

High level arsenic resistance in bacteria present in biooxidation tanks used to treat gold-bearing arsenopyrite concentrates: A review

D. E. RAWLINGS

Department of Microbiology, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa

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Abstract: The microbial consortium used in continuous-flow, stirred tank processes to treat gold-bearing arsenopyrite concentrates became adapted to high concentrations of arsenic over a number of years. The dominant microorganisms, *Acidithiobacillus caldus* and *Leptospirillum ferriphilum*, were found to contain two sets of arsenic resistance genes. One set of *ars* genes was present in all isolates of a species irrespective of whether they were highly arsenic resistant or not. A second set of *ars* genes was present on Tn21-like transposons and was found in all strains tested that had been adapted to high concentrations of arsenic. The arsenic resistance transposons present in *At. caldus* and *L. ferriphilum* were closely related, but sufficiently different for them to have been acquired independently rather than having been passed from one bacterium to the other. The transposons were transpositionally active in *Escherichia coli* and were shown to confer higher levels of arsenic resistance than the chromosomally-located *ars* genes where it was possible to test this. Transposons containing arsenic resistance genes that were identical or closely related to the transposon from *L. ferriphilum*, originally found in South Africa, were also found in both *L. ferrooxidans* and *L. ferriphilum* isolates from South America and Europe. An *arsB* gene knockout of *At. caldus* was produced by homologous recombination that demonstrated both the ability of the chromosomal *ars* genes to confer low levels of arsenic resistance in *At. caldus* and the development of a genetic system for the creation of knock-out mutants.

Key words: *Acidithiobacillus caldus*; *Leptospirillum*; arsenic resistance genes; transposons; microbial consortia; biooxidation tanks

1 Introduction

Continuous flow, stirred-tank based biooxidation processes for the recovery of gold from gold-bearing arsenopyrite concentrates were developed during the 1980s. A number of large-scale industrial plants that use these biooxidation process as a pre-treatment step prior to the recovery of gold by cyanidation have been built in several countries[1–2]. When arsenopyrite concentrates are biooxidized, levels of arsenic frequently reach saturation and soluble arsenate concentrations of 12 g/L and arsenite of 3–6 g/L have been reported[3]. During the early laboratory stages of development of the biooxidation process, oxidation of the arsenopyrite concentrate was slow and inefficient, partly because the bacteria were sensitive to the high concentrations of arsenic that were released. Arsenic inhibition was so severe that after a period of aeration, the mineral suspension was transferred to an unaerated tank where the pH was adjusted to 3.5 and the precipitated arsenic

was removed by settling. The mineral suspension was then transferred to a fresh tank where aeration was continued. Initially more than one arsenic precipitation step was required. As the separation of arsenic from the insufficiently oxidized arsenopyrite was complicated, the process was uneconomic. During the operation of a continuous-flow tank aeration system, fast-growing arsenopyrite-biooxidizing cells displaced the more readily washed out slow-growing cells and this resulted in the selection of highly arsenic resistant microbes[4–5]. Over a period of about two years the bacterial consortium became sufficiently resistant to arsenic so that no arsenic precipitation steps were required. This enabled the residence time of the mineral concentrate in the series of aeration tanks to be reduced from about 12 to less than 7 days.

In 1986, the first commercial arsenopyrite biooxidation plant was built at the Fairview gold mine near the town of Barberton in South Africa. After three years of operation, not only had the total residence time of the mineral in the biooxidation tanks been reduced to

a little over 3 days but also the quantity of mineral concentrate in suspension had been increased from 10% to 19% (w/v)[4]. Subsequently, several additional arsenopyrite biooxidation plants were built in other countries e.g. Brazil (Sao Bento), Australia (Wiluna and Harbour lights) and Ghana (Ashanti Sansu) Tamboraque (Lima, Peru) was built[1–2].

Continuous-flow tanks that are used for the biooxidation of arsenopyrite concentrates that operate at 40 °C are dominated by a mixture of the sulphur-oxidizing bacterium, *Acidithiobacillus caldus*, and the iron-oxidizing bacterium, *Leptospirillum ferriphilum* [6–7]. We wished to investigate what genetic changes had taken place that permitted a consortium of bacteria to increase their tolerance to total arsenic in solution from less than 1 g/L to 13 g/L. Unfortunately, no samples of the early biooxidation bacterial consortium were maintained and therefore it was not possible to compare the bacteria in the original arsenic-sensitive with the arsenic-tolerant consortia. However, isolates of the same species of bacteria as found in the biooxidation tanks that are unlikely to have been exposed to high levels of arsenic can be compared to the highly arsenic tolerant bacteria. In this work, investigations into how the *At. caldus* and *L. ferriphilum* strains present in arsenopyrite biooxidation tanks became highly arsenic resistant are reviewed.

2 Arsenic resistance mechanisms

In biological systems, arsenic typically exists in two oxidation states, arsenite (As III) and arsenate (As V). Arsenic resistance mechanisms have been reviewed extensively[8–11]. Typically, the minimal set of genes

that confers resistance to both arsenite and arsenate are known as the *arsR*, *arsB* and *arsC* genes. The gene product, ArsR, is an arsenic-responsive gene regulator, ArsB, a membrane-located arsenite efflux pump and ArsC an arsenate reductase required to reduce arsenate to arsenite prior to its export from the cell by ArsB. Other frequently encountered arsenic resistance-associated genes are *arsA* and *arsD*. ArsA is an ATPase that forms a complex with ArsB and assists in the export of arsenite, while ArsD was reported to be a second regulator that controls the upper level of *ars* gene expression. More recently, ArsD has been found to act as an arsenite binding protein or metallochaperone[12] that sequesters intracellular arsenite and delivers it to ArsA before being pumped out of the cell through the action of the ArsAB membrane efflux pump (Fig.1).

3 Transposons containing arsenic resistance genes from *At. caldus* and *L. ferriphilum*

Strains of *At. caldus* and *L. ferriphilum* were isolated from the arsenopyrite biooxidation tanks at the Fairview mine and gene banks were constructed[3, 13]. The gene banks were transformed into an arsenic sensitive *Escherichia coli* strain ACSH50Iq and the transformants screened for arsenate and arsenite resistance[3, 13]. A Tn21-like transposon containing gene for arsenic resistance was isolated from both *At. caldus* and *L. ferriphilum* (Fig.1). The transposons were sequenced and named TnAtcArs and TnLfArs, respectively.

The transposon-associated arsenic resistance genes from *At. caldus* were contained on a 12 444 bp DNA fragment that has 40 bp long, Tn21-like inverted repeat

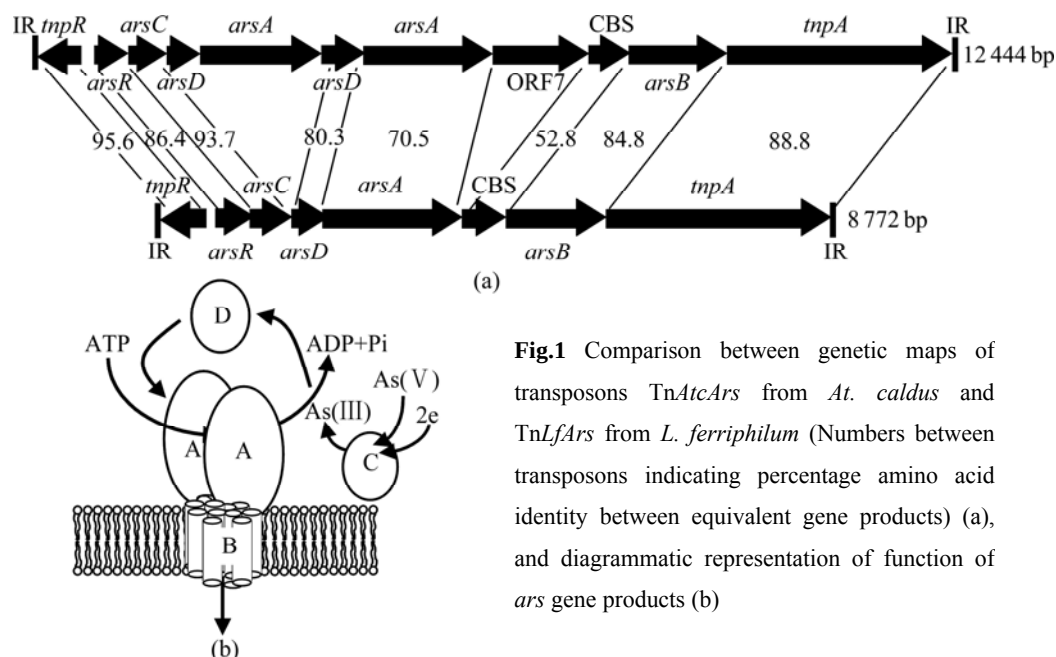


Fig.1 Comparison between genetic maps of transposons TnAtcArs from *At. caldus* and TnLfArs from *L. ferriphilum* (Numbers between transposons indicating percentage amino acid identity between equivalent gene products) (a), and diagrammatic representation of function of *ars* gene products (b)

(IR) sequences and genes for a Tn21 family-like resolvase (*tnpR*) and transposase (*tnpA*) (Fig.1). Seven ORFs closely related to arsenic resistance genes were located between the transposon resolvase and transposase genes. Arsenic resistance genes encoding products with high amino acid sequence homology to *arsR*, *arsC*, duplicate copies of *arsD* and *arsA* as well as an *arsB* were found. Between the second copy of *arsA* and the single copy of *arsB*, two open reading frames called ORF7 and ORF8 that had not previously been associated with *ars* genes were identified. The 470 aa long predicted product of ORF7 was reported to have the clear homology to the 466 aa NADH oxidase (46% identity/60% similarity) from the bacterium *Lactobacillus plantarum*. The product of ORF8 (158 aa) was reported to have the highest homology (54% identity/72% similarity) over approximately its entire length to what is known as the 146 aa CBS (cystathione- β -synthase)[14] domain of a membrane protein from *Desulfotobacterium hafniense*. Inactivation of either ORF7 or ORF8 did not appear to have a detectable effect on arsenite or arsenate resistance in *E. coli* and their function on Tn*AtcArs* is not known.

The arsenic gene containing transposon, Tn*LfArs*, isolated from *L. ferriphilum* was clearly related to Tn*AtcArs* from *At. caldus* (Fig.1). The *ars* genes of Tn*LfArs* were located between divergently transcribed *tnpR* and *tnpA* genes in a manner similar to the Tn*AtcArs* transposon[13]. As for Tn*AtcArs*, the *ars* genes of Tn*LfArs* consisted of *arsR*, *arsC*, *arsD*, *arsA* and *arsB* all transcribed in the same direction. Between *arsA* and *arsB* there was an open reading frame equivalent to ORF8 of Tn*AtcArs*. Despite the overall similarities, there were some important differences between Tn*LfArs* and Tn*AtcArs*. Tn*LfArs* was only 8 772 bp in length compared with 12 444 bp for Tn*AtcArs*. The main reason for the size difference was that ORF7 and the *arsDA* duplication of Tn*AtcArs* were absent from Tn*LfArs*. The Tn*LfArs* 43 bp left and right invert repeats (IR) differed from one another by 1 bp and were 3 bp longer than those for Tn*AtcArs*. Furthermore, although the amino acid sequences of several gene products were highly conserved (TnpR, ArsC and TnpA had 96%, 94% and 89% amino acid sequences with identity, respectively), others were much less conserved. For example, ORF8 and ArsA had only 53% and 71% amino acid sequence identity, respectively (Fig.1).

The differences between the two *ars* transposons suggested that it was highly unlikely that the transposons had been passed from *At. 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 were much too substantial for these to have arisen in the ten to fifteen

years or less since the biooxidation plant at Fairview mine began operation and the time of sample collection. This suggested that although the two types of bacteria had acquired related transposons to increase their arsenic resistance, they had acquired the *ars* transposons independently of each other.

4 Tn*AtcArs* and Tn*LfArs* were transpositionally active but no evidence was obtained that transposition frequency was increased in presence of arsenic

Both Tn*AtsArs* and Tn*LfArs* were tested to see whether they were still transpositionally active[3, 13] in *E. coli* by using plasmid pSa as a 'transposon trap'. A diagrammatic representation of the experiments is shown in Fig.2. Tn*AtcArs* was cloned into a non-conjugative plasmid (lacked an oriT origin of transfer) and it was shown that when present on its own in a cell, Tn*AtcArs* could not be conjugated to an *E. coli* rifampicin resistant recipient. However, when the conjugative plasmid pSa (chloramphenicol resistant) was present in the same cell as the non-mobilizable plasmid containing Tn*AtcArs*, the Tn*AtcArs* transposon could be conjugated into an *E. coli* rifampicin resistant mutant (Fig.2). The explanation for this was that Tn*AtcArs* had transposed into the pSa 'transposon trap' in the donor strain and that this pSa-Tn*AtcArs* plasmid had been transferred by conjugation to the *E. coli* recipient. Transposition of Tn*AtcArs* into pSa was confirmed by digestion of pSa and pSa-Tn*AtcArs* with the restriction endonuclease enzymes BglII and HindIII. It was found that size of one of the DNA fragments of pSa-Tn*AtcArs* was larger than pSa. Furthermore, whereas pSa gave no hybridization signal when using *arsB* of Tn*AtcArs* as probe, the pSa-Tn*AtcArs* plasmid did give a positive hybridization signal, indicating that the increase in size was due to the acquisition of arsenic resistance genes.

Similar results were obtained when Tn*LfArs* was tested for transposition activity in *E. coli* using the same type of experiment, indicating that both Tn*AtsArs* and Tn*LfArs* were transpositionally active[13].

Both transposons Tn*AtsArs* and Tn*LfArs* appeared to be dedicated to the transposition of the arsenic resistance genes located between the *tnpR* (resolvase) and *tnpA* (transposase) genes. Furthermore, the gene for the transposase was located downstream of the *arsB* gene and was transcribed in the same direction as the *ars* genes. This raised the question of whether transcriptional read-through from the *ars* genes might increase the level of expression of the *tnpA* gene and hence the frequency of transposition might increase in response to arsenic. An increased frequency of transposition in response to mercury has been reported in the mercury resistance

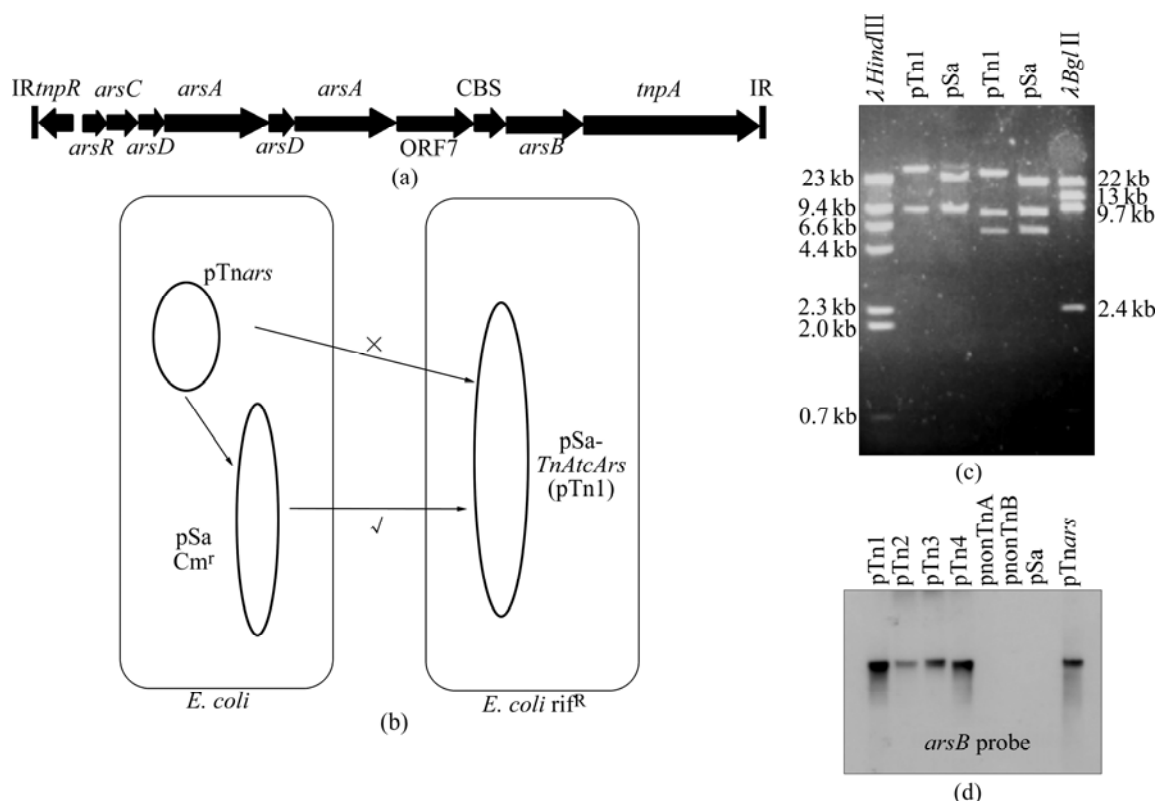


Fig.2 Representation of TnAtcArs inserted into plasmid pUC19 to produce non-mobilizable construct, pTnars (a); Diagrammatic illustration of experiment of TnAtcArs present on pTnars transposed into conjugative plasmid, pSa, before transfer to *E. coli* rifampicin resistant recipient (b); Restriction endonuclease analysis of pSa before and after insertion of TnAtcArs (pTn1) (c); Southern hybridization of pTn1 and three similar transposon insertions into pSa (pTn2-4) probed with *At. caldus* arsB (Lanes marked pnonTnA and pnonTnB containing plasmids from transconjugants where donor cells contained pSa but not pTnars) (d)

transposon Tn501[15]. However, we were unable to detect an increase in the frequency of transposition in response to arsenic in *E. coli*. This was possibly due to a fairly large scatter in the transposition frequency but northern hybridisation data showed that *tnpA* expression was not affected by the presence of arsenite, and therefore, increased transposition in response to arsenite probably does not occur[3].

5 Not all strains of *At. caldus* and *L. ferriphilum* contain ars resistance transposon

We had obtained six strains of *At. caldus*, three of which had been previously exposed to arsenic as they had previously been isolated from arsenopyrite concentrate biooxidation tanks. Although one strain was from a nickel ore biooxidation tank, the inoculum had originated from an arsenopyrite biooxidation tank some years previously. In addition, we obtained three strains that were unlikely to have been exposed to high levels of arsenic. These originated from Birch Coppice (BC13) and a coal spoil (KU) in the United Kingdom and from a bioreactor in Brisbane, Australia (C-SH12). All strains of

At. caldus previously exposed to arsenic were found to contain the TnAtcArs operon whereas none of the three strains not known to have been previously exposed to arsenic had the arsenic resistance transposon[16–17]. However, all six *At. caldus* strains contained chromosomally-encoded arsenic resistance genes.

A similar situation appeared to exist in *Leptospirillum*. Genomic DNA from three *L. ferrooxidans* strains (ATCC49879, DSM2705 and Chil-Lf2) and three *L. ferriphilum* strains (ATCC49881, Fairview and Warwick) was analysed for the presence of the transposon and chromosomal *ars* genes. When a probe with a fragment specific for TnLfArs was used in Southern hybridisation experiments, only *L. ferriphilum* Fairview gave a signal[13]. Both *L. ferriphilum* and *L. ferrooxidans* have chromosomal-type arsenic genes in addition to the Tn-related genes [18]. The *L. ferriphilum* chromosomal *ars* genes are atypical in that the *arsB* gene is preceded by an *arsR* and *arsC* and these two genes appeared to have been fused[13]. When the *arsRCB* chromosomal genes of *L. ferriphilum* was used as probe, all of the *L. ferriphilum* strains gave a hybridisation signal, but none of the *L. ferrooxidans* strains, even under lower stringency conditions. This suggested that

the chromosomal *ars* genes of *L. ferriphilum* and *L. ferrooxidans* had dissimilar DNA sequences. In subsequent experiments, the chromosomal *arsB* gene from *L. ferrooxidans* was isolated[18]. In *L. ferrooxidans*, the *arsB* appears to occur as a single gene, with a predicted amino acid sequence that is substantially different from *L. ferriphilum*. The absence of a gene for an ArsR regulator linked to *arsB* raised the question of how *arsB* expression is regulated in *L. ferrooxidans*. The finding suggested that either a gene for an *arsR* regulator lies elsewhere on the *L. ferrooxidans* chromosome or that the *arsB* gene that was isolated may be redundant.

6 Transposon encoded arsenic resistance genes confer much higher levels of arsenic resistance than chromosomally encoded arsenic resistance genes

The transposon-encoded arsenic resistance genes appeared to confer much higher levels of arsenic resistance than the chromosomally encoded arsenic resistance genes. Since no strains of *At. caldus* lacking the chromosomal arsenic resistance genes existed at the time, the levels of arsenic resistance were tested in *E. coli* after cloning the two types of resistance genes in an *E. coli* strain that lacked the usual chromosomal arsenic resistance genes[3, 19]. Whereas Tn*AtcArs* conferred resistance to *E. coli* *ars* mutants of at least 5 mmol/L arsenite, the cloned chromosomal resistance genes conferred resistance to only 0.25 mmol/L arsenite. Resistance to arsenate was more complicated to compare

because of interference by phosphate in the growth medium.

That an increase in arsenic resistance was conferred by Tn*AtcArs* was also tested in the normal host. *At. caldus* strain C-SH12 that lacked Tn*AtcArs* grew in medium containing up to approximately 20 mmol/L arsenite (Fig.3). After the transfer of Tn*AtcArs* to strain C-SH12 by conjugation, the transconjugant strain containing both the chromosomal- and transposon-encoded *ars* genes, grew in thiosulfate medium containing greater than 50 mmol/L arsenite at which point it was difficult to maintain arsenite in solution[19]. This illustrated the effectiveness of transposon-located *ars* genes in conferring arsenic resistance relative to a strain containing only the chromosomal *ars* genes.

With respect to *L. ferriphilum*, although the genes on Tn*LfArs* conferred arsenic resistance to *E. coli* when transferred to that bacterium, the cloned chromosomal genes were not functional in *E. coli*[13]. The relative effectiveness of the transposon-located and chromosome-located *ars* genes could therefore not be tested. Nevertheless, growth in arsenic of the *L. ferriphilum* Fairview strain containing Tn*LfArs*, was compared with growth in arsenic of the *L. ferriphilum* type strain ATCC49881 which lacked Tn*LfArs*. *L. ferriphilum* ATCC49881 grew in iron medium containing arsenic up to approximately 40 mmol/L of either As(V) or As(III) [13]. In contrast, *L. ferriphilum* Fairview was capable of growth and iron oxidation of up to 60 mmol/L of either As(V) or As(III). At higher concentrations it was difficult to keep the arsenic in solution and the resistance

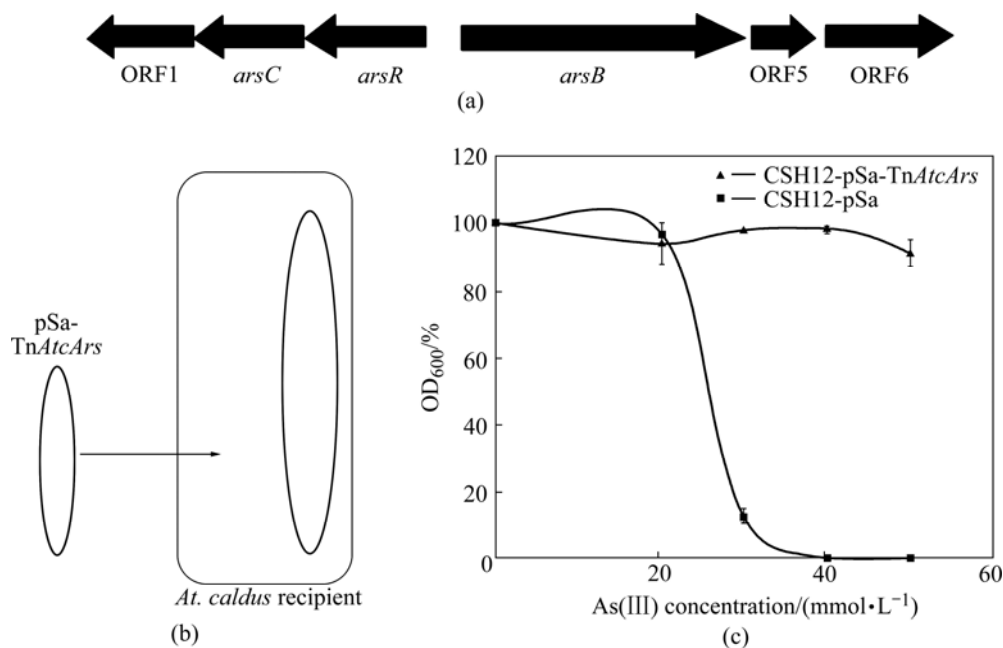


Fig.3 Genetic map of region including chromosomal *ars* genes of *At. Caldus* (a); Plasmid pSa-Tn*AtcArs* transferred by conjugation from *E. coli* to *At. caldus* (b); Growth after 19 d of incubation of *At. caldus* strain C-SH12 containing only pSa compared with growth of *At. caldus* strain C-SH12 containing pSa-Tn*AtcArs* in presence of increasing concentrations of arsenite (c) [19]

of this strain may therefore be higher. Since two different *L. ferriphilum* strains were used, this is not proof that the TnLfArs conferred increased arsenic resistance, but suggests that this might be the case.

7 Distribution of TnLfArs genes

As described above, it was found that the strains of *At. caldus* and *L. ferriphilum* that dominate the gold-bearing arsenopyrite biooxidation tanks at the Fairview mine had acquired related but different transposons of the Tn21-family that contained an operon that conferred arsenic resistance. Furthermore, only the three *At. caldus* strains and the *L. ferriphilum* Fairview strain that had previously been exposed to high levels of arsenic, contained an arsenic resistance transposon[13].

Many of the commercial arsenopyrite biooxidation processes that have been built in different parts of the world were inoculated with the microbial consortium from the biooxidation tanks of the Fairview mine. An exception to this is the biooxidation plant at Tamboraque (near Lima, Peru) where the consortium used in those tanks originated from a mixture of locally available iron and sulfur oxidizing microorganisms[20]. We wished to determine whether arsenic resistance in this consortium had occurred through the acquisition of a similar transposon as the Fairview consortium or by a different mechanism. Unfortunately we were not able to isolate a viable culture of a sulfur-oxidizing microorganism from the Tamboraque mineral sample following its transport to South Africa from Peru. However, we were able to isolate an iron oxidizer that turned out to be a strain of *L. ferrooxidans* rather than a *L. ferriphilum*[18]. We also had access to several isolates of both *L. ferriphilum* and *L. ferrooxidans* from Europe and these were also screened to detect whether they contained an arsenic resistance transposon related to those present in the Fairview strain even though they had not been selected for enhanced arsenic resistance. PCR and Southern

hybridization experiments indicated that arsenic resistance genes that were identical or closely related to those from South Africa were found in both *L. ferrooxidans* and *L. ferriphilum* isolates from South America and Europe [18].

The widespread occurrence of arsenic resistance transposons amongst different isolates of *L. ferriphilum* and *L. ferrooxidans* from at least three continents (Africa, South America and Europe) suggested that it should be possible to select for highly arsenic resistant biomining microorganisms from many different sources and that it was therefore probably unnecessary to import pre-adapted, arsenic resistant consortia.

8 Origin of Tn21-like arsenic resistance transposons

Since the TnAtcArs and TnLfArs transposons were transpositionally active and not present in all strains of *At. caldus*, *L. ferriphilum* or *L. ferrooxidans*, these transposons would be expected to have been acquired from the horizontal gene pool. This raised the question of whether they originated in bacteria such as the *At. caldus* and *Leptospirillum* isolates described here, or whether the arsenic resistance transposons originated in other organisms and were acquired by the 'biomining' bacteria by horizontal gene transfer.

A BLAST search using the *L. ferriphilum* ars genes indicated that they were most similar to transposon-related ars genes identified in *Methylobacillus flagellatus* [21] and *Alcaligenes faecalis* (AY297781)[13]. Even though this work was reported in 2006, the ars genes from the transposons found in *M. flagellatus* and *A. faecalis* remain the most closely related ars genes in sequence databases. A comparison of the structure of the TnLfArs ars transposon with that of *A. faecalis* and *Methylobacillus flagellatus* is shown in Fig.4 (structures of TnAtcArs and TnLfArs were compared in Fig.1). In general, the ars operon structures of TnLfArs, TnAtcArs

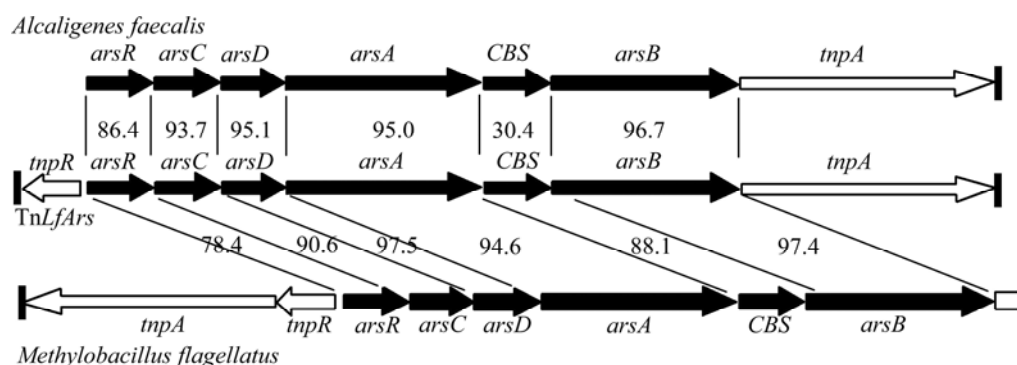


Fig.4 Diagrammatic representation of TnLfArs compared to related transposons found in *Alcaligenes faecalis* (AY297781) and *Methylobacillus flagellatus* (Numbers between transposons indicating percentage amino acid identity between equivalent ars gene products) [13]

and *A. faecalis* transposons are closely related. Exceptions are that *TnAtcArs* has an additional NADH oxidoreductase-like gene (ORF7) and in addition, the *M. flagellatus* *arsR* and *arsC* genes appear to have been fused. A comparison of the sequence identity of the *ars* gene products of the *M. flagellatus* and *A. faecalis* transposons with those of *TnLfArs* is also made in Fig.4. Some of predicted protein products within the *ars* operons are highly related to each other. For example, the ArsD, ArsA and ArsB proteins have 97.5%, 94.6% and 97.4% amino acid sequence identity, respectively.

With respect to the rest of the transposon structures, the *A. faecalis* transposon appears 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 Tn21-subfamily (and Tn3-family) transposons where they form a functional unit[22].

Together these results suggest that there exists a group of related Tn21-like transposons that carry arsenic resistance genes member of which are present in a number of microorganisms, most of which have still to be discovered[13]. Given current knowledge it is not yet possible to determine from which organisms *TnAtcArs* and *TnLfArs* originated or whether either transposon is the source of the related group of transposons. Further discoveries of transposons from this group might clarify this.

9 Construction of *At. caldus* chromosomal *ars* gene knockout mutants

Arsenic resistance genes have proved useful in another context, of that a protocol for creating *At. caldus* chromosomal gene knockout mutants is established. The usefulness of the *At. caldus* chromosomal *ars* genes was that the creation of a knockout mutant was not expected to be lethal to the host. To create an *arsB* knockout mutant, the *arsB* gene of *At. caldus* was cloned into a *At. caldus* suicide cloning vector. This vector contained the origin of transfer (*oriT*) sequence of the broad-host-range conjugative plasmid, pSa, and could replicate in *E. coli* but not in *At. caldus*[23]. The cloned *arsB* gene was inactivated by the deletion of a small piece of the gene and replacing it with a kanamycin resistance gene. This construct containing the inactivated *arsB* gene was transferred by conjugation from a strain of *E. coli* containing the conjugative plasmid pSa to *At. caldus*. Following conjugation, the only means by which the marker on the suicide plasmid could be recovered was by homologous recombination with the *ars* genes present on the *At. caldus* chromosome. Transconjugants were selected on a thiosulfate medium containing kanamycin and those grown were screened using Southern

hybridization with a probe made from the kanamycin resistance gene. This confirmed that the kanamycin gene had integrated into the *At. caldus* chromosome probably by means of the inactivated *arsB* chromosomal gene. As both double and single cross-over insertions within the *arsB* gene were possible, the presence of single cross-over mutants was screened by Southern hybridization using the small fragment deleted from the *arsB* gene as probe[23]. This deleted fragment would be missing from double cross-over but not single cross-over mutants. Of the 140 kanamycin resistant colonies checked, only six were double cross-over mutants where the wild type *arsB* gene had been replaced by the inactivated *arsB* gene. The other *At. caldus* mutants were single cross-over mutants where the conjugated suicide plasmid had been integrated into the chromosome such that the transconjugants contained both an active and an inactive copy of the *arsB* gene. When tested for arsenic resistance, isolates containing a single inactivated *arsB* gene were extremely sensitive to arsenite. This indicated that *arsB* gene knockouts had been successfully made and that the chromosomal *At. caldus* *ars* genes contributed to low-level arsenic resistance[23].

10 Conclusions

1) All strains of *At. caldus*, *L. ferriphilum* and *L. ferrooxidans* tested contain *ars* genes present on their chromosomes. In the case of *At. caldus*, these were shown to be functional in both *At. caldus* and *E. coli*. The chromosomal genes of both species of *Leptospirillum* were atypical and non-functional in *E. coli*. It is uncertain whether these chromosomal genes are functional in *Leptospirillum*. This is especially so in the case of *L. ferrooxidans* where only a single *arsB* was found without any sign of an *arsC* gene (arsenate reductase) or *arsR* regulator.

2) Beside chromosomal *ars* genes, *At. caldus*, *L. ferriphilum* and *L. ferrooxidans* strains isolated from biooxidation tanks treating gold-bearing arsenopyrite ores contain a second set of arsenic resistance genes that are present on the Tn21-like transposons, *TnAtcArs* and *TnLfArs*. In the case of *At. caldus*, these genes conferred high levels of resistance to arsenite and arsenate in both *At. caldus* and *E. coli*. In the case of *L. ferriphilum*, high levels of arsenic resistance occurred when the *TnLfArs* transposon was present in *E. coli*, but gene transfer techniques did not exist to test to what extent arsenic resistance was enhanced in strains of *Leptospirillum* that lacked the transposon.

3) Both the *TnAtcArs* and *TnLfArs* transposons are transpositionally active in *E. coli*, but no evidence was obtained that the rate of transposition was increased in the presence of arsenic.

4) The *TnLfArs* transposon or a transposon highly similar to *TnLfArs* is present in isolates of *L. ferriphilum* and *L. ferrooxidans* from at least three continents including some isolates not known to have been previously exposed to high levels of arsenic. This suggests that it should be possible to isolate consortia of arsenic resistant microorganisms without having to resort to obtaining such consortia from plants that have previously been exposed to arsenic such as commercially operating arsenopyrite biooxidation plants.

5) Although it is not yet possible to determine from where the *Tn21*-like *ars* gene containing transposons originated, a family of related transposons appears to exist in a variety of bacteria including typical heterotrophic microorganisms.

6) *At. caldus* strains in which the chromosomal *ars* genes were inactivated using a kanamycin resistance gene became highly sensitive to arsenic. This demonstrated that the chromosomal *ars* genes conferred not only the low levels of arsenic resistance to *At. caldus*, but also the development of a genetic system for the creation of knock-out mutants in *At. caldus*.

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