Journal of Bacteriology, May 2007, p. 3705–3711 Vol. 189, No. 10
0021-9193/07/$08.00+0 doi:10.1128/JB.01913-06
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

Regulation of Arsenate Resistance in *Desulfovibrio desulfuricans* G20 by an *arsRBCC* Operon and an *arsC* Gene▽†

Xiangkai Li[1] and Lee R. Krumholz[1,2]*

Department of Botany and Microbiology[1] and Institute for Energy and Environment,[2] University of Oklahoma, Norman, Oklahoma 73019-0245

Received 19 December 2006/Accepted 26 February 2007

Desulfovibrio desulfuricans **G20 grows and reduces 20 mM arsenate to arsenite in lactate-sulfate media. Sequence analysis and experimental data show that** ***D. desulfuricans*** **G20 has one copy of** ***arsC*** **and a complete** ***arsRBCC*** **operon in different locations within the genome. Two mutants of strain G20 with defects in arsenate resistance were generated by nitrosoguanidine mutagenesis. The** ***arsRBCC*** **operons were intact in both mutant strains, but each mutant had one point mutation in the single** ***arsC*** **gene. Mutants transformed with either the** ***arsC1*** **gene or the** ***arsRBCC*** **operon displayed wild-type arsenate resistance, indicating that the two** ***arsC*** **genes were equivalently functional in the sulfate reducer. The** ***arsC1*** **gene and** ***arsRBCC*** **operon were also cloned into** ***Escherichia coli*** **DH5α independently, with either DNA fragment conferring increased arsenate resistance. The recombinant** ***arsRBCC*** **operon allowed growth at up to 50 mM arsenate in LB broth. Quantitative PCR analysis of mRNA products showed that the single** ***arsC1*** **was constitutively expressed, whereas the operon was under the control of the** ***arsR*** **repressor protein. We suggest a model for arsenate detoxification in which the product of the single** ***arsC1*** **is first used to reduce arsenate. The arsenite formed is then available to induce the** ***arsRBCC*** **operon for more rapid arsenate detoxification.**

Arsenic is present in natural systems as arsenite (AsO_3^{3-}) and arsenate (AsO_4^{3-}) (18, 23), and both are toxic to microorganisms (17, 23). Due to the natural abundance of arsenic in geologic systems, a variety of environmental microorganisms have developed enzyme-catalyzed arsenic transformation systems (1, 16, 21). Among the microorganisms that live in arsenate-containing environments, sulfate-reducing bacteria are known to carry out arsenate reduction and are likely to be important mediators of this process. *Desulfosporosinus auripigmenti* can respire arsenate with resultant arsenite production (20). Several members of the order *Desulfovibrionales* have also been shown to reduce arsenate (16). *Desulfomicrobium* sp. strain Ben-RB will grow with lactate and arsenate, while another arsenate-reducing strain, *Desulfovibrio* sp. strain Ben-RA, cannot grow with arsenate as an electron acceptor, suggesting that arsenate reduction is simply a mechanism for detoxification by strain Ben-RA (16). It is still unclear whether the ability to reduce arsenate is universally present in members of sulfate-reducing bacteria and whether arsenate reduction is typically used for respiration or detoxification.

The best-studied arsenate resistance system in prokaryotes is the *ars* operon. The *ars* operon on the *Escherichia coli* plasmid R773 (the most thoroughly studied *ars* operon) contains the genes *arsRDABC* in that order (3, 4, 8, 29). However, the majority of *ars* operons contain only *arsRBC* (33). The protein product of *arsR* is a *trans*-acting repressor that binds the operon's promoter and shuts down transcription of the operon. Arsenite is a known inducer of the *ars* operon, acting by inactivation of ArsR (28). The gene product of *arsD* is a metallochaperone transferring arsenite to ArsA (14). The *arsA* gene encodes a catalytic subunit of an oxyanion-translocating ATPase (26, 27). The *arsB* gene encodes a membrane protein that can function independently as a chemiosmotic arsenite transporter. The ArsA/ArsB complex can also be the primary ATP-driven arsenite transporter (7). The protein product of *arsC* is the cytoplasmic arsenate reductase, which converts intracellular arsenate to arsenite (2, 10). The *arsC* genes can be divided mainly into two families. The products of the *arsC* gene from the *E. coli* plasmid R773 family use glutaredoxin as a reductant (9), while gene products of pI258 and the *Bacillus subtilis* family use thioredoxin as a reductant (32). Protein sequences between the two families have less than 20% similarity to each other (33). The effect of the *ars* operon is cumulative, and multiple copies of the *ars* operon have been shown to increase resistance to arsenate (3).

The *ars* system is a detoxification system and is thought not to be involved in respiration (17, 33). However, a number of anaerobic microorganisms have another enzyme system that allows them to respire arsenate to arsenite. This system can be the only arsenate-transforming system or it may be present in addition to the above-mentioned detoxification system (30). The best characterized of these arsenate respiration systems contains the *arrAB* operon. The *arrA* gene encodes a molybdenum-containing enzyme within the dimethyl sulfoxide reductase family, and *arrB* encodes an iron-sulfur protein (30, 31).

In this report, the mechanisms for arsenate reduction in *Desulfovibrio desulfuricans* G20 were investigated. This strain is a genetically tractable derivative of the wild-type strain G100A, which was originally isolated from an oil well corrosion site (35) and subsequently used as a model for

* Corresponding author. Mailing address: Department of Botany and Microbiology, University of Oklahoma, George Lynn Cross Hall, 770 Van Vleet Oval, Norman, OK 73019. Phone: (405) 325-0437. Fax: (405) 325-7619. E-mail: krumholz@ou.edu.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 2 March 2007.

TABLE 1. Primers used in this study

Primer	Sequence
PlinkRB	5′-AAGGTCCATGCCTTCCTCC-3′/5′-TCTTTGTCACTATCTCGAAACCAA-3′
PlinkBC	5′-AGGTCCATGCCTTCCTCC-3′/5′-CCCGTGCTCATAGCCCTA-3′
PlinkCC	5′-CCGCCAAAGGGTTTAGTC-3′/5′-CGGGAGACAAGAAGAAACTC-3′
ParsGSP1	5′-GAAGCTGGATGACCTTCTCG-3′
ParsGSP2	5′-GAACGGGATGCCAAGATAGA-3′
ParsGSP3	5′-CTTGACGTCCGGGTACAGAT-3′
PdTanchor	5′-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTTV-3′
Panchor	5′-GACCACGCGTATCGATGTCGAC-3′
ParsC1	5′-GGTTCATGTTCGCCTCTGCC-3′/5′-GGCCGTCTCTTTCGCGCTG-3′
ParsRBCC	5′-CCATTAGCTTGTTGCTACACC-3′/5′-GCCAAGGTGCTAATGAAATGA-3′
ParsC1RT[a]	5′-GGTTCATGTTCGCCTCTGCC-3′
ParsRRT[a]	5′-CGCAGCACGTTCAAAACTC-3′
ParsBRT[a]	5′-GACATGACAGACACCGGCAA-3′
ParsC2RT[a]	5′-CACCGAATTTGTCACAACGC-3′
ParsC3RT[a]	5′-TCGATGGTCTTTGACTCTTGC-3′
P16SRT[a]	5′-CTGCTGGCACGGAGTTAGC-3′
ParsC1RealT[b]	5′-GTCAAAGCTGGTTTCCGAACTG-3′/5′-GGCTTTCGGCAGGAAAAAA-3′
ParsRRealT[b]	5′-TCACAGAAGCCTTAGCTTTGC-3′/5′-ATCCATTTTCCGCTCCTTCT-3′
ParsBRealT[b]	5′-ATCGCCATCGGCTTGATTC-3′/5′-GACAATGCGAGCACCTTGAA-3′
ParsC2RealT[b]	5′-ATTCCGCTGGTGTGAAAAAG-3′/5′-TCGATGGTCTTTGACTCTTGC-3′
ParsC3RealT[b]	5′-ACTCCTGTCGAAGCCAGATG-3′/5′-GGGTTTAGTCCGTGCTTTTTC-3′
P16SRealT[b]	5′-GGGTGAGTAACGCGTGGATT-3′/5′-AGCAGAGGCCCCCTTTACC-3′

[a] Primers were generated manually based on the sequence at the end of each gene.
[b] Primers were generated by Primer Express (ABI Prism).

sulfate-reducing bacteria. (NCBI genome accession number, NZ_AABN00000000). Here, we have characterized the arsenate transformation system in strain G20 and propose a regulatory mechanism for arsenate detoxification.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media and conditions. *D. desulfuricans* G20 and pSC27 (35) were obtained from Judy Wall, University of Missouri, Columbia. The anaerobic lactate-sulfate medium used for growth of *D. desulfuricans* G20 was prepared as previously described (12) under N_2 and contained sodium lactate (62 mM), Na_2SO_4 (50 mM), $MgSO_4$ (8 mM), NH_4Cl (5 mM), HEPES (25 mM), K_2HPO_4 (2.2 mM), $CaCl_2.2H_2O$ (0.6 mM), yeast extract (0.1%), and trace amounts of other minerals and vitamins (34). The pH was adjusted with NaOH to 7.2 prior to autoclaving. Agar (1.5%) was added to the solid media. $NaHCO_3$ (8 mM) and 0.025% cysteine HCl (for liquid media) or 0.005% $PdCl_2$ (for solid media) was added after autoclaving. The last two were added as reductants. Solid media were dispensed after the addition of antibiotics. Plates were cooled, dried overnight in a laminar-flow hood, and reduced overnight in an anaerobic glove box (Coy Laboratory Products, Inc., Grass Lake, MI). A lactate-sulfate medium containing between 1 μM and 1 mM arsenite ($NaAsO_2$) or between 1 mM and 50 mM arsenate (Na_3AsO_4) was used for determination of arsenic resistance. The lactate-arsenate medium was similar to the lactate-sulfate medium except that Na_3AsO_4 (20 mM for liquid or 25 mM for solid media) was substituted for Na_2SO_4 and $MgCl_2$ was substituted for $MgSO_4$. Arsenic from filter-sterilized stock solutions was added to the media after autoclaving. Typically, 0.1 ml of a 24-h growing culture was inoculated into 10 ml of sterile medium. Kanamycin was added to all media (1,050 μg/ml for liquid media; 175 μg/ml for solid media) used for screening and maintaining G20 transformants (G20 has displayed increased kanamycin resistance in liquid medium). *E. coli* strain DH5α was grown in Luria-Bertani (LB) broth with 100 μg/ml kanamycin (if needed). LB broth with arsenate concentrations ranging from 1 to 20 mM was used for determining levels of arsenate resistance in *E. coli*. All cultures were incubated at 37°C, and cell densities in cultures were determined in a Spectronic-20 spectrophotometer and reported as optical density at 600 nm (OD_{600}).

Arsenite detection and arsenite and arsenate quantification. Arsenite was detected qualitatively by adjusting the pH of sulfate-grown cultures to 3.0 with HCl, allowing orpiment (As_2S_3) to form (20, 21). Cultures grown without sulfate were treated with 0.1 ml of a solution of 2% $Na_2S \cdot 9H_2O$ and subsequently adjusted to pH 3.0. Arsenate and arsenite were quantified spectrophotometrically using the method of Johnson and Pilson (11).

Genome sequence and phylogenetic analysis. The *D. desulfuricans* G20 genomic sequence, along with other proteobacterial sequences, was obtained from http://genome.jgi-psf.org/mic_home.html; alignment and other bioinformatic analyses were carried out with CLUSTALW 1.82 and NNPP promoter finder 2.2 and through the NCBI website (http://www.ncbi.nlm.nih.gov), the BCM searchlauncher website (http://searchlauncher.bcm.tmc.edu), and VIMSS computational genomics (http://www.microbesonline.org).

Analysis of the *arsRBCC* operon. In order to determine the start of the *arsRBCC* operon, rapid amplification of 5′ cDNA ends (5′ RACE) was performed with the 5′/3′ RACE kit (Roche, Mannheim, Germany). Cells were grown in lactate-sulfate medium to an OD_{600} of 0.2. Then, the cells were treated with 20 mM arsenate to increase expression of the *arsRBCC* operon. Total RNA was isolated at an OD_{600} of 0.4 with the RNeasy minikit (QIAGEN Inc., Valencia, CA) following the kit's manual. RNA (1 μg) was used to synthesize the single-stranded cDNA with ParsGSP1 (Table 1) and transcriptor reverse transcriptase. The single-stranded cDNAs were cleaned with the High Pure PCR purification kit (included in the 5′/3′ kit), and a poly(A) tail was added at the 5′ end, using terminal transferase. Then, two rounds of PCR were performed with the cDNA using *Taq* polymerase and ParsGSP2 and PdTanchor (Table 1) (first round) and ParsGSP3 and Panchor (Table 1) (second round). PCR products were purified with the High Pure PCR purification kit and sequenced with ParsGSP3 and Panchor (Table 1).

In order to be sure that the *arsR arsB* and *arsC2 arsC3* genes belonged to one operon, three sets of primers (PlinkRB, PlinkBC, and PlinkCC) (Table 1) were designed to amplify the three gaps in these four genes. The RNA samples described above (1 μg) were used to synthesize double-stranded cDNA with Superscript reverse transcriptase II and ParsC3RT (Table 1). PCR was performed using the Plink primers and *Taq* polymerase. The PCR products were checked by gel electrophoresis.

Real-time PCR for quantification of mRNAs. Cells were grown in lactate-sulfate medium to an OD_{600} of 0.2. One set of replicate cultures was treated with 20 mM arsenate to determine its effect on induction of mRNA. After the cells had reached an OD_{600} of 0.4 (about 4 h), cells were harvested by centrifugation (5,000 × *g* for 5 min at 4°C). RNA was subsequently isolated with the RNeasy minikit. RNA samples (49 μl) were mixed with 8.5 μl DNase and 8.5 μl buffer (Promega Corporation, Madison, WI). The mixtures were incubated at 37°C for 1 h to eliminate the DNA contamination, and RNA was again purified with the QIAGEN RNeasy minikit. For real-time PCR analysis, each of the five *ars* genes' cDNAs was first synthesized with 2 pmol gene-specific primers (Table 1), 2 μg total RNA as a template, and 1 μl deoxynucleoside triphosphates (10 mM each). Superscript reverse transcriptase II was used as described in the manual (Invitrogen Corporation, Carlsbad, CA). Real-time PCR was carried out as previ-

ously described (24) with the ABI Prism 7000 system (Applied Biosystems, Foster City, CA). Primer sequences were designed by ABI Prism 7000 SDS Software, and amplification was performed by a standard protocol. Each amplicon was 101 bp. Relative quantification of mRNA expression was calculated using the Pfaffl method (24). 16S rRNA was used as a reference gene. DNA contamination of RNA samples was tested by running PCRs as described above but omitting the reverse transcriptase.

MNNG mutagenesis. *D. desulfuricans* G20 cells were mutated, and arsenate-sensitive mutants were identified. G20 cells were first grown in lactate-sulfate medium to an OD_{600} of 0.4. A 1-ml aliquot of cells was treated with 50 μl *N*-methyl-*N*′-nitro-nitrosoguanidine (MNNG) solution (2.5 μg/ml) (22) for 4 hours. Surviving cells were recovered on lactate-sulfate plates. The killing rate was 99.7%. Mutants (1,920 colonies) were transferred into parallel 96-well microtiter plates (with and without 20 mM arsenate), and growth was determined after 2 days (13). The ability to reduce arsenate was determined by the formation of a yellow precipitate (orpiment) (19, 20). Potential mutants were subsequently transferred to parallel serum tubes with and without 20 mM arsenate to confirm the loss of arsenate resistance.

General molecular methods. Plasmids were isolated with the Qiaprep Mini Prep kit (QIAGEN Inc., Valencia, CA). Chromosomal DNA was isolated with the Easy DNA kit (Invitrogen Corp., Carlsbad, CA). PCR was performed by using the *Taq* DNA polymerase system (Invitrogen Corp.) with *Pfu* polymerase (Takara Bio Inc., Otsu, Shiga, Japan) to obtain blunt-ended PCR products. T4 DNA ligase (Invitrogen Corporation, Carlsbad, CA) was used to ligate (16 h) the PCR product into pSC27 (100 ng of each). Plasmid constructs were transformed into *E. coli* DH5α using standard procedures (5). The plasmids were subsequently isolated and transformed into *D. desulfuricans* G20 as follows. Competent cells of strain G20 were prepared by growing the culture to early stationary phase (OD_{600}, 0.8) and centrifuging the cells under N_2 in sealed bottles at 6,000 × *g* for 10 min at 4°C. The cell pellet was resuspended in 50 ml of an ice-cold solution containing sucrose (400 mM) and magnesium chloride (1 mM) previously sparged with N_2 to remove oxygen. This process was repeated twice, and the cells were stored on ice. Cells (85 to 95 μl) were then mixed with 0.5 to 2.5 μg of plasmid DNA to a total volume of 100 μl and treated in an ECM 399 electroporator (BTX Harvard Apparatus Inc., Holliston, MA) at 2,500 V in an anaerobic glove box. Cells were recovered in lactate-sulfate medium for 4 h and then plated out on solid medium with 175 μg/ml kanamycin to select for transformants.

Cloning and sequencing of arsenic resistance genes. The *arsC1* (1.8-kbp) and the *arsRBCC* operon (2.7-kbp) regions of the chromosomes of these two mutants and strain G20 were amplified with the sequence-specific primers ParsC and ParsRBCC (Table 1). The arsenic resistance genes were cloned into pSC27 as follows. pSC27 was digested with SmaI following the manufacturer's protocol (New England Biolabs, Ipswich, MA), and the 1.8-kbp and 2.7-kbp blunt-ended PCR products were each blunt-end ligated into pSC27. Reconstructed plasmids were chemically transformed (5, 6) into *E. coli* DH5α by selecting for kanamycin resistance. The plasmids containing PCR products of *D. desulfuricans* G20's arsenic resistance genes (*arsC1* and *arsRBCC*) were named pXL10c and pXL11op, respectively (Table 1). Genes were sequenced by cloning the same PCR products directly into the pCR4-TOPO vector (TOPO TA cloning kit; Invitrogen Corp., Carlsbad, CA). DNA sequencing was carried out by the dideoxynucleotide chain termination method at the Oklahoma Medical Research Foundation (Oklahoma City, OK).

RESULTS AND DISCUSSION

***D. desulfuricans* G20 growth and arsenate reduction.** Strain G20 cultures grew and reduced arsenate in liquid media with lactate as an electron donor and sulfate as an electron acceptor at arsenate concentrations up to 20 mM (Fig. 1A). Cultures whose growth was inhibited with erythromycin (*Desulfovibrio* strains are sensitive to erythromycin; the MIC of strain G20 is 25 μg/ml [unpublished data]) did not detectably reduce any arsenate. G20 cultures typically reach an optical density of 1.1 with or without 20 mM arsenate in lactate-sulfate medium. Although we did not measure lactate consumption or sulfate reduction, it is likely that the 62 mM lactate in the media provided adequate reducing equivalents to reduce all of the arsenate and as much sulfate as was reduced in the control. In

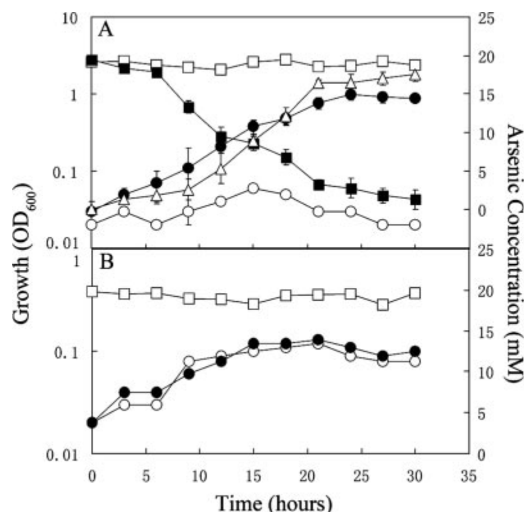

FIG. 1. Growth and arsenate reduction by *D. desulfuricans* strain G20. (A) Growth and arsenate reduction in lactate-sulfate medium with 20 mM initial arsenate. Growth (●); arsenate concentration (■); arsenite concentration (△). Erythromycin (50 ng/ml) inhibited growth (○) and arsenate reduction (□). The error bars indicate standard deviations. (B) Growth and arsenate reduction in the absence of sulfate. Growth with lactate-arsenate (●); arsenate concentration (□); growth in lactate only medium as a control (○).

the absence of arsenate, the lag phase in lactate-sulfate media was 5.5 h (data not shown) compared to 10.5 h with arsenate (Fig. 1A). Doubling times under both conditions were 3.8 h. This result suggests that arsenate reduction is not an energy-yielding process. The arsenate concentration was reduced to less than 0.1 mM after 24 h (Fig. 1A). Orpiment did not form naturally in these cultures, as either lower sulfide concentrations or lower pH is required for its formation (19). We have also observed that two related species, *Desulfovibrio vulgaris* Hildenborough and *Desulfovibrio* sp. strain ASR (a marine strain obtained from Brad Tebo), similarly reduce arsenate (data not shown). Based on our work and previously published studies describing the ability of *Desulfovibrio* sp. strain Ben-RA and *Desulfomicrobium* sp. strain Ben-RB (16) to grow with and reduce arsenate, it appears that this ability is likely broadly or universally present within the *Desulfovibrionales* cluster.

The ability of strain G20 to grow and reduce arsenate when present as the sole electron acceptor was also tested. Controls were grown without arsenate. Neither growth nor arsenate reduction was observed (Fig. 1B) under these conditions, suggesting that *D. desulfuricans* G20 reduces arsenate as a detoxification mechanism rather than as a means of respiration.

When *Desulfovibrio* sp. strain Ben-RA was grown in lactate-sulfate medium with 9.2 mM arsenate, the arsenate concentration was reduced to about 6 mM after 60 h of incubation, and under the same conditions, *Desulfomicrobium* sp. strain Ben-RB reduced 8.2 mM arsenate to about 2 mM. The two strains can tolerate arsenate concentrations similar to those tolerated by G20 but exhibit a lower arsenate reduction rate (16). *Desulfomicrobium* sp. strain Ben-RB also grows with and reduces 3.8 mM arsenate as an electron acceptor in lactate-arsenate medium, a process which neither strain Ben-RA nor strain G20 is able to carry out. This suggests that even though

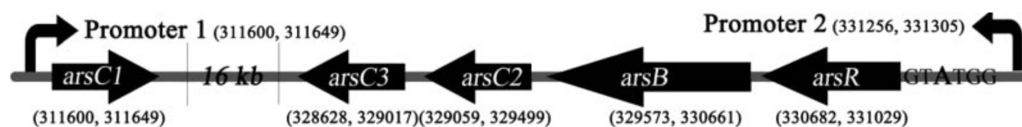

FIG. 2. Diagram showing the genomic locations of arsenate reductase genes of *D. desulfuricans*. Shown are the locations of the ORFs in the *D. desulfuricans* genome. The numbers in parentheses are the nucleotide numbers from the NCBI *D. desulfuricans* genome sequence (NZ_AABN02000000). The base A in boldface is the transcriptional start site of the *arsRBCC* operon based on 5′ RACE analysis.

these three strains can reduce arsenate, they may use different mechanisms.

Genome sequence and phylogenetic analysis. In order to further explore the mechanism of arsenate transformation in *D. desulfuricans* G20, we searched the genome for both nucleotide and amino acid sequences of *arsR*, *-D*, *-A*, *-B*, and *-C*, as well as *arrA* and *arrB* from the NCBI nr database. In the *D. desulfuricans* G20 genome, three open reading frames (ORFs) were identified that displayed similarity to *arsC*s from pI258 and the *B. subtilis* family (17, 32): they are designated here *arsC1* (gi:23473897; annotated in NCBI), *arsC2* (gi:53691679; NCBI), and *arsC3* (gi: 53691680; NCBI) (Fig. 2). There are five other predicted similar *arsC* genes within currently available genomic sequences of δ-proteobacteria (see Fig. S1 in the supplemental material) (http://microbesonline.gov). The *arsC1* and *arsC2* genes are most closely related to each other and similarly related to the *arsC* genes from *Desulfotalea psychrophila* LSV54 and *Desulfuromonas acetoxidans* DSM 684. The *arsC3* gene, on the other hand, has only 57% protein similarity to the other two G20 *arsC* genes and is more closely related to the *Wolinella succinogenes arsC* (gi: 34556458; NCBI) (68% protein similarity) (see Fig. S1 in the supplemental material). Seven δproteobacterial strains have genomic sequences available. Six strains have predicted *arsC* genes within the pI258/*B. subtilis* family, while *Bdellovibrio bacteriovorus* HD100 has a predicted *arsC* within the R773 family (gi:42521650) (http://microbesonline.gov). The presence of three copies of *arsC* in this configuration is quite unusual. The only other known multilocus arsenate resistance system is present in *Pseudomonas aeruginosa* (3, 33). The *P. aeruginosa* genome contains an *arsC* gene (accession number, gi:15597475) by itself, while it also has an *arsRBC* operon (3). These two systems are far apart on the chromosome, as in G20, and both are thought to be functional (33). It is also known that multiple *ars* resistance genes increase arsenate resistance, based on work with *E. coli* (3). As *arsC* is responsible for reduction of cytoplasmic arsenate to arsenite, the presence of three copies of *arsC* likely provides increased levels of arsenate reduction. This search also revealed one ORF in the G20 genome (gi:23473907; NCBI) whose protein sequence is 86% similar to that of ArsB (gi:116584655) of *Bacillus cereus* and one ORF whose protein sequence is 68% similar to the putative arsenic efflux pump regulator protein (gi:27464265) of *Enterobacter cloacae*. Four other predicted *arsB* genes were detected in genomic sequences available for δproteobacteria: (gi: 68001681 in *Geobacter metallireducens* GS-15, gi:39998045 in *Geobacter sulfurreducens* PCA, gi:68178276 in *Desulfuromonas acetoxidans* DSM 684, and gi:50876667 in *D. psychrophila* LSV54). In addition, three predicted *arsR* genes were detected in those genomes: (gi:68001683 in *G. metallireducens* GS-15, gi:39998043 in *G. sulfurreducens* PCA, and gi:95930854 in *D. acetoxidans* DSM 684).

The four ORFs, *arsC2*, *arsC3*, and those related to *arsR* and *arsB*, form a putative *arsRBCC* operon with an 88% possible promoter upstream of *arsR*. The *arsC1* gene has a 66% possible upstream promoter of its own (Fig. 2). The only other strain of *Desulfovibrio* in which arsenic resistance has been studied genetically is *Desulfovibrio* sp. strain Ben-RA (16). In strain Ben-RA, arsenate reduction does not support growth and likely involves a chromosomal gene that hybridizes to *arsC* of the R773 system (18, 33).

Based on genomic analysis, there are at least three other members of the δproteobacteria with an *ars* operon in their genomes—*G. sulfurreducens* PCA (gi:39998043, 39998044, and 39998045), *G. metallireducens* GS-15 (gi:68001681, 68001682, and 68001683), *D. acetoxidans* DSM 684 (*arsR*; gi:68178275 and 68178276)—suggesting a common mechanism of arsenate detoxification among these δproteobacteria. However, the order of the genes in these other *ars* operons is *RCB*. *D. vulgaris* has an *arsC* homolog and reduces high arsenate levels (data not shown). However, the lack of *arsB* and *arsR* indicates that *D. vulgaris* may utilize a unique mechanism for detoxifying arsenate. There are no strong homologs to *arrA* and *arrB* in the G20 genome, providing additional evidence that strain G20 utilizes a detoxification rather than a respiration process to reduce arsenate.

Operon analysis. In order to prove that the *arsRBCC* operon detected by sequence analysis is a functional operon, the following tests were carried out. 5′ RACE was performed to determine the transcriptional start of the operon. Sequence analysis has shown that base 331029 (NCBI) is the start of transcription of the *arsRBCC* operon (Fig. 2). To prove that *arsR*, *arsB*, *arsC1*, and *arsC2* are transcribed as a single mRNA, gap amplification PCR was carried out and the PCR products were visualized by gel electrophoresis. Clear PCR products were formed using the PlinkRB, PlinkBC, and PlinkCC primer sets (data not shown), indicating that the entire *arsRBCC* operon is transcribed as a unit.

Real-time PCR. The roles of the two genetic units, *arsC1* and the *arsRBCC* operon, in arsenate resistance remained to be determined. To address this issue, transcriptional analysis was carried out. Expression of *arsC1* and *arsRBCC* was monitored at the transcript level in strain G20 and in the AS2 recombinant (pXL11op) grown with or without arsenate (Fig. 3). In both strains, expression of *arsC1* was only marginally increased (24%) upon the addition of arsenate. The regulator *arsR* was similarly induced by only 18%. On the other hand, expression of *arsB* was induced about 17-fold and *arsC2* and *arsC3* were increased about 3-fold (Fig. 3). When transformed with pXL11op, expression patterns were similar but at a slightly higher level (Fig. 3). These results showed that *arsB*, *arsC2*, and *arsC3* in *D. desulfuricans* are regulated by arsenic, likely as a direct result of the arsenite produced by reduction of arsenate (28, 29); however, expression of *arsC1* appears constitutive.

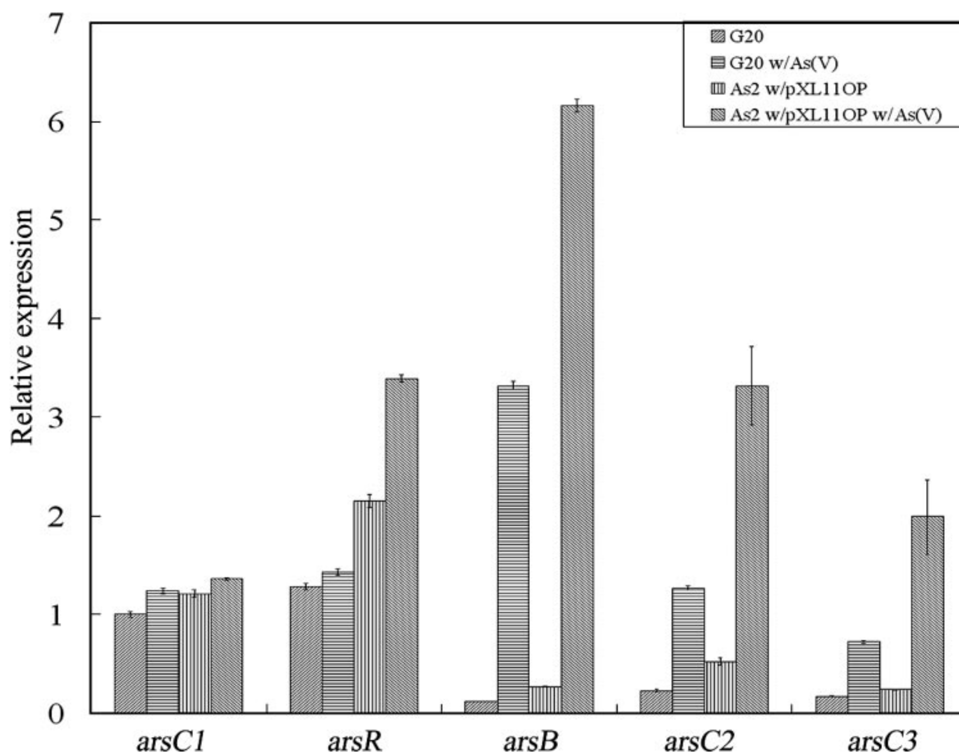

FIG. 3. Expression levels of *arsC1*, *arsR*, *arsB*, *arsC2*, and *arsC3* during arsenate treatment. G20 w/As(V), G20 treated with arsenate; As2 pXL11op w/As(V), As2 pXL11op treated with arsenate. Expression was determined by real-time PCR and normalized with 16S rRNA. The relative expression of *arsC1* in the absence of arsenate treatment was converted to a value of 1 as a reference. The error bars show the standard deviations for triplicate samples.

The polar expression effect observed with the *arsRBCC* operon has been previously reported (15). In the cyanobacterium *Synechocystis* sp. strain PCC 6803, expression of the *arsB* gene was increased about 12-fold, while expression of a downstream *arsC* within the same operon was increased about 2-fold (15). A comparison of expression levels of strain G20 with the pXL11op transformant (Fig. 3) suggests that two or three copies of the plasmid are present in strain G20.

Characterization of arsenate-sensitive mutants of strain G20. We sequenced the *arsRBCC* operon and *arsC1* of As1 and As2, the two MNNG mutant strains determined to be most sensitive to arsenate. The *arsRBCC* operons in both mutants are 100% identical to the original strain; there is a single point mutation in each mutant in the *arsC1* region, which could have caused dramatic structural changes. In As1, the phenylalanine (TTC) at position 33 has been changed to a serine (TCC), and in As2, the arginine (CGG) at position 64 has been changed to a tryptophan (TGG). These two mutants were subsequently tested for the ability to grow at lower arsenate concentrations. Although neither could tolerate 10 mM arsenate, both could grow at 5 mM arsenate (As1, data not shown; As2, Fig. 4A), likely as a result of residual arsenate reductase activities associated with the intact *arsC2* and *arsC3*. Previous work has shown that the loss of *arsC* function results in arsenate but not arsenite sensitivity (25). Inactivation of each of the other genes in the *arsRBC* operon is known to result in different phenotypes (36). Deletion of *arsR* results in overexpression of *arsB* and *arsC*, while the loss of *arsB* results in sensitivity to both arsenate and arsenite. Both As1 and As2 strains are more sensitive to arsenate than the parental strain but are similarly sensitive to arsenite (Fig. 4B).

Expression of the cloned arsenate reductase in *E. coli* DH5α and G20 mutant As2. In order to confirm that the identified *ars*

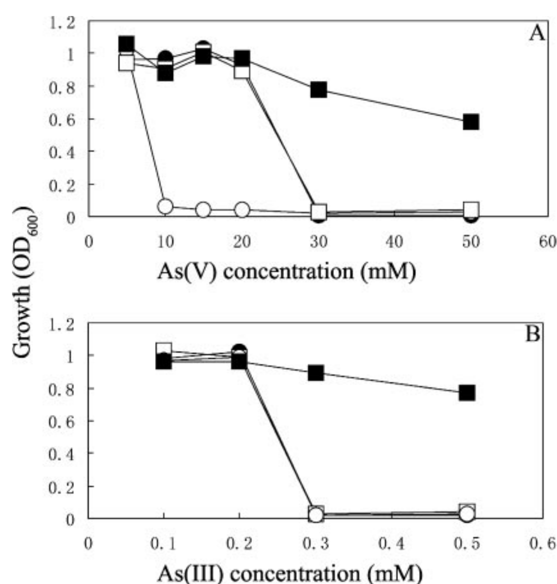

FIG. 4. Max OD_{600} after 48 h of incubation for recombinant strains of *D. desulfuricans* with increasing levels of (A) arsenate and (B) arsenite. *D. desulfuricans* G20 (●); As2 (○); As2 pXL10c (□); As2 pXL11op (■).

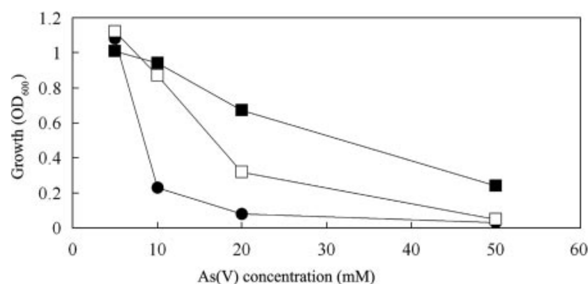

FIG. 5. Maximum growth of *E. coli* DH5α recombinants in LB broth with arsenate. *E. coli* DH5α (●); DH5α with pXL10c (□); DH5α with pXL11op (■).

genes were those involved in arsenate reduction in G20, the genes were cloned into *E. coli* and into the arsenate-sensitive mutant As2. Recombinant strains were subsequently tested to determine whether plasmid-borne genes had conferred arsenate resistance on these strains. *E. coli* strain DH5α is derived from strain K-12, known to have a chromosomal *arsRBC* operon (4); however, it would not grow with 20 mM arsenate (Fig. 5). *E. coli* pXL11op transformants (*arsRBCC*) were able to grow in LB broth containing 20 mM arsenate to an OD_{600} of 0.6 in 12 h and tolerated up to 50 mM arsenate with a lower cell density (OD_{600}) (Fig. 5). Orpiment was formed in tubes following growth (after decreasing the pH and addition of sulfide). The pXL10c (*arsC1*) transformants grew to only a low level with 20 mM arsenate and not at 50 mM (Fig. 5). These transformants lacked additional *arsB*, the arsenite pump, and therefore were not likely able to remove arsenite as effectively from cells after reduction. In this test, the *arsRBCC* operon's effect seemed cumulative. A previous study had also shown that introducing an *E. coli* chromosomal *ars* operon-containing plasmid into *E. coli* increased arsenate resistance 2- to 10-fold (3).

The two *ars* gene-containing plasmids were also transformed into the *D. desulfuricans* mutant As2 (Fig. 4). Although As2 can tolerate only 5 mM arsenate, the complemented mutants (with pXL10c) can tolerate up to 20 mM. It has been shown that MNNG can introduce mutations at multiple sites (22). However, the results of the complementation experiment provide strong evidence that the specific mutation in the *arsC1* region of As2 is responsible for loss of arsenate resistance. Surprisingly, As2 complemented with pXL11op can grow at concentrations exceeding 50 mM arsenate (Fig. 4A), suggesting that both copies of *arsRBCC* are functional in this construct. These results also confirm the fact that both *arsC1* and *arsRBCC* are involved in the response to arsenate toxicity in G20 cells. Mutant As2 pXL10c and strain G20 have similar resistances to arsenite (0.2 mM) (Fig. 4B), confirming that the *arsB* and *arsR* genes are intact in As2. The fact that mutant As2(pXL11op) (containing two copies of *arsRBCC*, one on the chromosome and one on the plasmid) is tolerant of up to 0.8 mM arsenite indicates that additional copies of *arsB* and *arsR* can confer extraordinary levels of arsenite resistance.

Freshly inoculated cells were inhibited by an arsenite concentration of 0.3 mM (Fig. 4B), whereas growing cells produced millimolar levels of arsenite that appeared to have little effect on them. It is therefore likely that energized cells are less sensitive to arsenite, as they have the ATP necessary to remove arsenite from cells.

Model for response to arsenate. Based upon the experimental data, a model is proposed for the observed arsenate reduction response (Fig. 6). In this model, the constitutively expressed *arsC1* allows a rapid response to an influx of arsenate into the cell, as the arsenate is reduced by ArsC1. Arsenite formed in the reaction then inactivates ArsR, allowing the *arsRBCC* operon to be transcribed, with the two copies of *arsC* allowing high levels of arsenate reduction. It is likely that the multilocus arsenate resistance system in *P. aeruginosa* is controlled in a manner similar to that of the system described here,

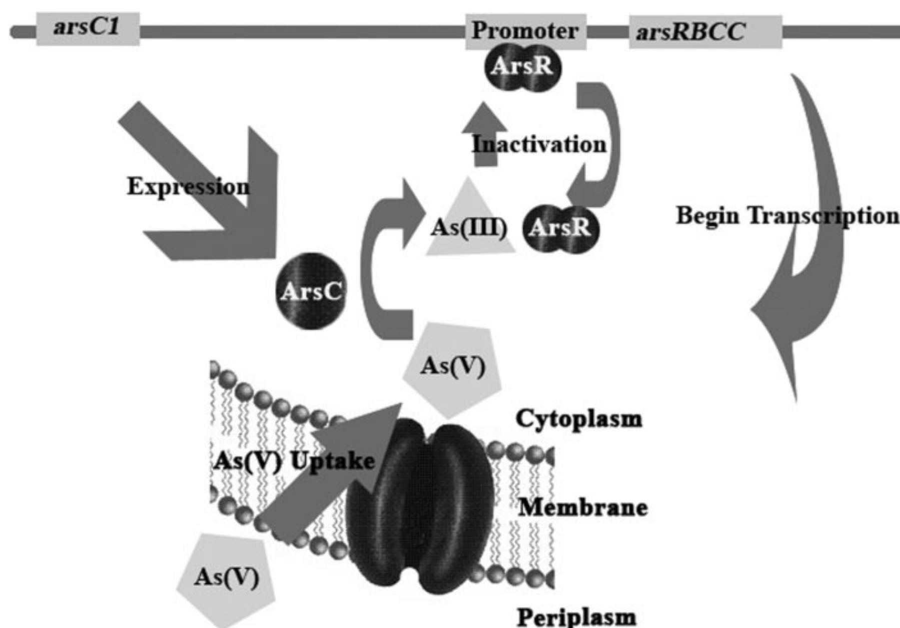

FIG. 6. Proposed model for an arsenate reduction system of *D. desulfuricans* G20.

although expression studies have not been carried out (3). This model is supported by data showing the need for both As resistance genetic units when cells are treated with 20 mM arsenate. We then predicted that we could bypass the need for *arsC1* by pretreating cells with lower levels of arsenate, directly inducing the *arsRBCC* operon. An experiment was carried out comparing As1 and As2 mutant cells pregrown to an OD_{600} of 0.1 with or without 5 mM arsenate and then challenged with 20 mM arsenate. In both mutants, cells pretreated with arsenate were able to continue to grow with 20 mM arsenate, whereas cells in which the *arsRBCC* operon was not induced showed no further growth after the challenge (see Fig. S2 in the supplemental material). The maintenance of a relatively complex arsenate detoxification system such as has been observed here suggests that G20 cells growing in the natural environment must be equipped to deal with rapidly changing and perhaps relatively high levels of arsenate.

ACKNOWLEDGMENTS

This research was supported by a grant from the Environmental Remediation Science Program (ERSP) of the Office of Biological and Environmental Research of the U.S. Department of Energy Office of Science.

REFERENCES

1. **Ahmann, D., A. L. Roberts, L. R. Krumholz, and F. M. Morel.** 1994. Microbe grows by reducing arsenic. Nature **371:**750.
2. **Bennett, M. S., Z. Guan, M. Laurberg, and X. D. Su.** 2001. *Bacillus subtilis* arsenate reductase is structurally and functionally similar to low molecular weight protein tyrosine phosphatases. Proc. Natl. Acad. Sci. USA **98:**13577–13582.
3. **Cai, J., K. Salmon, and M. S. DuBow.** 1998. A chromosomal *ars* operon homologue of *Pseudomonas aeruginosa* confers increased resistance to arsenic and antimony in *Escherichia coli*. Microbiology **144:**2705–2713.
4. **Carlin, A., W. Shi, S. Dey, and B. P. Rosen.** 1995. The *ars* operon of *Escherichia coli* confers arsenical and antimonial resistance. J. Bacteriol. **177:**981–986.
5. **Cohen, S. N., A. C. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69:**2110–2114.
6. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. A manual for genetic engineering: advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
7. **Dey, S., and B. P. Rosen.** 1995. Dual mode of energy coupling by the oxyanion-translocating ArsB protein. J. Bacteriol. **177:**385–389.
8. **Diorio, C., J. Cai, J. Marmor, R. Shinder, and M. S. DuBow.** 1995. An *Escherichia coli* chromosomal *ars* operon homolog is functional in arsenic detoxification and is conserved in gram-negative bacteria. J. Bacteriol. **177:** 2050–2056.
9. **Gladysheva, T. B., K. L. Oden, and B. P. Rosen.** 1994. Properties of the arsenate reductase of plasmid R773. Biochemistry **33:**7288–7293.
10. **Ji, G., and S. Silver.** 1992. Reduction of arsenate to arsenite by the ArsC protein of the arsenic resistance operon of *Staphylococcus aureus* plasmid pI258. Proc. Natl. Acad. Sci. USA **89:**9474–9478.
11. **Johnson, D. L., and M. E. Pilson.** 1975. The oxidation of arsenite in seawater. Environ. Lett. **8:**157–171.
12. **Krumholz, L. R., and M. P. Bryant.** 1986. *Eubacterium oxidoreducens* sp. nov. requiring H_2 or formate to degrade gallate, pyrogallol, phloroglucinol, and quencetin. Arch. Microbiol. **144:**8–14.
13. **Kuai, L., A. A. Nair, and M. F. Polz.** 2001. Rapid and simple method for the most-probable-number estimation of arsenic-reducing bacteria. Appl. Environ. Microbiol. **67:**3168–3173.
14. **Lin, Y. F., A. R. Walmsley, and B. P. Rosen.** 2006. An arsenic metallochaperone for an arsenic detoxification pump. Proc. Natl. Acad. Sci. USA **103:** 15617–15622.
15. **Lopez-Maury, L., F. J. Florencio, and J. C. Reyes.** 2003. Arsenic sensing and resistance system in the *Cyanobacterium synechocystis* sp. strain PCC 6803. J. Bacteriol. **185:**5363–5371.
16. **Macy, J. M., J. M. Santini, B. V. Pauling, A. H. O'Neill, and L. I. Sly.** 2000. Two new arsenate/sulfate-reducing bacteria: mechanisms of arsenate reduction. Arch. Microbiol. **173:**49–57.
17. **Mukhopadhyay, R., and B. P. Rosen.** 2002. Arsenate reductases in prokaryotes and eukaryotes. Environ. Health Perspect. **110**(Suppl. 5):745–748.
18. **Mukhopadhyay, R., B. P. Rosen, T. Phung Le, and S. Silver.** 2002. Microbial arsenic: from geocycles to genes and enzymes. FEMS Microbiol. Rev. **26:** 311–325.
19. **Newman, D. K., D. Ahmann, and F. M. Morel.** 1998. A brief review of microbial arsenate respiration. Geomicrobiology **15:**255–268.
20. **Newman, D. K., T. J. Beveridge, and F. M. Morel.** 1997. Precipitation of arsenic trisulfide by *Desulfotomaculum auripigmentum*. Appl. Environ. Microbiol. **63:**2022–2028.
21. **Newman, D. K., E. K. Kennedy, J. D. Coates, D. Ahmann, D. J. Ellis, D. R. Lovley, and F. M. Morel.** 1997. Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum* sp. nov. Arch. Microbiol. **168:**380–388.
22. **Oeschger, M. P., and M. K. Berlyn.** 1979. A simple procedure for localized mutagenesis using nitrosoguanidine. Mol. Gen. Genet. **134:**77–83.
23. **Oremland, R. S., and J. F. Stolz.** 2003. The ecology of arsenic. Science **300:**939–944.
24. **Pfaffl, M. W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:**e45.
25. **Rosen, B. P.** 2002. Biochemistry of arsenic detoxification. FEBS Lett. **529:** 86–92.
26. **Rosen, B. P.** 1999. Families of arsenic transporters. Trends Microbiol. **7:**207–212.
27. **Rosen, B. P.** 2002. Transport and detoxification systems for transition metals, heavy metals and metalloids in eukaryotic and prokaryotic microbes. Comp. Biochem. Physiol. A **133:**689–693.
28. **Rosenstein, R., K. Nikoleit, and F. Gotz.** 1994. Binding of ArsR, the repressor of the *Staphylococcus xylosus* (pSX267) arsenic resistance operon to a sequence with dyad symmetry within the *ars* promoter. Mol. Gen. Genet. **242:**566–572.
29. **Rosenstein, R., A. Peschel, B. Wieland, and F. Gotz.** 1992. Expression and regulation of the antimonite, arsenite, and arsenate resistance operon of *Staphylococcus xylosus* plasmid pSX267. J. Bacteriol. **174:**3676–3683.
30. **Saltikov, C. W., A. Cifuentes, K. Venkateswaran, and D. K. Newman.** 2003. The *ars* detoxification system is advantageous but not required for arsenate respiration by the genetically tractable *Shewanella* species strain ANA-3. Appl. Environ. Microbiol. **69:**2800–2809.
31. **Saltikov, C. W., and D. K. Newman.** 2003. Genetic identification of a respiratory arsenate reductase. Proc. Natl. Acad. Sci. USA **100:**10983–10988.
32. **Sato, T., and Y. Kobayashi.** 1998. The *ars* operon in the skin element of *Bacillus subtilis* confers resistance to arsenate and arsenite. J. Bacteriol. **180:**1655–1661.
33. **Silver, S.** 1996. Bacterial resistances to toxic metal ions—a review. Gene **179:**9–19.
34. **Steger, J. L., C. Vincent, J. D. Ballard, and L. R. Krumholz.** 2002. *Desulfovibrio* sp. genes involved in the respiration of sulfate during metabolism of hydrogen and lactate. Appl. Environ. Microbiol. **68:**1932–1937.
35. **Wall, J. D., T. Murnan, J. Argyle, R. S. English, and B. J. Rapp-Giles.** 1996. Transposon mutagenesis in *Desulfovibrio desulfuricans*: development of a random mutagenesis tool from Tn*7*. Appl. Environ. Microbiol. **62:**3762–3767.
36. **Wu, J., and B. P. Rosen.** 1993. Metalloregulated expression of the *ars* operon. J. Biol. Chem. **268:**52–58.