The Chromosomal Arsenic Resistance Genes of *Thiobacillus ferrooxidans* Have an Unusual Arrangement and Confer Increased Arsenic and Antimony Resistance to *Escherichia coli*

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ABSTRACT

The chromosomal arsenic resistance genes of the acidophilic, chemolithoautotrophic, biomining bacterium *Thiobacillus ferrooxidans* were cloned and sequenced. Homologues of four arsenic resistance genes, *arsB*, *arsC*, *arsH*, and a putative *arsR* gene, were identified. The *T. ferrooxidans arsB* (arsenite export) and *arsC* (arsenate reductase) gene products were functional when they were cloned in an *Escherichia coli ars* deletion mutant and conferred increased resistance to arsenite, arsenate, and antimony. Therefore, despite the fact that the *ars* genes originated from an obligately acidophilic bacterium, they were functional in *E. coli*. Although *T. ferrooxidans* is gram negative, its ArsC was more closely related to the ArsC molecules of gram-positive bacteria. Furthermore, a functional *trxA* (thioredoxin) gene was required for ArsC-mediated arsenate resistance in *E. coli*; this finding confirmed the gram-positive ArsC-like status of this resistance and indicated that the division of ArsC molecules based on Gram staining results is artificial. Although *arsH* was expressed in an *E. coli*-derived in vitro transcription-translation system, ArsH was not required for and did not enhance arsenic resistance in *E. coli*. The *T. ferrooxidans ars* genes were arranged in an unusual manner, and the putative *arsR* and *arsC* genes and the *arsBH* genes were translated in opposite directions. This divergent orientation was conserved in the four *T. ferrooxidans* strains investigated.

Thiobacillus ferrooxidans is an acidophilic (optimum pH, 1.8 to 2.5), obligately chemolithotrophic bacterium that obtains its energy through oxidation of ferrous iron to ferric iron or oxidation of reduced inorganic sulfur compounds to sulfuric acid. It is a member of a consortium of bacteria (which includes *Thiobacillus caldus* and *Leptospirillum ferrooxidans*) that is used in commercial biooxidation processes to recover gold from arsenopyrite ores (22). Although recent analysis of microbial populations in continuous-flow biooxidation tanks has revealed that *T. ferrooxidans* may not be as dominant as was once thought, this organism is nevertheless usually present in such tanks (21). Total arsenic levels greater than 13 g liter^{-1} may be present in arsenopyrite biooxidation tanks, and therefore the microorganisms present must have a mechanism of resistance to arsenic (8).

Plasmid-associated arsenic efflux resistance mechanisms have been known for many years and have been extensively reviewed (5, 23, 30-32,35). Although the number of components of these systems varies, in the case of *Escherichia coli* plasmids R773 and R46, as well as *Acidiphilium multivorum* plasmid pKW301 (34), as many as five genes (*arsRDABC*) are present. In the case of R773, the genes are transcribed in a single operon. The *arsR* and *arsD* genes encode repressors that control the basal and upper levels of *ars* operon expression,

while the *arsABC* genes encode the structural components of the arsenic resistance mechanism. ArsA is an ATPase which forms a complex with ArsB, the transmembrane arsenite efflux pump. ArsC is a small, cytoplasmically located arsenate reductase which reduces arsenate to arsenite, which can then be pumped out of the cell. The ArsB protein is capable of exporting arsenite even in the absence of ArsA (9).

The arsenic resistance systems of *Staphylococcus* plasmids pSX267 and pI258, as well as the chromosomally located arsenic resistance systems of *E. coli* (4) and *Pseudomonas aeruginosa* (3), consist of only three genes, *arsRBC*. Nevertheless, the *ars* operons are capable of exporting arsenate, arsenite, and antimony oxyanions in the absence of *arsA* by using membrane potential rather than ATP as an energy source. Recently, an arsenic resistance system which consists of *arsRBC* and a fourth open reading frame (ORF) of unknown function was discovered in the *skin* element of *Bacillus subtilis* (28). An arsenic resistance system has been discovered in Tn*2502* located on plasmid pYV of *Yersinia enterocolitica*, and this system consists of *arsRBC*, as well as a divergently transcribed gene, *arsH* (17). The function of *arsH* is not known, but the presence of this gene either in *cis* or in *trans* is essential for arsenic resistance in *Y. enterocolitica*.

Here we describe isolation and analysis of the evolutionary relationships of the arsenic resistance genes of *T. ferrooxidans*. We found that these genes are functional in *E. coli* and have an unusual divergent *arsCRBH* operon structure, which appeared to be conserved in all of the *T. ferrooxidans* strains which we examined.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and media. The strains, plasmids, and primers used in this study are shown in Table 1. *E. coli* strains were grown on Luria-Bertani medium (25). *T. ferrooxidans* strains were grown in tetrathionate medium or iron sulfate medium (19) at 30°C. Ampicillin (100 µg/ml), chloramphenicol (20 µg/ml), and tetracycline (20 µg/ml) were used as required.

DNA techniques, sequencing, and analysis. A *T. ferrooxidans* ATCC 33020 gene bank consisting of 4- to 9-kb fragments obtained from a partial *Sau*3A digest cloned into the *Bgl*II site of the suicide vector pEcoR251 (20) was transformed into *E. coli ars* deletion mutant AW3110, which was made competent by the simple and efficient method (10). Plasmid preparation, restriction endonuclease digestion, gel electrophoresis, ligation, and Southern blot hybridization were carried out by using standard methods (20). Pulsed-field gel electrophoresis was carried out by using a Beckman Geneline transverse alternating field electrophoresis apparatus. Labeling of probes, hybridization, and detection were performed by using a dioxigenin-dUTP nonradioactive DNA labeling and detection kit (Roche). Sequences were determined by the dideoxy chain termination method (27) by using a thermosequenase fluorescently labeled primer cycle sequencing kit (Amersham Pharmacia Biotech UK Ltd.). Sequencing reactions were performed with an ALFexpress automated sequencer (Pharmacia Biotech, Uppsala, Sweden). Results were analyzed by using the VAX-based Genetics Computer Group Inc. sequence analysis package (version 7.1) and its associated programs and the PC-based DNAMAN software (version 4.1) from Lynnon BioSoft. Multiple sequence alignments were shaded by using the Genedoc Multiple Sequence Alignment Editor and Shading Utility (version 2.5.000). Comparison searches were performed by using the gapped-BLAST program of the National Center for Biotechnology Information (NCBI) (1). Homology trees were constructed by using the Multiple Sequence Alignment tool in DNAMAN.

Requirement for thioredoxin for arsenate resistance.A 7.1-kb *Hin*dIII-*Bgl*II fragment of pTfars1a, which contained the *ars* genes of *T. ferrooxidans*, was cloned into pACYC digested with *Hin*dIII and*Bam*HI. The resulting clone, pTfarsCRBH-Cm, was used to test pBluescriptSK-based plasmids in *trans. E. coli* BH2012, BH5262, and MC1061 were made competent with CaCl$_2$ and were transformed with pTfarsCRBH-Cm. *E. coli* BH5262 was also transformed with pTrx6, pTTn1, and pTT150 (Table 1). All of the strains mentioned above were streaked onto Luria agar plates (24) containing 0, 2, 5, 7, 10, and 15 mM sodium arsenate and were incubated at 37°C overnight.

PCR.A PCR was performed with the primers described in Table1, which were synthesized at the Synthetic DNA Laboratory, Department of Biochemistry, University of Cape Town. The reaction was performed with a Biometra Personal cycler by using Redhot *Taq* DNA polymerase (Advanced Biotechnologies). After an initial denaturation step consisting of 60 s at 94°C, 25 cycles consisting of 30 s at 94°C, 30 s at 57°C (for primers BBARSB and BBARSC) or 63°C (for primers ARSHF and ARSHR), and 90 s at 72°C were performed. A final extension step consisting of 120 s at 72°C before cooling to 25°C completed the reaction.

Arsenic and antimony resistance assays.Constructs were transformed into competent *E. coli* AW3110 cells. Assays to determine resistance to arsenite and antimonite were carried out in Luria broth (LB). In assays to determine resistance to arsenate, cells were grown in low-phosphate medium (18) supplemented with 2 mM K$_2$HPO$_4$. Overnight cultures were diluted 100-fold into fresh medium containing the appropriate antibiotics and different concentrations of sodium arsenite, potassium antimonite, or sodium arsenate. The cultures were incubated at 37°C for 5 h in the case of LB or for 12 h in the case of low-phosphate medium, and the absorbance at 600 nm was determined. The incubation times used corresponded to the end of the log phase of growth of controls under the same conditions.

In vitro transcription-translation analysis.A prokaryotic DNA-directed, *E. coli* S30 extract-based, in vitro transcription-translation kit for circular DNA (Promega) was used to analyze the polypeptides synthesized from clones that conferred arsenic resistance and subclones. The [^{35}S]methionine-labeled translation products were separated on a sodium dodecyl sulfate-polyacrylamide electrophoresis gel and detected by autoradiography.

Nucleotide sequence accession number.The nucleotide sequence determined in this study has been deposited in the GenBank database under accession no. AF173880.

RESULTS

Cloning of the *ars* genes of *T. ferrooxidans.* The *E. coli ars* operon deletion mutant (AW3110) was transformed with a partial *Sau*3A plasmid bank, and colonies were selected on the basis of their ability to complement the mutant on LB plates containing 0.5 mM sodium arsenite. One plasmid (pTfars1a), which contained a 7.4-kb insert and which retransformed *E. coli* AW3110 to arsenite resistance at a high frequency, was selected for further study. The plasmid was mapped to determine the positions of restriction endonuclease sites, as shown in Fig.1, and a 7.1-kb*Hin*dIII-*Bgl*II fragment was cloned into pBluescriptSK (pTfars1b). The source of the insert DNA was confirmed by Southern hybridization. A 5.3-kb internal*Hin*dIII-*Stu*I fragment was labeled and used to probe the chromosomal DNA of *T. ferrooxidans* ATCC 33020 and pTfars1b digested with *Pst*I. The sizes of *Pst*I fragments that were 3.5 and 2.0 kb long and were inside the insert of pTfars1b corresponded exactly to the sizes of *Pst*I fragments of *T. ferrooxidans* ATCC 33020 chromosomal DNA (data not shown). This indicated that the insert DNA originated from *T. ferrooxidans* ATCC 33020, that a single copy was

present, and that no rearrangements in the region which included the two *Pst*I fragments occurred during cloning. Chromosomal DNA was also digested with two rarely cutting 8-bp recognition sequence restriction enzymes, *Pac*I and *Swa*I, as well as with a combination of both of these enzymes. Restriction fragments were separated by using pulsed-field gel electrophoresis and were hybridized to a *T. ferrooxidans arsBH* probe. Signals of hybridization to *Pac*I, *Swa*I, and *Pac*I-*Swa*I fragments that were approximately 450, 620, and 280 kb long, respectively, were obtained (data not shown). During chromosomal mapping experiments (unpublished data), DNA fragments that were the same sizes hybridized to a *T. ferrooxidans ntrBC* chromosomal gene probe. This indicated that the two sets of genes are located within 280 kb of each other and that the *T. ferrooxidans ars* genes are located on the chromosome.

Sequence analysis of pTfars1b. The entire insert DNA was sequenced in both directions, and nine ORFs or partial ORFs were identified (Fig. 1). The characteristics of the predicted products of the nine ORFs are shown in Table 2. ORF 2 and ORF 3 were homologues of the *arsB* and *arsC* genes of other bacteria, but unlike other systems, in which the *arsC* gene is downstream of the *arsB* gene, the *T. ferrooxidans arsC* gene was upstream of *arsB* and the genes were divergently transcribed (Fig. 1). A fourth ORF was found between the *arsC* and *arsB* genes, and this ORF was also divergent with respect to *arsB*. This ORF exhibited weak but clear homology (30 to 40% identity) to many transcriptional regulators, including some members of the ArsR family. Although the putative ArsR of *T. ferrooxidans* contains two possible helix-turn-helix motifs, the positions of these motifs do not correspond to the position of the helix-turn-helix DNA binding domain identified in other ArsR proteins that have been studied. The putative ArsR of *T. ferrooxidans* also does not contain the conserved motif that includes two cysteine residues (ELCVCDL), which has been shown to be required for binding of the arsenite inducer (29). Downstream of the *arsB* gene was a homologue of a gene previously identified as *arsH*. This gene was first discovered in *Y. enterocolitica* (17) and has been reported to be essential for arsenic resistance, although its function is not known. By using a BLASTN search of the nonredundant GenBank-EMBL-DDBJ-PDB database at the NCBI, we found a third homologue of ArsH on the chromosome of *Synechocystis* sp. The predicted amino acid sequence of the *T. ferrooxidans* ArsH-like homologue was 68% identical to the predicted amino acid sequence of the *Y. enterocolitica* molecule and 65% identical to the amino acid sequence of the hypothetical protein of *Synechocystis* sp. Alignment and phylogenetic analysis of all of the ArsC proteins in the NCBI nonredundant database showed that ArsC of *T. ferrooxidans* is most closely related to ArsC of the *P. aeruginosa* chromosomal *ars* operon (Fig. 2). Both *T. ferrooxidans* and *P. aeruginosa* are unusual in that although their ArsB proteins cluster with the ArsB proteins of other gram-negative organisms (data not shown), their ArsC proteins are more closely related to the ArsC proteins of gram-positive bacteria. The ArsC arsenate reductases of gram-positive bacteria have four conserved cysteine residues (of which two are essential [13]), whereas two cysteines (of which one is essential [15]) are present in the ArsC proteins of gram-negative bacteria. The putative ArsC protein of *T. ferrooxidans* contains four cysteine residues with spacing similar to the spacing in the ArsC proteins of gram-positive bacteria (data not shown).

Thioredoxin is required for arsenate reduction by *T. ferrooxidans* ArsC. A major difference between the ArsC proteins of gram-positive bacteria and the ArsC proteins of gram-negative

bacteria is the source of the reducing power used for reduction of arsenate. It has been shown that reduction of arsenate by a gram-positive ArsC is coupled to thioredoxin (11) and that reduction by a gram-negative ArsC is coupled to glutathione (18). Since ArsC of *T. ferrooxidans* was clearly like the ArsC molecules of gram-positive bacteria, thioredoxin and not glutathione should have been required for arsenate reduction. To investigate this, *E. coli* strains with mutations in the thioredoxin gene (*trxA*) or both the thioredoxin gene and the γ-glutamylcysteinyl synthetase gene (*gshA*; responsible for the synthesis of glutathione) were examined to determine their resistance to arsenate. The *trxA* mutant strain was resistant to arsenate and was able to grow in the presence of 15 mM sodium arsenate, while the double mutant (*trxA gshA*) was sensitive to arsenate and was not able to grow in the presence of 2 mM sodium arsenate. This indicated that the glutathione-requiring *E. coli* chromosomal *ars* genes were able to confer resistance to arsenate in the absence of thioredoxin but not in the absence of glutathione. When the *E. coli* double mutant strain was transformed with the *T. ferrooxidans ars* genes (pTfarsCRBH-Cm), a similar result was obtained because neither the *E. coli ars* genes nor the *T. ferrooxidans ars* genes were functional in the double mutant. However, when a plasmid containing the thioredoxin gene from *T. ferrooxidans*, pTrx6, was added in *trans* together with the *T. ferrooxidans ars* genes to the *E. coli* double mutant (*trxA gshA*), resistance to arsenate was restored. If the double mutant was transformed with only pTrx6, the cells remained sensitive to arsenate. This result provided genetic evidence that reduction of arsenate by ArsC of *T. ferrooxidans* is coupled to thioredoxin.

Conservation of the unusual *ars* operon structure in other *T. ferrooxidans* strains. To determine whether the divergent arrangement of the *arsBH* and putative *arsR* and *arsC* genes found in *T. ferrooxidans* ATCC 33020 was unique to this strain, primers BBARSB and BBARSC (Table 1) were designed within the 5' ends of the *arsB* and *arsC* genes, which allowed amplification of the 450-bp putative *arsR*-promoter region (Fig. 3A). If other strains of *T. ferrooxidans* also have divergent *arsBH* and putative *arsR* and *arsC* genes, the primers would be orientated towards each other, and amplification of a 450-bp fragment would occur. If the genes were not divergently arranged, the primers would face in the same direction, and no amplification would be detected. A PCR product of the predicted size was obtained from chromosomal preparations of *T. ferrooxidans* ATCC 33020, ATCC 23270, ATCC 19859, and ATCC 13598 but not from chromosomal preparations of other acidophilic bacteria, such as *L. ferrooxidans* DSM 2705, *Thiobacillus thiooxidans* ATCC 19377, and *T. caldus* MNG, or from *E. coli* DH5α (Fig. 3B). PCR experiments performed with primers which amplified the *arsH* gene (primers ARSHF and ARSHR [Table 1]) yielded a product of the predicted size for all of the *T. ferrooxidans* strains tested but not for the other bacteria (data not shown). This indicated that other strains of *T. ferrooxidans* also possessed the *arsH* gene.

Expression of *T. ferrooxidans ars* gene products by using an *E. coli* in vitro transcription-translation system. Before investigating which of the predicted ORFs described in Table 2 contributed to arsenic resistance in *E. coli*, we examined which polypeptides were expressed in an *E. coli* in vitro transcription-translation system. Compared with the vector (Fig. 4, lanes 5 and 10), the pTfars1b clone yielded additional polypeptides at approximately 45, 41, 35, 27, 25, 18, 14, and 12 kDa (Fig. 4, lane 6). The 18-kDa protein was clearly identified as ArsC. The size of this protein was consistent with the size predicted from the sequence (18.2 kDa);

this protein was present only when *arsC* was included in a test construct and was the only polypeptide produced by pTfarsC (data not shown). The 27-kDa protein was identified as ArsH; its size was close to the predicted size (26.7 kDa), it was synthesized only when an *arsH* gene was present, and it was the only polypeptide synthesized by pTfarsH (Fig. 4B, lane 9). A broad band at about 35 kDa was the only polypeptide band produced by pTfarsB and was always present when *arsB* was present. This protein appeared to be smaller than the 48.5-kDa protein predicted, but membrane-located proteins often migrate anomalously (2, 12, 24, 26) and ArsB must have been synthesized since all of the cells containing *arsB* constructs were resistant to arsenite. The 14-kDa band corresponded to the putative ArsR protein as it was the only additional band (compared to the vector) produced by pTfarsR (Fig. 4, lane 7). Based on the polypeptides produced by pTfars1b and the predicted sizes of the ORFs, the 41- and 45-kDa polypeptides were products of ORFs 1 and 5, respectively. We presumed that the 25- and 12-kDa proteins present in pTFars1b but not pTfarsCRBH were synthesized from genes downstream of ORF 5.

Ability of the cloned *T. ferrooxidans ars* gene products to confer increased resistance to arsenic compounds and antimonite in *E. coli* AW3110. Constructs pTfars1b and pTfarsCRBH conferred equivalent levels of resistance to arsenite [As(III)] and arsenate [As(V)] in *E. coli* AW3110 (data not shown). The abilities of pTfarsCRBH and subclones to confer resistance to arsenite in *E. coli* AW3110 were tested further, as shown in Fig. 5A. A construct containing only the *arsB* gene (pTfarsB) conferred levels of resistance to arsenite similar to the levels of resistance conferred by pTfarsCRBH or pTfarsCRB. This experiment was repeated four times, and although the results of the experiments varied, we obtained no clear evidence that arsenite resistance in *E. coli* was improved by the presence of *arsC* or *arsH*. Therefore, only *arsB* was required for resistance to arsenite in *E. coli*. The cloned *T. ferrooxidans arsB* gene was also required to enhance the resistance of *E. coli* to antimony, and this resistance was not enhanced further by the presence of *arsC* or *arsH* (data not shown). As expected, the *arsC* and *arsB* genes were essential for increased resistance of *E. coli* to arsenate, and this resistance was not increased by the presence of *arsH* (Fig. 5B).

DISCUSSION

During biooxidation of arsenopyrite ores and concentrates large quantities of arsenic are released into the surrounding solution. Since *T. ferrooxidans* is a member of the consortium of bacteria involved in arsenopyrite biooxidation, we expected that *T. ferrooxidans* would possess arsenic resistance genes. However, the unusual arrangement of the *T. ferrooxidans ars* operon was not expected. No *arsD* gene or *arsA*-like gene (ATPase subunit) was found in the immediate vicinity of the arsenic resistance genes, and only *arsC, arsB*, and *arsH*-like genes were identified based on initial sequence comparisons. More careful analysis resulted in identification of a putative regulator between the *arsB* and *arsC* genes. However, the predicted protein exhibited only relatively weak homology to the ArsR proteins produced by previously described *ars* operons. This protein also lacked the conserved metal-binding box to which the arsenite inducer binds, which causes a conformational change in the helix-turn-helix domain and results in dissociation of the repressor from the DNA (29). Recently, the regulator of the chromosomal zinc resistance operon of *Staphylococcus aureus*, ZntR, which appears to belong to the ArsR family of transcriptional regulators, was also found to lack the

metal-binding motif (33). The binding reaction of the ZntR protein and the *znt* promoter was, however, still inhibited by the presence of 25 µM Zn^{2+} or Co^{2+}. There is, therefore, some indication that there may be other unknown interactions involved in induction of the operon. The orientation of the putative *arsR* and *arsC* genes and the *arsBH* genes indicated that the genes must be divergently transcribed in a manner that has not been observed before. Furthermore, the divergent gene arrangement was conserved in *T. ferrooxidans* strains that originated from Canada, the United States, and Japan but was not found in *L. ferrooxidans*, *T. thiooxidans*, or *T. caldus*. In such a divergent arrangement, it is possible that transcription of *arsC* and transcription of *arsB* are regulated separately. It has been suggested that TCAT-N7-TTTG

may represent a consensus binding site for the *E. coli* chromosomal ArsR and R773 ArsR repressors (36), but no corresponding sequences were detected in the region between the *T. ferrooxidans arsC* and *arsB* genes (data not shown). Work is in progress to investigate regulation of the *T. ferrooxidans ars* operon. In addition to Northern blotting and transcript mapping, this work will involve construction of *arsR-*, *arsC-*, and *arsB-lacZ* promoter-fusion reporter genes, a p*tac-arsR* IPTG (isopropyl-β-d-thiogalactopyranoside)-controlled expression construct, and an *E. coli lac-Δars* mutant strain. This study should reveal how the divergent operon is regulated and whether the activity of the putative ArsR protein of *T. ferrooxidans* is affected by arsenic.

An ORF with homology to the *arsH* gene was located immediately downstream of the *arsB* gene. The *arsH* gene was first identified in *Y. enterocolitica*, in which it is divergently transcribed from the *arsRBC* genes (17). Although it has been reported that the *arsH* gene is necessary for arsenic resistance, the function of the gene is unknown, and Neyt et al. (17) hypothesized that it might act as some type of regulator. When BLAST, Prosite (http://www.expasy.ch/prosite), and pfam (http://pfam.wustl.edu/pfam) were used, ArsH proteins exhibited no clearly discernable overall or motif similarity to other proteins. The finding that the *T. ferrooxidans arsH*-like gene was expressed in an *E. coli* in vitro transcription-translation system but was not required for resistance to arsenite, arsenate, or antimony in *E. coli* is in apparent contrast to data obtained for the arsenic resistance genes present on the *Y. enterocolitica* pYV virulence plasmid. However, the effect of pYV *arsH* was studied in *Y. enterocolitica*, while the effect of the *T. ferrooxidans arsH* was studied in a heterologous *E. coli* host. It is possible that *arsH* has an effect on arsenic resistance in *T. ferrooxidans* that is not observed when *arsH* is cloned in *E. coli*. The presence of an *arsH*-like gene appears to be a feature of *T. ferrooxidans*, as such a gene was detected in all four strains examined. Whether ArsH plays a role in *ars* regulation will be examined in another study.

As an obligately chemolithotrophic acidophilic bacterium, *T. ferrooxidans* comes from an environment which could be predicted to be genetically more isolated than the environments of most other bacteria in which *ars* genes have been studied. Therefore, it was interesting to compare the predicted amino acid sequences of the *T. ferrooxidans* ArsB and ArsC proteins with the amino acid sequences of proteins of other bacteria. *T. ferrooxidans* ArsB clearly grouped with the ArsB proteins of other gram-negative bacteria. The predicted amino acid sequence of *T. ferrooxidans* ArsC was most closely related to the amino acid sequence of ArsC of *P. aeruginosa*. However, unlike *P. aeruginosa* ArsC, which did not reduce arsenate when *arsC* was cloned in *E. coli* (3), cloned *arsC* of *T. ferrooxidans* was expressed and functional in the heterologous host. In contrast to the ArsB proteins, the ArsC proteins of *T. ferrooxidans* and *P. aeruginosa* were more similar to ArsC proteins of gram-

positive bacteria. Genetic evidence that ArsC of *T. ferrooxidans* is biochemically like the thioredoxin-requiring gram-positive type of ArsC proteins supports this grouping. Therefore, it is clear that there are at least two groups of ArsC proteins, a four-conserved-cysteine thioredoxin-requiring ArsC group and a two-conserved-cysteine glutathione-requiring ArsC group (31). Since *T. ferrooxidans* is the second gram-negative bacterium found to have a four-conserved-cysteine thioredoxin-requiring ArsC, correlation of this type of ArsC with gram-positive bacteria, which appeared to be valid based on the initial data for several of the arsenic resistance genes examined, appears to be an oversimplification.

Like other workers, we found that the ArsB protein is difficult to detect unequivocally in an *E. coli* in vitro system (34). Nevertheless, the *arsB* and *arsC* genes of *T. ferrooxidans* were clearly expressed and functional in *E. coli*. As found with other *ars* operons, the *T. ferrooxidans arsB* gene on its own was able to confer resistance to arsenite and antimony, but *arsC* was required in addition to *arsB* for arsenate resistance (Fig.5). The ability of the *T. ferrooxidans ars* system to function in *E. coli* is noteworthy when how arsenic resistance systems may be energized is considered. It is thought that in arsenic resistance systems which lack the ArsA (ATPase) membrane potential rather than ATP, hydrolysis is the energy source (9, 12,35). The obligately acidophilic organism *T. ferrooxidans* has an internal pH of about 6.5, and when cells are growing on Fe^{2+} at pH 2.0, they maintain a ΔpH of 4.5 U (7). Unlike other bacteria, acidophilic bacteria with a large ΔpH have a positive inside membrane potential rather than a negative inside membrane potential, which subtracts from the H^+ gradient instead of augmenting it (16). Nevertheless, in spite of the fact that it originated from a bacterium that has a positive inside membrane potential, the *T. ferrooxidans* ArsB arsenite efflux pump membrane was functional in *E. coli*.

This initial investigation of the *T. ferrooxidans* arsenic resistance system was performed with *E. coli*. In future work we will have to include studies of arsenic resistance and gene expression in *T. ferrooxidans*, which, because of the rudimentary genetic system available, the lack of mutants, and the difficulty of readily obtaining large quantities of cells, will present a considerable challenge.
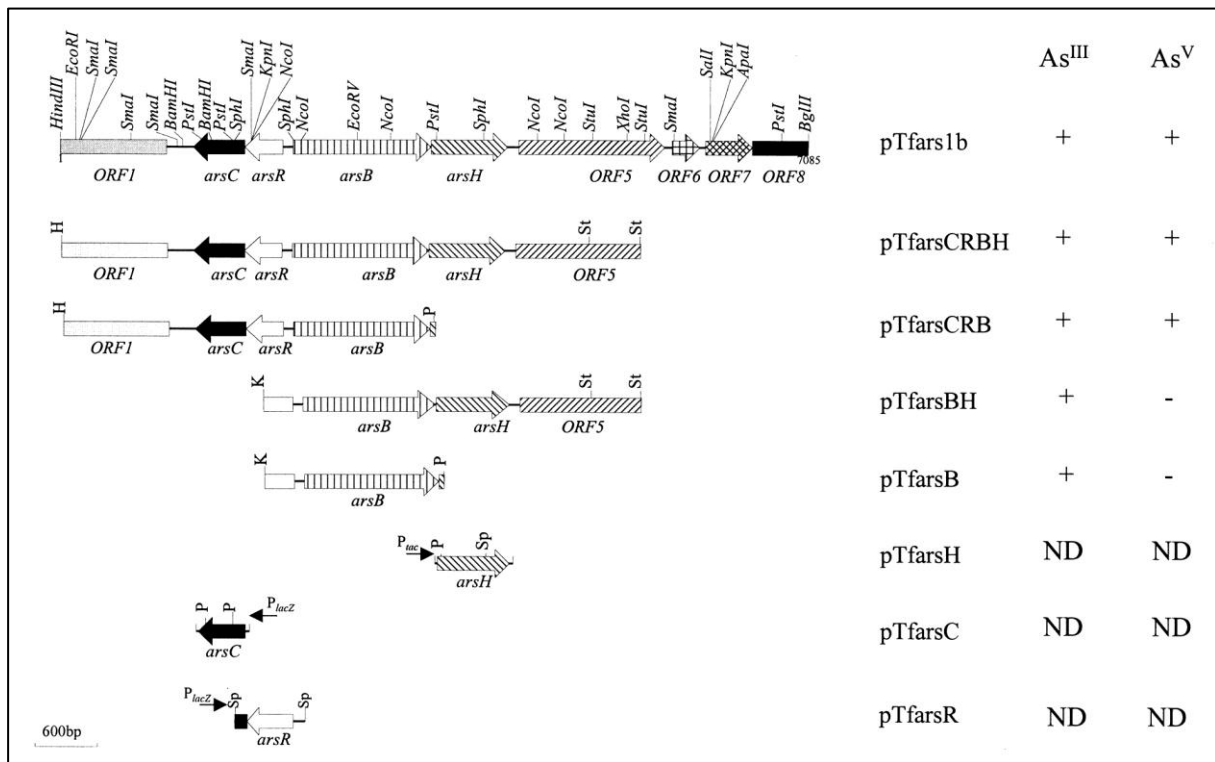
ACKNOWLEDGMENTS

Figures

Figure 1

pTfars1b and deletion clones constructed in this study. The diagram shows the genes, ORFs, restriction endonuclease map, and whether the clones were resistant to 0.5 mM arsenite in Luria agar (AsIII) or 0.2 mM arsenate in low-phosphate medium (AsV). ND, not determined. The directions and types of vector promoters are indicated by labeled arrows.
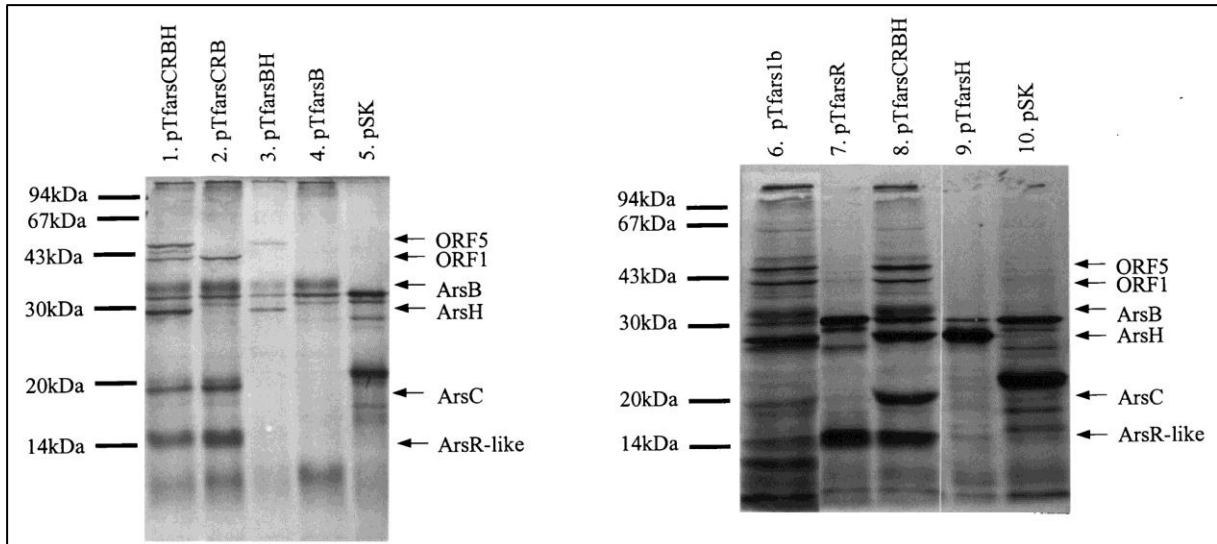
Figure 2
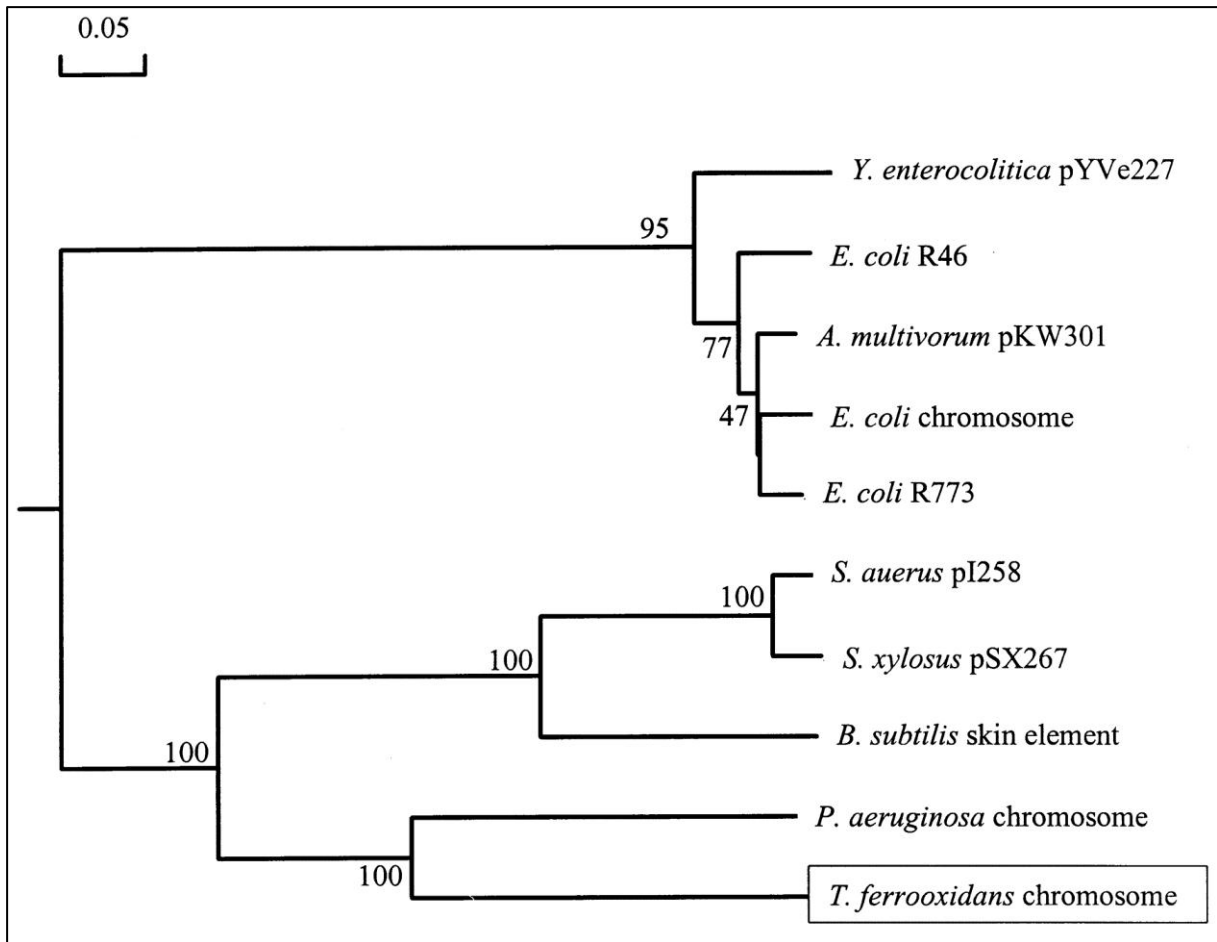
Phylogenetic tree based on ArsC proteins. Bootstrap values (expressed as percentages) based on 100 attempts are indicated at the branch points. The accession numbers for the protein sequences are as follows: Y. enterocolitica pYVe227, AAD16858;E. coli R46, AAB09628; A. multivorum pKW301, BAA24824; E. coli chromosome, AAC76528; E. coli R773, AAA21096; S. aureus pI258, AAA25638;Staphylococcus xylosus pSX267, AAA27589; B. subtilis skin element, BAA06970; P. aeruginosa chromosome, AAC69644.

Figure 3
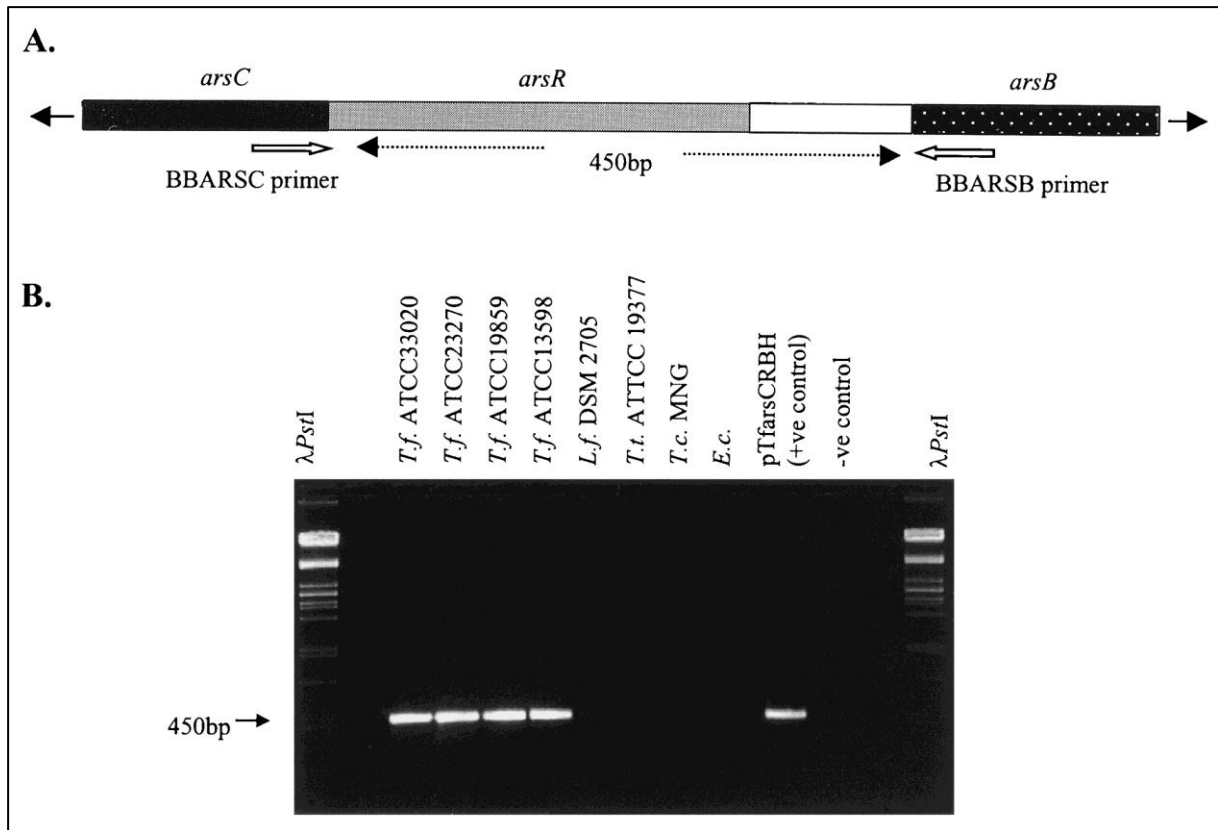
(A) Locations of primers used to determine the divergent arrangement of the putative arsR and arsC genes and the arsBH genes in different strains of T. ferrooxidans and other biomining bacteria. Primers located within the arsB and arsC genes were used to amplify the 450-bp region between these two genes. (B) Ethidium bromide-stained agarose gel of the PCR amplification products, prepared by using chromosomal DNA from different biomining bacteria. Abbreviations:T.f., T. ferrooxidans; L.f., L. ferrooxidans; T.t., T. thiooxidans;T.c., T. caldus; E.c., E. coli. +ve, positive; -ve, negative.

Figure 4

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the proteins expressed from pTfars1a and subclones by using an E. coli-derived in vitro transcription-translation system.

Figure 5
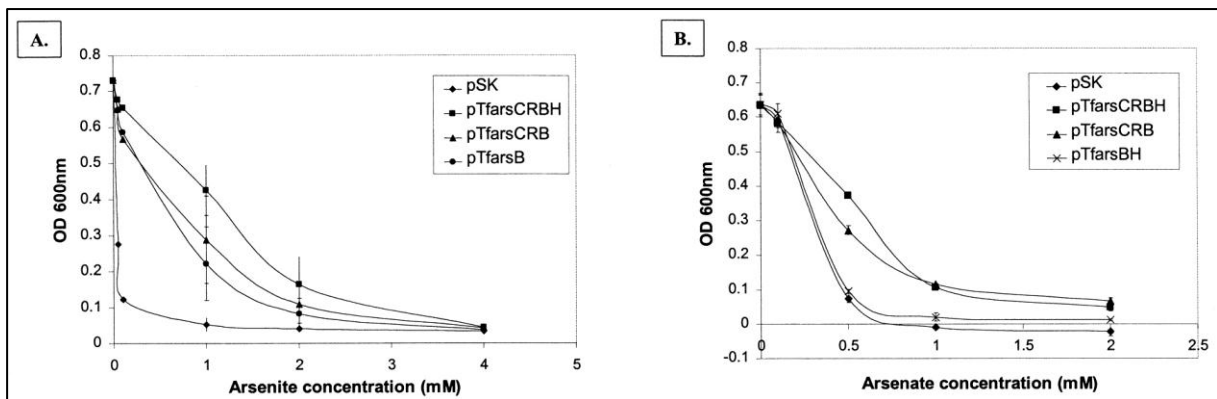
Growth of E. coli AW3110 containing pSK, pTfarsCRBH, pTfarsCRB, pTfarsBH, and pTfarsB in the presence of arsenite (A) and arsenate (B). Each data point represents the results of three assays of three independent experiments. The error bars indicate standard deviations. OD 600 nm, optical density at 600 nm.

Tables

Table 1.
Strains, plasmids, and primers used

Strain, plasmid, or primer	Description	Reference or source[a]
Strains		
Escherichia coli strains		
W3110	K-12 F⁻IN(*rrnD-rrnE*)	Rosen
AW3110	W3110 Δ*ars*::*cam*	4
MC1061	K-12 F⁻ *araD139*? *galU galK hsdR rpsL*	Persson
BH5262	K-12*araD139*? *galU galK hsdR rpsL argH1 trxA7004 ilvC*::Tn*10 gshA*	14
BH2012	K-12 *araD139*? *galU galK hsdR rpsL metA46 argH1 trxA7004 ilvC*::Tn*5*	14
Thiobacillus ferrooxidans strains		
ATCC 33020	Wild type	ATCC
ATCC 23270	Wild type	ATCC
ATCC 19859	Wild type	ATCC
ATCC 13598	Wild type	ATCC
*Thiobacillus caldus*MNG	Wild type	This laboratory
Thiobacillus thiooxidans ATCC 19377	Wild type	ATCC
Leptospirillum ferrooxidans DSM 2705	Wild type	DSM
Plasmids		
pBluescript(SK)	Amp^r *lacZ'*, ColE1 replicon, cloning vector	Stratagene
pUCBM21	Amp^r *lacZ'*, ColE1 replicon, cloning vector	Stratagene
pACYC184	Tc^rCm^r, p15A replicon, cloning vector	6
pTfars1a	Ap^r (from *T. ferrooxidans*plasmid library, 7-kb *Sau*3A1 fragment cloned into pEcoR251 digested with *Bgl*II)	Library of R. S. Ramesar
pTfars1b	Ap^r (6.7-kb*Hin*dIII-*Bgl*II fragment of pTfars1a cloned into pBluescript)	This study
pTfarsCRBH	Ap^r(5.3-kb *Hin*dIII-*Stu*I fragment of pTfars1b cloned into pBluescript cut with *Eco*RV and*Hin*dIII)	This study
pTfarsCRB	Ap^r (*Eco*RV-*Xba*I fragment of pTfars1b, which contained the 5' end of *arsB* and all of *arsH*, was replaced with the*Eco*RV-*Xba*I fragment of pBB08, which contained only the 5' end of *arsH*)	This study
pTfarsBH	Ap^r (*Kpn*I deletion of pTfarsCRBH)	This study
pTfarsB	Ap^r(*Pst*I deletion of pTfarsBH)	This study
pTfarsH	Ap^r (PCR product obtained with primers ARSHF and ARSHR, cloned into pBluescript)	This study

Strain, plasmid, or primer	Description	Reference or source[a]
pTfarsC	Ap[r] (PCR product obtained with primers ARSCF and ARSR, cloned into pBluescript)	This study
pTfarsR	Ap[r] (0.55-kb *Sph*I fragment of pTfarsCRBH, cloned into pUCBM21)	This study
pBB08	Ap[r] (0.8-kb *Eco*RV-*Pst*I fragment of pTfars1b, cloned into pBluescript)	This study
pTfarsCRBH-Cm	Cm[r] (6.7-kb*Hin*dIII-*Bgl*II fragment of pTfars1a, cloned into the *tet* gene of pACYC184)	This study
pTrx6	Ap[r] (contains *T. ferrooxidans*ATCC 33020 *trxA* gene, cloned into pBluescript)	19
Primers		
BBARSB	(*Bam*HI) 5′-GCGGATCCAGGGTGACGAGAAATATGGC-3′	This study
BBARSC	(*Bam*HI) 5′-GCGGATCCGGGGTTTTCATCACTGG-3′	This study
ARSHF	(*Eco*RI) 5′-GCGAATTCTGGTGGCTGCCGCTGGCTTG-3′	This study
ARSHR	(*Hin*dIII) 5′-GAAAGCTTGCGTACCCCCAACCTCATGCC3′	This study
BBARSCF	(*Hin*dIII) 5′-GCAAGCTTCGGTGAAACCCGCTCTCCCT-3′	This study
BBARSCR	(*Eco*RI) 5′-GCGAATTCTGTGCGCGCCTTGATGGGTGGC-3′	This study

a Rosen, B. Rosen, Wayne State University, Detroit, Mich.; Persson, Britt Persson, University of Umea, Umea, Sweden; ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; Stratagene, Stratagene, La Jolla, Calif.

Table 2
Locations and sizes of ORFs in pTfars1b

ORF	Position	No. of amino acids	Size (kDa)	Closest relationship to a known protein[a]	Product detected[b]
ORF 1	1014-ND[c]	338	ND	41% identity and 57% similarity to putative membrane protein of *E. coli* (accession no. ACC73882)	Yes
arsC	1752-1261	163	18.1	53% identity and 65% similarity to *P. aeruginosa* ArsC (accession no. AAC69644)	Yes
arsR	1752-2108	118	13.1	35% identity and 47% similarity to *Mycobacterium tuberculosis* hypothetical protein Rv2640c (accession no. CAB02346)	Yes
arsB	2202-3512	436	48.4	57% identity and 70% similarity to ArsB from *E. coli* plasmid R773 (accession no. P08691)	Yes
arsH	3518-4240	240	26.7	68% identity and 81% similarity to *Y. enterocolitica* ArsH (accession no. AAB42206)	Yes
ORF 5	4348-5724	458	44.7	58% identity and 72% similarity to signal recognition particle protein (fifty-four homologue) (P48) of *E. coli* (accession no. BAA16495)	Yes
ORF 6	5795-6055	86	9.56	66% identity and 79% similarity to 30S ribosomal protein S16 of *Haemophilus influenzae* Rd KW20 (accession no. P44382)	No
ORF 7	6111-6554	147	12.6	44% identity and 58% similarity to 16S rRNA-processing protein RIMM of *Haemophilus influenzae*Rd KW20 (accession no. P44568)	No

ORF	Position	No. of amino acids	Size (kDa)	Closest relationship to a known protein[a]	Product detected[b]
ORF 8	6554-ND	94	ND	34% identity and 52% similarity to tRNA (guanine-N1)-methyltransferase of *Salmonella typhimurium* (accession no. P36245)	No

a The identity and similarity values are based on amino acid sequence data. The accession numbers are GenBank-EMBL database accession numbers.

b Protein products were detected by using an in vitro *E. coli* transcription-translation system.

c ND, not determined. The ORF was incomplete, and the molecular mass was not calculated; however, the number of amino acids is indicated.

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