1471-2180-8-95>.
- Desoeuvre A, Casiot C, Hery M. 2016. Diversity and distribution of arsenic-related genes along a pollution gradient in a river affected by acid mine drainage. *Microb Ecol* 71:672–685. <https://doi.org/10.1007/s00248-015-0710-8>.
- Xiao QQ, Li LG, Ma LP, Zhang SY, Bao P, Zhang T, Zhu YG. 2016.

- Metagenomic analysis revealed highly diverse microbial arsenic metabolism genes in paddy soils with low-arsenic contents. *Environ Pollut* 211:1–8. <https://doi.org/10.1016/j.envpol.2015.12.023>.
25. Zhang SY, Zhao FJ, Sun GX, Su JQ, Yang XR, Li H, Zhu YG. 2015. Diversity and abundance of arsenic biotransformation genes in paddy soils from southern China. *Environ Sci Technol* 49:4138–4146. <https://doi.org/10.1021/acs.est.5b00028>.
  26. Cuebas M, Villafane A, McBride M, Yee N, Bini E. 2011. Arsenate reduction and expression of multiple chromosomal *ars* operons in *Geobacillus kaustophilus* A1. *Microbiology* 157:2004–2011. <https://doi.org/10.1099/mic.0.048678-0>.
  27. Li X, Krumholz LR. 2007. Regulation of arsenate resistance in *Desulfovibrio desulfuricans* G20 by an *arsRBC* operon and an *arsC* gene. *J Bacteriol* 189:3705–3711. <https://doi.org/10.1128/JB.01913-06>.
  28. Saltikov CW, Wildman RA, Jr, Newman DK. 2005. Expression dynamics of arsenic respiration and detoxification in *Shewanella* sp. strain ANA-3. *J Bacteriol* 187:7390–7396. <https://doi.org/10.1128/JB.187.21.7390-7396.2005>.
  29. Murphy JN, Saltikov CW. 2009. The *ArsR* repressor mediates arsenite-dependent regulation of arsenate respiration and detoxification operons of *Shewanella* sp. strain ANA-3. *J Bacteriol* 191:6722–6731. <https://doi.org/10.1128/JB.00801-09>.
  30. Achour-Rokbani A, Cordi A, Poupin P, Bauda P, Billard P. 2010. Characterization of the *ars* gene cluster from extremely arsenic-resistant *Microbacterium* sp. strain A33. *Appl Environ Microbiol* 76:948–955. <https://doi.org/10.1128/AEM.01738-09>.
  31. Fernandez M, Udaondo Z, Niqui JL, Duque E, Ramos JL. 2014. Synergic role of the two *ars* operons in arsenic tolerance in *Pseudomonas putida* KT2440. *Environ Microbiol Rep* 6:483–489. <https://doi.org/10.1111/1758-2229.12167>.
  32. Hery M, Gault AG, Rowland HAL, Lear G, Polya DA, Lloyd JR. 2008. Molecular and cultivation-dependent analysis of metal-reducing bacteria implicated in arsenic mobilisation in South-east Asian aquifers. *Appl Geochem* 23:3215–3223. <https://doi.org/10.1016/j.apgeochem.2008.07.003>.
  33. 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.0506836103>.
  34. Carlin A, Shi WP, Dey S, Rosen BP. 1995. The *ars* operon of *Escherichia coli* confers arsenical and antimicrobial resistance. *J Bacteriol* 177:981–986. <https://doi.org/10.1128/jb.177.4.981-986.1995>.
  35. Wysocki R, Bobrowicz P, Ulaszewski S. 1997. The *Saccharomyces cerevisiae* ACR3 gene encodes a putative membrane protein involved in arsenite transport. *J Biol Chem* 272:30061–30066. <https://doi.org/10.1074/jbc.272.48.30061>.
  36. Aaltonen EK, Silow M. 2008. Transmembrane topology of the ACR3 family arsenite transporter from *Bacillus subtilis*. *Biochim Biophys Acta* 1778:963–973. <https://doi.org/10.1016/j.bbame.2007.11.011>.
  37. Fu HL, Meng Y, Ordonez E, Villadangos AF, Bhattacharjee H, Gil JA, Mateos LM, Rosen BP. 2009. Properties of arsenite efflux permeases (Acr3) from *Alkaliphilus metalliredigens* and *Corynebacterium glutamicum*. *J Biol Chem* 284:19887–19895. <https://doi.org/10.1074/jbc.M109.011882>.
  38. Ji G, Silver S. 1992. Reduction of arsenate to arsenite by the *ArsC* protein of the arsenic resistance operon of *Staphylococcus aureus* plasmid p1258. *Proc Natl Acad Sci U S A* 89:9474–9478. <https://doi.org/10.1073/pnas.89.20.9474>.
  39. Butcher BG, Deane SM, Rawlings DE. 2000. The chromosomal arsenic resistance genes of *Thiobacillus ferrooxidans* have an unusual arrangement and confer increased arsenic and antimony resistance to *Escherichia coli*. *Appl Environ Microbiol* 66:1826–1833. <https://doi.org/10.1128/AEM.66.5.1826-1833.2000>.
  40. Messens J, Van Molle I, Vanhaesebrouck P, Limbourg M, Van Belle K, Wahni K, Martins JC, Loris R, Wyns L. 2004. How thioredoxin can reduce a buried disulphide bond. *J Mol Biol* 339:527–537. <https://doi.org/10.1016/j.jmb.2004.04.016>.
  41. McInerney MJ, Struchtemeyer CG, Sieber J, Mouttaki H, Stams AJ, Schink B, Rohlin L, Gunsalus RP. 2008. Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. *Ann N Y Acad Sci* 1125:58–72. <https://doi.org/10.1196/annals.1419.005>.
  42. Klein M, Friedrich M, Roger AJ, Hugenholtz P, Fishbain S, Abicht H, Blackall LL, Stahl DA, Wagner M. 2001. Multiple lateral transfers of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing prokaryotes. *J Bacteriol* 183:6028–6035. <https://doi.org/10.1128/JB.183.20.6028-6035.2001>.
  43. Friedrich MW. 2002. Phylogenetic analysis reveals multiple lateral transfers of adenosine-5'-phosphosulfate reductase genes among sulfate-reducing microorganisms. *J Bacteriol* 184:278–289. <https://doi.org/10.1128/JB.184.1.278-289.2002>.
  44. Zverlov V, Klein M, Lucker S, Friedrich MW, Kellermann J, Stahl DA, Loy A, Wagner M. 2005. Lateral gene transfer of dissimilatory (bi)sulfite reductase revisited. *J Bacteriol* 187:2203–2208. <https://doi.org/10.1128/JB.187.6.2203-2208.2005>.
  45. Shi W, Wu J, Rosen BP. 1994. Identification of a putative metal binding site in a new family of metalloregulatory proteins. *J Biol Chem* 269:19826–19829.
  46. Dong H, Nilsson L, Kurland CG. 1995. Gratuitous overexpression of genes in *Escherichia coli* leads to growth inhibition and ribosome destruction. *J Bacteriol* 177:1497–1504. <https://doi.org/10.1128/jb.177.6.1497-1504.1995>.
  47. Coppi MV, Leang C, Sandler SJ, Lovley DR. 2001. Development of a genetic system for *Geobacter sulfurreducens*. *Appl Environ Microbiol* 67:3180–3187. <https://doi.org/10.1128/AEM.67.7.3180-3187.2001>.
  48. Smith AH, Lingas EO, Rahman M. 2000. Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. *Bull World Health Organ* 78:1093–1103.
  49. van Geen A, Rose J, Thoral S, Garnier J, Zheng Y, Bottero J. 2004. Decoupling of As and Fe release to Bangladesh groundwater under reducing conditions. Part II. Evidence from sediment incubations. *Geochim Cosmochim Acta* 68:3475–3486. <https://doi.org/10.1016/j.gca.2004.01.026>.
  50. Lovley DR, Phillips EJ. 1988. Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Appl Environ Microbiol* 54:1472–1480.
  51. Anderson RT, Vrionis HA, Ortiz-Bernad I, Resch CT, Long PE, Dayvault R, Karp K, Marutsky S, Metzler DR, Peacock A, White DC, Lowe M, Lovley DR. 2003. Stimulating the in situ activity of *Geobacter* species to remove uranium from the groundwater of a uranium-contaminated aquifer. *Appl Environ Microbiol* 69:5884–5891. <https://doi.org/10.1128/AEM.69.10.5884-5891.2003>.
  52. Hobbie JE, Daley RJ, Jasper S. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl Environ Microbiol* 33:1225–1228.
  53. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. 2001. *Current protocols in molecular biology*, vol 3. John Wiley & Sons, Inc, Hoboken, NJ.
  54. Aklujkar M, Lovley DR. 2010. Interference with histidyl-tRNA synthetase by a CRISPR spacer sequence as a factor in the evolution of *Pelobacter carbinolicus*. *BMC Evol Biol* 10:230. <https://doi.org/10.1186/1471-2148-10-230>.
  55. Marx CJ, Lidstrom ME. 2001. Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria. *Microbiology* 147:2065–2075. <https://doi.org/10.1099/00221287-147-8-2065>.