Resistance Determinants of a Highly Arsenic-Resistant Strain of *Leptospirillum ferriphilum* Isolated from a Commercial Biooxidation Tank

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Two sets of arsenic resistance genes were isolated from the highly arsenic-resistant *Leptospirillum ferriphilum* Fairview strain. One set is located on a transposon, Tn*lfArs*, and is related to the previously identified Tn*AtcArs* from *Acidithiobacillus caldus* isolated from the same arsenopyrite biooxidation tank as *L. ferriphilum*. Tn*lfArs* conferred resistance to arsenite and arsenate and was transpositionally active in *Escherichia coli*. Tn*lfArs* and Tn*AtcArs* were sufficiently different for them not to have been transferred from one type of bacterium to the other in the biooxidation tank. The second set of arsenic resistance genes conferred very low levels of resistance in *E. coli* and appeared to be poorly expressed in both *L. ferriphilum* and *E. coli*.

Processes for the biooxidation of gold-bearing arsenopyrite concentrates were developed in the 1980s and are now used in several countries (17). These are among the largest commercial fermentation processes known. Biooxidation is a mineral pretreatment process during which the molecular structure of the arsenopyrite mineral is broken down, exposing the gold and allowing its extraction by cyanide (7). During this process, large quantities of arsenic are released into continuous-flow aeration tanks in which the biooxidation takes place. The majority of arsenopyrite biooxidation processes operate at 40°C and are dominated by a mixture of the sulfur-oxidizing bacterium *Acidithiobacillus caldus* and the iron-oxidizing bacterium *Leptospirillum ferriphilum*. During the first few years of operation, the continuous-flow nature of the processes resulted in the selection of highly arsenic-resistant bacteria.

Studies to investigate what genetic changes had taken place that accompanied this high level of arsenic resistance have been carried out with *A. caldus*. Highly arsenic-resistant strains of *A. caldus* have been found to contain an unusual Tn21-like *ars* operon that is not present in less-resistant strains (6, 23, 24). The 12-kb Tn*AtcArs* is unusual in that the *tnpR* (transposase) and *tnpR* (resolvase) genes occur on opposite ends of the transposon, unlike other transposons of the Tn21 and Tn7 family, where they form an adjacent unit. These two transposon genes flank a series of genes (*arsRCDADA orf7 orf8B*) associated with arsenic resistance that are themselves unusual. Genes for an *ArsR* (negative regulator) (20, 27) and *ArsC* (arsenate reductase) (10, 11) are followed by a tandem duplication of the genes for *ArsD* (a second repressor) (28) and *ArsA* (an ATPase that associates with *ArsB* and links arsenite export to ATP hydrolysis) (22). These genes are followed by genes encoding ORF7 (an NADH-like oxidoreductase), ORF8 (a cystathionine-β-synthase [CBS] domain-containing protein), and *ArsB*, the arsenite efflux pump. Deletion of one copy of *arsDA* readily occurs, but this deletion does not appear to affect resistance when cloned on a multicopy plasmid in *Escherichia coli* (24). Similarly, the inactivation or deletion of ORF7 and ORF8 did not affect arsenic resistance in *E. coli*. Some strains of highly arsenic-resistant *A. caldus* have a second transposon that is identical to Tn*AtcArs* except that it extends from the inverted repeat adjacent to *tnpR* to *orf7*, at which point it is truncated. This truncated transposon lacks an *arsB* but nevertheless confers low-level arsenic resistance when cloned on its own in *E. coli* (23).

As the dominant iron-oxidizing bacterium (5), it was anticipated that *L. ferriphilum* strains from commercial arsenopyrite biooxidation tanks would also possess effective arsenic resistance mechanisms. Whereas *A. caldus* is a γ-proteobacterium (9), *L. ferriphilum* is a member of the nitrospirae (12), and we wished to determine whether these two phylogenetically distant but highly arsenic-resistant bacteria isolated from the same biooxidation process would have similar mechanisms for arsenic resistance. More specifically, we wished to determine whether the Tn*AtcArs* transposon found in *A. caldus* (24) was also present in highly arsenic-resistant strains of *L. ferriphilum*. We therefore examined the arsenic resistance mechanisms of a strain of *L. ferriphilum* isolated from a commercial biooxidation tank treating a gold-bearing arsenopyrite concentrate at the Fairview mine (South Africa) and compared these mechanisms with those of other *L. ferriphilum* isolates.

Cloning of the *ars* genes of *L. ferriphilum*. *L. ferriphilum* strain Fairview isolated from the biooxidation tanks at the Fairview mine, South Africa (Table 1), was cultured at 30°C in basal medium with FeSO₄ (18), and genomic DNA was isolated as described previously (24). Partially digested 5- to 10-kb Sau3A DNA fragments were used to construct a gene bank in the BglII site of the positive selection vector pEcoR252 (Table 1). *E. coli* DH5α (Table 1) cells were transformed with the ligation mix and plated onto Luria agar plus ampicillin (100 μg/ml). Colonies were scraped off and used to prepare the gene bank. The bank was transformed into the *E. coli* arsenic mutant ACSH50Iq, and transformants able to grow on LB plates containing 0.5 mM arsenite were selected. Twelve arsenic-resistant transformants!
were isolated, their plasmids were mapped, and all were found to have regions in common. One of these, pLfTnArs, was selected for further analysis. The source of insert DNA was confirmed by Southern hybridization using three different fragments as a probe. Total DNA of *L. ferrooxidans* Fairview and pLfTnArs digested with various restriction enzyme combinations generated fragments that were the same size as the cloned fragments, indicating that the insert DNA originated from *L. ferrifilimum* Fairview (data not shown). Furthermore, the signals obtained suggested that there were two copies of the transposon present on 10-kb and 12-kb BamHI fragments. *L. ferrifilimum* Fairview genomic DNA was digested with BamHI, and fragments in the 8- to 14-kb size range were used to generate a mini gene bank in the same way as described above. This gene bank was screened in *E. coli* ACSH50Iq for arsenite resistance to isolate the 10-kb and 12-kb transposon-containing fragments. Restriction mapping confirmed

### TABLE 1. Bacterial strains, plasmids, and primers used in this study

| Strains | Description and/or source | Source or reference |
|---------|---------------------------|---------------------|
| **Strains** | | |
| *L. ferrooxidans* | Romania | W. Sand |
| ATCC 49879 | Markosyan strain (1972), Cu mine, Armenia | DSMZ, Braunschweig, Germany |
| DSM2705 | Cu mine, Chile | B. Johnson |
| Chil-L2 | | |
| *L. ferrooxidans* | Romania | W. Sand |
| ATCC 49881 | South Africa | E. Lawson |
| Warwick | Warwick, United Kingdom | P. Norris |
| **E. coli** | | |
| DH5α | | Promega |
| ACSH50Iq | rpsL Δ(lac-pro) Δars::cam | 3 |
| ACSH50Iq-Rif | | 24 |
| **Plasmids** | | |
| pEcoR252 | Ap<sup>r</sup>; EcoRI inactivation cloning vector | 30 |
| pBluescript SK | Ap<sup>r</sup> lac<sup>Z</sup>; ColE1 replicon, cloning vector | Stratagene |
| pUC19 | Ap<sup>r</sup> lac<sup>Z</sup>; ColE1 replicon, cloning vector | 29 |
| pGEM-T | Ap<sup>r</sup>; T-tailed PCR product cloning vector | Promega |
| pGL10 | Km<sup>r</sup>; RK2/Rp4 replicon, cloning vector | 4 |
| pMCI1403 | Ap<sup>r</sup>; promoterless lac<sup>YZ</sup> operon, ColE1 replicon | Pharmacia |
| pKK223-3 | Ap<sup>r</sup>; P<sub>lac</sub> ColE1 replicon, cloning vector | 21 |
| pSa | Km<sup>r</sup> Cm<sup>r</sup>; IncW replicon, mobilizing plasmid | |
| pEcoBlunt | Ap<sup>r</sup>; pEcoR252 blunted at the BglII site and relegated to inactivate the EcoRI endonuclease | 24 |
| pLfTnArs | Ap<sup>r</sup>; from the *L. ferrifilimum* Fairview gene bank<sup>a</sup> | This study |
| pLiArs | Ap<sup>r</sup>; from the *L. ferrifilimum* Fairview gene bank<sup>a</sup> | This study |
| pLfTnpUC1 | Ap<sup>r</sup>; 13-kb HindIII fragment from pLfTnArs cloned into pUC19 | This study |
| pLfTn1, pLfTn2 | As<sup>r</sup>; Km<sup>r</sup>; transposon transconjugants isolated from the mating experiment performed in *E. coli* | This study |
| pKKLAr R | Ap<sup>r</sup>; 380-bp PCR product of *arsR* obtained using LfArsRF/LfArsRR primers, cloned into pKK223.3 | This study |
| pKKLAr R | Ap<sup>r</sup>; 380-bp PCR product of *arsR* obtained using LfArsRF/LfArsRR primers, cloned into pKK223.3 | This study |
| pKKLAr R | Ap<sup>r</sup>; 380-bp PCR product of *arsR* obtained using LfArsRF/LfArsRR primers, cloned into pKK223.3 | This study |
| ptac | Km<sup>r</sup>; 1.4-kb SphI-PvuI fragment from pKK223.3 in pGL10 | 24 |
| ptacLAr R | Km<sup>r</sup>; 1.6-kb BamHI-PvuI fragment from pKKLAr R in pGL10 | This study |
| ptacLAr R | Km<sup>r</sup>; 2.1-kb BamHI-PvuI from pKKLAr R in pGL10 | This study |
| pLfArsRLacZ | Ap<sup>r</sup>; 350-bp PCR product of *arsR* promoter obtained with LfArsRLacZF/LfArsRLacZR primers in MC1403 | This study |

### Primers<sup>a</sup>,<sup>b</sup>,<sup>c</sup>,<sup>d</sup>

| Primers | Description | Source |
|---------|-------------|-------|
| IMArsF | 5′-AAGGAGCATTTTTTAAAAACAGGC-3′ | This study |
| IMArsR | 5′-ACGCAGAAGATCCAGATGAGG-3′ | This study |
| LfArsRLacZF | 5′-GGATTCGATCGATCTGAAATG-3′ | This study |
| LfArsRLacZR | 5′-TATACGATCCGCTGGAGATTGATCAT-3′ | This study |
| LfArsRF (EcoRI) | 5′-GGATTCGATCGATCTGAAATG-3′ | This study |
| LfArsRR (HindIII) | 5′-GGCAAGCTTAAAGAGATGCTATG-3′ | This study |
| LfArsR (HindIII) | 5′-GGCAAGCTTAAAGAGATGCTATG-3′ | This study |
| LfArsR (HindIII) | 5′-GGCAAGCTTAAAGAGATGCTATG-3′ | This study |
| LfArsR (HindIII) | 5′-GGCAAGCTTAAAGAGATGCTATG-3′ | This study |
| LfArsR (HindIII) | 5′-GGCAAGCTTAAAGAGATGCTATG-3′ | This study |

<sup>a</sup> DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen.<br><sup>b</sup> Restriction enzyme sites incorporated into primers are indicated in parentheses and are shown in boldface type in the primer sequences.<br><sup>c</sup> An A→T mutation was incorporated into the primer (underlined) so as to introduce a stop codon for *arsR*.<br><sup>d</sup> See Fig. 1.
that the two transposons were identical between the invert repeats but differed in the flanking regions, indicating that they occurred in two different locations on the *L. ferriphilum* genome.

During a metagenome sequencing project for an environmental acid mine drainage microbial community from Iron Mountain (Richmond, Calif.), the near-complete genome sequence of bacteria identified as *Leptospirillum* group II (i.e., *L. ferriphilum*) was assembled (25). Analysis of this gapped sequence identified putative arsenic resistance genes, which appeared to be different from those described above. Primers IMArsF and IMArsR (Table 1) based on the sequence of the *ars* genes of the *L. ferriphilum* composite genome were used to successfully amplify a 2.6 kb-fragment from *L. ferriphilum* Fairview genomic DNA in the following reaction mixture: 100 ng of genomic DNA in a 50-μl volume containing 2 mM MgCl₂, 0.25 μM of each primer, 200 μM each deoxynucleoside triphosphate, and 1 U TaqI polymerase. Partial sequencing of this fragment confirmed the presence of *ars* genes. Colony hybridizations of the *L. ferriphilum* Fairview Sau3A gene bank in *E. coli* ACSH50I³ were performed using this fragment as a probe, and a plasmid named pLfArs was isolated.

**Sequence analysis of the *L. ferriphilum* Fairview *ars* genes.**

The inserts of plasmids pLfArs and pLFTnArs were sequenced in both directions, and the open reading frames (ORFs) identified are shown in Fig. 1. The arsenic genes present on pLfArs and pLFTnArs were located between divergently transcribed *tnpR* and *tnpA* genes in a manner similar to that of the recently reported Tn*AtcArs* transposon from *A. caldus* (24). Therefore, the name Tn*LfArs* was given to the transposon from *L. ferriphilum*. The *ars* genes consist of *arsR*, *arsC*, *arsD*, *arsA*, and *arsB* all transcribed in the same direction. Between *arsA* and *arsB* is an ORF (157 amino acids [aa]) that encodes a protein with a CBS domain. CBS domains are small domains of unknown function that usually occur in two to four copies per protein and dimerize to form a stable globular structure (2). A similar protein of 158 aa occurs in Tn*AtcArs* that was inactivated without affecting arsenic resistance in *E. coli* (24). The *ars* genes are flanked by divergent *tnpR* (resolvase) and *tnpA* (transposase) genes (Fig. 1). The amino acid sequences of TnpR,
Table 2. Comparison of the *L. ferriphilum* Fairview Ars proteins to those in other organisms

| Protein | % Identity to homologue in *L. ferriphilum* TnlfArs* | A. caldus TnA6Ars | Alcaligenes faecalis | M. flagellatus TnArs | L. ferriphilum Charsb |
|---------|-----------------------------------------------------|--------------------|---------------------|---------------------|----------------------|
| TnpR    | 95.6                                                | NA                 | 89.0                | NA                  | NA                   |
| ArsR    | 86.4                                                | 86.4               | 78.4                | 17.7                | NA                   |
| ArsC    | 93.7                                                | 93.7               | 90.6                | 29.0                | NA                   |
| ArsD    | 80.3                                                | 95.1               | 97.5                | NA                  | NA                   |
| ArsA    | 70.5                                                | 95.0               | 94.6                | NA                  | NA                   |
| ArsB    | 52.8                                                | 30.4               | 88.1                | NA                  | NA                   |
| TnpA    | 84.8                                                | 96.7               | 97.4                | 58.2                | NA                   |
| TnpR    | 88.8                                                | 94.7               | 96.8                | NA                  | NA                   |

* Boldface type indicates to which organism the TnlfArs homologue shows the highest sequence identity. GenBank accession numbers are as follows: TnA6Ars, AY821803; Alcaligenes faecalis, AY297781; M. flagellatus Chars, NZ_AAAX01000013. NA, not applicable.

b The chromosomal (Ch) *ars* genes isolated on plasmid pLFArs. Sequence data indicate that the *ArsR* and *ArsC* proteins have been fused but have been artificially separated for comparison purposes.

TnpA, and all of the *ars* gene products were highly conserved, with ArsA being the least conserved (70.5% sequence identity) and TnpR being the most conserved (95.6% sequence identity) (Table 2).

Despite the overall similarities, there were nevertheless some important differences between TnlfArs and TnA6Ars. TnlfArs is only 8,772 bp in length, compared with 12,444 bp for TnA6Ars. The main reason for the size difference is that ORF7 and the *arsD* duplication of TnA6Ars were absent from TnlfArs. Furthermore, the amino acid sequences of the two CBS domain-containing proteins were not as well conserved as the other gene products (52.8% sequence identity) (Table 2). The TnlfArs 43-bp left and right invert repeats differed from one another by 1 bp and were 3 bp longer than those for TnA6Ars (not shown).

A BLAST search using the *L. ferriphilum* *ars* genes indicated that they were most similar to transposon-related *ars* genes recently identified in *Methylobacillus flagellatus* (GenBank accession number NZ_AAAX01000013) and *Alcaligenes faecalis* (accession number AY297781), although studies of these *ars* transposons have not been reported. A comparison of the operon structures of the four *ars* transposons identified thus far is shown in Fig. 1. In general, the operon structures of the TnlfArs, TnA6Ars, and the *Alcaligenes faecalis* transposons are most closely related, with TnA6Ars having an additional NADH oxidoreductase-like ORF7 and the *Alcaligenes faecalis* transposon appearing to have undergone a deletion of tnpR and an inverted repeat. The structure of the *M. flagellatus* transposon is different in that the tnpA and tnpR genes are adjacent to each other as for most other Tn2I subfamily (and TnI family) transposons where they form a functional unit (13). In addition, the *M. flagellatus* arsR and arsC genes appear to have been fused. Interestingly, the last 39 amino acids of TnpA are repeated downstream of *M. flagellatus* arsR, reading in the same orientation as arsB (Fig. 1). This remnant could be the link between the arrangement of the tnpA and tnpR genes in the TnA6Ars, TnlfArs, and *Alcaligenes faecalis* operons compared to the gene layout in the *M. flagellatus* operon.

A comparison of the sequence identities of all of the *ars* transposon-associated gene products with those of TnlfArs is made in Table 2. Unexpectedly, although TnlfArs and TnA6Ars originated from bacteria from the same environment, the amino acid sequences of their gene products were not necessarily the most closely related. TnpR of TnlfArs was most related to that of TnA6Ars, while ArsR and ArsC were equally related to both TnA6Ars and the transposon of *Alcaligenes faecalis*. However, the predicted ArsD, ArsA, ArsB, and TnpA proteins of TnlfArs were much more related to those of the *Alcaligenes faecalis* and *M. flagellatus* transposons than to those of TnA6Ars.

At first glance, the finding of two similar *ars* transposons in the two types of acidophilic bacteria is not surprising, as transposons, and particularly those of the Tn2I family, are very effective at distributing the genes they carry (13). This finding is consistent with the prediction, reported previously by Woese (26), that “cosmopolitan genes,” of which arsenic resistance genes are a typical example, are more likely to be a characteristic of particular environments than of particular organismal lineages. If cosmopolitan genes are not restricted to certain lineages, they might be expected to pass between different types of bacteria within an environment that selects for them. It is highly unlikely that the *ars* transposons have been passed from *A. caldus* to *L. ferriphilum* or vice versa in the biooxidation tank environment. The differences in DNA sequences between the genes and the amino acid sequences of their products are far too substantial for them to have arisen in the 10 to 15 years or less since the biooxidation plants began operation and the time of sample collection. This suggests that although the two types of bacteria have arrived at similar solutions to their need for arsenic resistance, they have acquired the *ars* transposons independently of each other.

Restriction mapping (Fig. 1) and partial sequence analysis of the chromosomal *ars* genes present on pLFArs revealed three ORFs with sequence identity to *arsR, arsC*, and *arsB* genes as found in the *L. ferriphilum* acid mine drainage isolate whose genome was partially sequenced (25). Surprisingly, in spite of the acid mine drainage sample having originated from California and the Fairview isolate having originated from South Africa, the nucleotide sequences of these *ars* genes were identical. The *arsC* genes are atypical in that they form one continuous open reading frame and seem to be translated as a fusion protein. The predicted amino acid sequences of these gene products and the equivalent proteins of TnlfArs were not highly conserved (Table 2).

Resistance conferred by the *L. ferriphilum* Fairview *ars* genes in *E. coli*. The ability of the constructs pLTFtArs and pLFArs to confer resistance to arsenite and arsenate was tested in *E. coli* ACSH501P. Cultures grown overnight were diluted 100-fold into fresh medium (Luria broth for arsenate assays and low-phosphate medium [15] supplemented with 2 mM K2HPO4 for arsenate assays) containing various concentrations of sodium arsenite/arsenate and incubated at 37°C for 5 h, and the absorbance at 600 nm was determined. Cells containing the transposon-located *ars* genes (pLTFtArs) were more resistant to both arsenite and arsenate but were considerably more resistant to arsenite than the control cells (pEcoBlunt) (Fig. 2). The chromosomal *ars* genes (pLFArs) conferred only slightly more arsenic resistance than the control. The very weak resistance conferred by pLFArs could be due to weak promoter expression in *E. coli*. Gene expression analyses were therefore performed to investigate this further.
Regulation of expression of the *L. ferriphilum* Fairview *ars* genes. To determine whether the *ars* genes on pLfArs were expressed in *E. coli*, mRNA was analyzed using reverse transcription (RT)-PCR. Total RNA was isolated (24) from 50 ml of mid-exponential-phase cultures of *E. coli* ACISH50⁹ carrying pLfArs grown in LB containing 25 μM arsenate, 25 μM arsenite, or no arsenic and with antibiotic selection. For isolation from *L. ferriphilum* Fairview, cells were first grown without arsenic and then diluted 100-fold into fresh medium containing 0.1 mM arsenite/arsenate or no arsenic. RNA was isolated using the same method as that used for *E. coli*. A first-strand cDNA synthesis kit (AMV; Roche) was used for cDNA synthesis from mRNA. A PCR containing 2 μl of the 20-μl (total volume) reverse transcriptase reaction mixture was performed. The LfArsBrFR primer was used for cDNA synthesis from *arsB* mRNA (Table 1) and was used in combination with the LfArsBrFR primer for the PCR. A 330-bp *arsB-arsRC* product was obtained, indicating that *arsB* expression was obtained in *E. coli* and that *arsB* was cotranscribed with *arsRC* (Fig. 3). However, as this determination was not quantitative, the level of expression was unknown.

Further analyses were performed on the same mRNA described above using slot blot hybridization. Total mRNA was transferred onto a Hybond-N⁺ nylon membrane (Amersham) using a slot blot apparatus (Hoeffer Scientific) and hybridized according to the manufacturer’s instructions by using digoxigenin-labeled DNA probes. Probes specific for the chromosomal *arsB* transcript (0.6-kb PvuI-NcoI) and the 16S rRNA gene (as an internal control) were used. Signal intensities were measured with a Macbeth TD 109 transmission densitometer. In both *E. coli* and *L. ferriphilum* Fairview, weak induction (less than twofold) of *arsB* expression was detected from arsenate- and arsenite-induced samples, and this varied substantially between experiments (not shown). This confirmed that the *ars* genes were expressed in *E. coli*, presumably from a promoter upstream of *arsRC*.

To further test this, a translational *lacZ* fusion of the putative promoter region for *arsRCB* in pLfArs was made, and β-galactosidase expression was determined in *E. coli* ACISH50⁹. A region of the *ArsR* protein that included 344 bp upstream of the ATG start codon was amplified by PCR using the LfArsRlacZF/LfArsRlacZR primer pair (Table 1). The PCR products were digested with BamHI-EcoRI and ligated into the promoterless *lacZ* reporter gene of pMC1403, resulting in the construct pLfArsRlacZ. Cultures of *E. coli* ACISH50⁹ cells harboring the various constructs grown overnight were diluted 1:200 into fresh medium containing the appropriate antibiotics, sodium arsenate or sodium arsenite (25 μM) when indicated, and 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated at 30°C to an optical density at 600 nm of 0.5. The β-galactosidase activities were measured using a method described previously by Miller (14). Constructs in which ArsRC (ptacLfArsRC) (Table 1) was expressed from a tac promoter as well as ArsR (ptacLfArsR) (with an artificial translational stop codon inserted between ArsR and ArsC) (Table 1) were added in trans. Similar β-galactosidase activities of approximately 120 units were obtained for the *arsR-lacZ* construct in the absence or presence of 25 μM arsenate or arsenite, ArsR, and ArsRC (not shown). Taken together with the arsenic growth studies using *E. coli*, it was concluded that the *L. ferriphilum* *ars* genes present on pLfArs were poorly expressed and regulated in both *E. coli* and *L. ferriphilum*. One could speculate that this provided the selection pressure for *L. fer-
LfArs Fairview to acquire TnLfArs to enhance its ability to survive in an arsenic-rich environment, like the biooxidation tanks from which it was isolated.

Expression and regulation analyses of the transposon ars genes were not performed, as a similar study was conducted for the closely related TnMtcArs genes identified in A. caldus in which 25 μM arsenate or arsenite resulted in a fourfold induction (24), and both of these transposons conferred similar resistance to arsenic in E. coli (not shown).

Screening of Leptospirillum isolates for ars genes. Genomic DNA from three L. ferrooxidans strains (ATCC 49879, DSM2705, and Chil-L2) and three L. ferriphilum strains (ATCC 49881, Fairview, and Warwick) was isolated as described previously (24) and was analyzed for the presence of the transposon and chromosomal ars genes by using Southern hybridization. When probing was performed with a fragment specific for TnLfArs, only L. ferriphilum Fairview gave a signal (data not shown). This suggested that this organism likely acquired the ars transposon in the biominning environment via horizontal gene transfer. When hybridization was performed with a 1-kb PvuI-StuI fragment from pLfArs containing the arsRCB genes, all the L. ferriphilum strains gave a hybridization signal, but none of the L. ferrooxidans strains gave a hybridization signal, even under lower-stringency conditions (data not shown). The L. ferriphilum ATCC 49881 and Fairview chromosomal ars genes were located on the same 4-kb EcoRV fragment, indicating that their ars genes and flanking regions are likely identical. A hybridization signal was only obtained from L. ferrooxidans ATCC 49879 when it was probed with an A. caldus chromosomal arsB-specific fragment and under low-stringency conditions (data not shown), suggesting that the isolates of L. ferrooxidans must have a set of ars genes that are different from those of the L. ferriphilum group.

As only the L. ferriphilum strain originally isolated from the Fairview mine had the TnLfArs, we tested whether this strain was more resistant to growth in arsenic than the L. ferriphilum type strain ATCC 49881 and two L. ferrooxidans strains, DSM2705 and ATCC 49879. L. ferriphilum Fairview was capable of growth and iron oxidation in up to 60 mM As(V) as well as As(III) (data not shown). At higher concentrations, it was difficult to keep the arsenic in the solution, and the resistance of this strain may therefore be higher. L. ferrifilum ATCC 49881 and L. ferrooxidans DSM2705 grew up to approximately 40 mM As(V) and As(III), while L. ferrooxidans ATCC 49879 grew up to 30 mM As(III) and 20 mM As(V). Therefore, L. ferrifilum Fairview had maintained its ability to grow in higher concentrations of arsenic than other strains of Leptospirillum even though it had not been exposed to arsenic during 8 to 10 years of subculture in the laboratory.

TnLfArs is capable of transposition in E. coli. To test whether TnLfArs was transpositionally active in E. coli, a similar strategy used previously was used here (24). Briefly, cells containing a nonmobilizable plasmid, pLtTnpUC1 (containing the L. ferriphilum ars transposon genes) (Table 1), and the conjugative plasmid pSa were mated with an E. coli ACH5014-Rif recipient and plated onto selective medium. Plasmid DNA was isolated from two As class Km” Ap” transconjugants (pLtTn1 and pLtTn2), digested with BamHI-HindIII restriction enzymes, and analyzed by a trans-alternating field electrophoresis gel (Fig. 4A) and Southern hybridization (Fig. 4B) using an arsD-containing fragment as a probe. Hybridization signals were obtained for both transconjugant plasmids but not for pSa alone, suggesting that the transposon had jumped to pSa and was conjugated into the recipient cell. Furthermore, the size of the largest pSa fragment was increased (from 23 kb to approximately 36 kb), confirming the insertion of the transposon (Fig. 4A, compare lanes 2 and 3).

As both the L. ferriphilum and A. caldus ars transposons have been found to be capable of transposition in E. coli, it is likely that Tn4A is functional in A. caldus and that TnLfArs is functional in L. ferrifilum. The observation that one type of bacterium is unlikely to have acquired the transposon from the other might indicate that horizontal gene transfer does not easily occur between these two types of bacteria or that the biooxidation tanks are not a suitable environment for this to occur.

Exactly how these transposons may have evolved relative to each other is difficult to determine given this limited set of data. However, the discovery of four arsenic resistance-associated transposons in widely different geographical locations in bacteria that are physiologically and phylogenetically diverse suggests that other related transposons are likely to be found in other arsenic-rich environments. An understanding of the evolutionary development of these transposons awaits their discovery.

Nucleotide sequence accession number. The sequence of inserts of plasmid pLtTnArs was deposited in the GenBank database under accession number DQ057986.

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REFERENCES

1. Reference deleted.

2. Bateman, A. 1997. The structure of a domain common to archaeabacteria and the homocystinuria disease protein. Trends Biochem. Sci. 22:12–13.

3. Butcher, B. G., and D. E. Rawlings. 2002. The divergent chromosomal
operon of Acidithiobacillus ferrooxidans is regulated by an atypical ArsR protein. Microbiology 148:3983–3992.
4. Casadaban, M. J., A. Martinez-Arias, S. K. Shapira, and J. Chou. 1983. \(\beta\)-Galactosidase gene fusions for analyzing gene expression in Escherichia coli and yeast. Methods Enzymol. 100:293–308.
5. Coram, N. J., and D. E. Rawlings. 2002. Molecular relationship between two groups of the genus Leptospirillum and the finding that L. ferripilum sp. nov. dominates South African commercial biooxidation tanks that operate at 40°C. Appl. Environ. Microbiol. 68:838–845.
6. de Groot, P., S. M. Deane, and D. E. Rawlings. 2003. A transposon-located arsenic resistance mechanism from a strain of Acidithiobacillus caldus isolated from commercial, arsenopyrite biooxidation tanks. Hydrometallurgy 71:115–123.
7. Dew, D. W., E. N. Lawson, and J. L. Broadhurst. 2001. Genus II Leptospirillum. In G. Garrity (ed.), Bergey’s manual of comparative bacteriology, vol. I. Williams & Wilkins, Baltimore, Md.
8. Reference deleted.
9. Hallberg, K. B., and E. B. Lindström. 1994. Characterization of Thiobacillus caldus sp. nov., a moderately thermophilic acidophile. Microbiology 140:3451–3456.
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 89:7974–7978.
11. Ji, G., E. A. Garber, L. G. Ames, C.-M. Chen, J. A. Fuchs, and S. Silver. 1994. Arsenate reductase of Staphylococcus aureus plasmid pI258. Biochemistry 33:7294–7299.
12. Johnson, D. B. 2001. Genus II Leptospirillum Hippe 2000 (ex Markosyan 1972, 26). p. 443–447. In G. Garrity (ed.), Bergey’s manual of comparative bacteriology, vol. I. Williams & Wilkins, Baltimore, Md.
13. Liebert, C. A., R. M. Hall, and A. O. Summers. 1999. Transposon Tn21, flagship of the floating genome. Microbiol. Mol. Biol. Rev. 63:507–522.
14. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.
15. Oden, K. L., T. B. Gladysheva, and B. P. Rosen. 1994. Arsenate reduction mediated by the plasmid-encoded ArsC protein is coupled to glutathione. Mol. Microbiol. 12:301–306.
16. Reference deleted.
17. Rawlings, D. E., D. Dew, and C. du Plessis. 2003. Biominalerization of metal-containing ores and concentrates. Trends Biotechnol. 21:38–44.
18. Rawlings, D. E., N. J. Coram, M. N. Gardner, and S. M. Deane. 1999. Thiobacillus caldus and Leptospirillum ferripilum are widely distributed in continuous flow biooxidation tanks used to treat a variety of ores and concentrates, p. 777–786. In R. Amils and A. Ballester (ed.), Biophyrometallurgy and the environment towards the mining of the 21st century, part A. Elsevier, Amsterdam, The Netherlands.
19. Reference deleted.
20. Shi, W., J. Wu, and B. P. Rosen. 1994. Identification of a putative metal binding site in a new family of metalloregulatory proteins. J. Biol. Chem. 269:19826–19829.
21. Tait, R. C., R. C. Lundquist, and C. I. Kado. 1982. Genetic map of the crown gall suppressive IncW plasmid pSa. Mol. Gen. Genet. 186:10–15.
22. Tisa, L. S., and B. P. Rosen. 1989. Molecular characterization of an anion pump: the ArsB protein is the membrane anchor for the ArsA protein. J. Biol. Chem. 265:190–194.
23. Tuñin, I. M., P. de Groot, S. M. Deane, and D. E. Rawlings. 2004. Multiple sets of arsenic resistance genes are present within highly arsenic resistant industrial strains of the biomining bacterium, Acidithiobacillus caldus. Int. Cong. Ser. 1275:165–172.
24. Tuñin, I. M., P. de Groot, S. M. Deane, and D. E. Rawlings. 2005. An unusual Tn21-like transposon containing an unusual ars operon is present in highly arsenic-resistant strains of the biomining bacterium Acidithiobacillus caldus. Microbiology 151:3027–3039.
25. Tyson, G. W., J. Chapman, P. Hugenholtz, E. E. Allen, R. J. Ram, P. M. Richardson, V. V. Solovyev, E. M. Rubin, D. S. Rokhsar, and J. F. Banfield. 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature 428:37–43.
26. Woese, C. R. 2004. A new biology for a new century. Microbiol. Mol. Biol. Rev. 68:173–186.
27. Wu, J. H., and B. P. Rosen. 1991. The ArsR protein is a trans-acting regulatory protein. Mol. Microbiol. 5:1331–1336.
28. Wu, J. H., and B. P. Rosen. 1993. The arsD gene encodes a second trans-acting regulatory protein of the plasmid-encoded resistance operon. Mol. Microbiol. 5:615–623.
29. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strain: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119.
30. Zabeau, M., and K. K. Stanley. 1982. Enhanced expression of the cro-\(\beta\)-galactosidase fusion proteins under the control of the Pm promoter of bacteriophage lambda. EMBO J. 1:1217–1224.