Cloning and characterization of the chromosomal arsenic resistance genes from *Acidithiobacillus caldus* and enhanced arsenic resistance on conjugal transfer of *ars* genes located on transposon TnAtcArs

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All strains of the moderately thermophilic, acidophilic, sulphur-oxidizing bacterium *Acidithiobacillus caldus* that have been tested contain a set of chromosomal arsenic resistance genes. Highly arsenic-resistant strains isolated from commercial arsenopyrite bio-oxidation tanks contain additional transposon-located (TnAtcArs) arsenic resistance genes. The chromosomal *At. caldus* *ars* genes were cloned and found to consist of *arsR* and *arsC* genes transcribed in one direction, and *arsB* in the opposite direction. The *arsRC* genes were co-transcribed with ORF1, and *arsB* with ORF5 in both *At. caldus* and *Escherichia coli*, although deletion of ORFs 1 and 5 did not appear to affect resistance to arsenate or arsenite in *E. coli*. ORFs 1 and 5 have not previously been reported as part of the *ars* operons, and had high amino acid identity to hypothetical proteins from *Polaromonas naphthalenivorans* (76 %) and *Legionella pneumophila* (60 %), respectively. Reporter-gene studies showed that the arsenic operon of transposon origin (TnAtcArs) was expressed at a higher level, and was less tightly regulated in *E. coli* than were the *At. caldus* *ars* genes of chromosomal origin. Plasmid pSa-mediated conjugal transfer of TnAtcArs from *E. coli* to *At. caldus* strains lacking the transposon was successful, and resulted in greatly increased levels of resistance to arsenite.

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INTRODUCTION

Acidithiobacillus caldus is a moderately thermophilic, acidophilic, sulphur-oxidizing, Gram-negative bacterium (Hallberg & Lindström, 1994). Together with the iron-oxidizing bacterium *Leptospirillum ferriphilum*, it has been reported to dominate the microbial populations in commercial arsenopyrite concentrate bio-oxidation tanks that operate at 40 °C and pH 1.6 (Rawlings *et al.*, 1999). These vigorously aerated tanks are used in a pretreatment process to decompose and open up the structure of gold-bearing arsenopyrite concentrates to facilitate the extraction of the gold by cyanide (Rawlings *et al.*, 2003).

Southern hybridization experiments using the *arsB* genes from the *Escherichia coli* plasmid R773, or the *arsC* gene of *Acidithiobacillus ferrooxidans* (Butcher *et al.*, 2000) as probes have indicated that all strains of *At. caldus* tested contain a set of chromosomal arsenic resistance genes (Dopson *et al.*,

2001; Tuffin *et al.*, 2004). Studies on *At. caldus* strain KU have shown that arsenic resistance is inducible by arsenite [As(III)], arsenate [As(V)] and antimony, resulting in an energy-dependent decrease in accumulation of these toxic ions (Dopson *et al.*, 2001). This, together with the demonstration of arsenate reductase activity, suggests that *At. caldus* contains an arsenic resistance mechanism similar to those described for other bacteria. However, the chromosomal arsenic resistance genes have not been isolated.

The large quantities of toxic arsenic compounds released during the bio-oxidation of arsenopyrite concentrates (Dew *et al.*, 1997) result in the acquisition, by the *At. caldus* isolates found in these tanks, of an additional set of arsenic resistance genes that are not present in the less arsenic-resistant isolates of the same species (Tuffin *et al.*, 2005). These additional genes are located on an unusual Tn21-like transposon, TnAtcArs, which is transpositionally active in laboratory strains of *E. coli*, and appears to be dedicated to the spread of arsenic resistance.

Here, we report the cloning and characterization of the chromosomal arsenic resistance genes present in all strains

Abbreviation: NCBI, National Centre for Biotechnology Information.

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of *At. caldus*. We examined the levels of arsenic resistance conferred by both the chromosomal and *TnAtcArs* *ars* genes when cloned in laboratory strains of *E. coli*, independently and when present together in the same host. Furthermore, we tested whether there was any interaction between the two arsenic resistance operons at the regulation level. We transferred the arsenic resistance genes present on *TnAtcArs* by conjugation from *E. coli* to *At. caldus*, and demonstrated increased arsenic resistance in the recipient *At. caldus*. This is believed to be the first report of the transfer of genes to *At. caldus*, and could serve as a basis for the development of a genetic system for the bacterium.

METHODS

Media, bacterial strains and plasmids. *At. caldus* bacterial strains and plasmid constructs used in this study are shown in

Table 1. *pEcoR252* (Zabeau & Stanley, 1982), *pBluescript SK* (Stratagene), *pGem-T* (Promega), *pGL10* (Km^r , RK2/RP4 replicon, cloning vector; A. Toukdarian, University of California, San Diego), *pUCBM21* (Boehringer Mannheim), *pMC1403* (Casadaban *et al.*, 1983), *pKK223-3* (Pharmacia), *pSa* (Tait *et al.*, 1982) and *pEcoBlunt* (Tuffin *et al.*, 2005) have been described previously. *E. coli* strains (DH5 α , Promega; ACSH50I q , Butcher & Rawlings, 2002; HB101, Bolivar *et al.*, 1977; CSH56, Cold Spring Harbor Laboratory) were grown in Luria–Bertani (LB) broth medium (Sambrook *et al.*, 1989), with ampicillin (100 $\mu\text{g ml}^{-1}$) or kanamycin (100 $\mu\text{g ml}^{-1}$) added as required. *At. caldus* strains were grown at 37 °C in tetrathionate medium (5 mM), sterilized and adjusted to pH 2.5 (Rawlings *et al.*, 1999); sodium thiosulphate medium (5 mM), sterilized and adjusted to pH 4.6 (sodium thiosulphate replaced the tetrathionate); or elemental sulphur (S^0) medium, sterilized and adjusted to pH 4.6. For the S^0 medium, elemental sulphur was sterilized by adding 0.5 g S^0 to 5 ml water, and heating to 105 °C for 1 h on two successive days. The sterilized sulphur was then added to the basal medium (0.5 g l^{-1}).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>At. caldus</i>		
#6	Fairview mine, Barberton, South Africa	Rawlings <i>et al.</i> (1999)
BC13 (ATCC 51756)	Birch coppice, Warwickshire, UK	Hallberg & Lindström (1994)
KU (DSM 8584)	Kingsbury Coal Spoil, UK	Hallberg & Lindström (1994)
C-SH12 (DSM 9466)	Continuous Bioreactor, Brisbane, Australia	Goebel & Stackebrandt (1994)
Plasmids		
pAtcars4	Ap^r ; 10 kb <i>Sau3A</i> fragment of <i>At. caldus</i> #6 cloned into the <i>Bgl</i> II site of <i>pEcoR252</i>	This study
pAtcars6	Ap^r ; pAtcars4 with <i>arsC</i> and ORF1 deleted	This study
pAtcarsCRB5	Ap^r ; blunted 2.9 kb <i>Pvu</i> II– <i>Hind</i> III fragment from pAtcars4 cloned into a blunted <i>Bgl</i> II site of <i>pEcoR252</i>	This study
pAtcarsCRB5GL	Km^r ; 2.9 kb <i>Pvu</i> II– <i>Hind</i> III fragment from pAtcars4 cloned into a <i>Hind</i> III– <i>Sma</i> I site of <i>pGL10</i>	This study
pAtcarsCRB	Ap^r ; blunted 2.6 kb <i>Bst</i> EII fragment from pAtcars4 cloned into a blunted <i>Bgl</i> II site of <i>pEcoR252</i>	This study
pAtcarsRB5	Ap^r ; deletion construct of pAtcars6 cut with <i>Bst</i> EII/ <i>Sph</i> I, blunted and ligated	This study
pAtcarsRB	Ap^r ; deletion construct of pAtcars6 cut with <i>Pvu</i> II/ <i>Sph</i> I, blunted and ligated	This study
ptacGL	Km^r ; 1.4 kb <i>Sph</i> I– <i>Pvu</i> I fragment from pKK223.3 in <i>pGL10</i>	Tuffin <i>et al.</i> (2005)
pGEM–ChArsR	Ap^r ; PCR product of <i>arsR</i> (bp 1049–1544) made using ChArsRF/ChArsRR primers, cloned in <i>pGEM-T</i>	This study
pKKChArsR	Ap^r ; 460 bp <i>Eco</i> RI– <i>Hind</i> III fragment from pGEM–ChArsR cloned in pKK223.3	This study
ptacChArsR	Km^r ; <i>arsR</i> containing blunted 1.58 kb <i>Bam</i> HI– <i>Sca</i> I fragment from pKKChArsR cloned into a blunted <i>Sma</i> I site in <i>pGL10</i>	This study
pChArsRLacZ	Ap^r ; PCR product of <i>arsR</i> promoter (bp 1455–1955) made with ChArsRLacZF/ChArsRLacZR primers in pMC1403	This study
pChArsBLacZ	Ap^r ; PCR product of <i>arsB</i> promoter (bp 1383–1586) made with ChArsBLacZF/ChArsBLacZR primers in pMC1403	This study
pTnArs1GL	Km^r ; ‘transposon-like’ arsenic operon isolated from <i>At. caldus</i> #6, truncated in the <i>tnpA</i> gene, in the cloning vector <i>pGL10</i>	Tuffin <i>et al.</i> (2005)
pTnArsRLacZ	Ap^r ; <i>At. caldus</i> transposon PCR product of <i>arsR</i> promoter in pMC1403 (also named ArsRLacZ)	Tuffin <i>et al.</i> (2005)
pTn2	As^r Km^r ; transposon construct from a transconjugant isolated from the mating experiment performed in <i>E. coli</i>	Tuffin <i>et al.</i> (2005)
ptacTnArsR	Km^r ; <i>At. caldus</i> transposon <i>arsR</i> in <i>pGL10</i> (also named ptacArsR)	Tuffin <i>et al.</i> (2005)

DNA isolation and manipulations. Plasmid preparation, restriction endonuclease digestion, gel electrophoresis, ligation and Southern blot hybridization were performed using standard methods (Sambrook *et al.*, 1989). Total DNA was extracted from *At. caldus* strain #6, which had been isolated from the arsenopyrite bio-oxidation plant at the Fairview Mine, Barberton, South Africa. For the construction of the *At. caldus* strain #6 gene bank containing large inserts, chromosomal DNA was isolated as follows. *At. caldus* cells were harvested by centrifugation, washed three times in acidified water (pH 1.8), and resuspended in Tris/EDTA (TE) buffer, pH 7.6. Lysis was with 1 % SDS in the presence of proteinase K (1 mg ml⁻¹) at 37 °C. Proteins were precipitated by centrifugation in the presence of 2 M ammonium acetate before DNA was precipitated with ethanol, washed twice in 70 % ethanol, and resuspended in TE buffer (pH 7.6). This DNA was partially digested with *Sau*3A, the fragments separated using a sucrose gradient, and fragments in the 10–25 kb size range were ligated into the *Bgl*II site of the positive-selection cloning vector pEcoR252. Approximately 9600 colonies were obtained by transforming the ligation mixture into *E. coli* DH5 α , and selecting for growth on Luria agar (LA) plus ampicillin (100 μ g ml⁻¹). These colonies were scraped from an LA plate, and used to prepare the *At. caldus* gene bank. Sequencing was by the dideoxy chain-termination method, using an ABI PRISM 377 automated DNA sequencer, and the sequence was analysed using a variety of software programs, but mainly a combination of the Glimmer 2 (www.tigr.org/softlab; Delcher *et al.*, 1999) and DNAMAN (Lynnon BioSoft) programs. Comparison searches were performed using the gapped-BLAST program at the National Centre for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>; Altschul *et al.*, 1997). The nucleotide sequence for the insert of pAtcars4 was deposited in the GenBank database under the accession number DQ810790.

PCR. PCR was performed using the primers described in Table 2, with 50 ng plasmid DNA in a 50 μ l volume containing 2 mM MgCl₂, 0.25 μ M each primer, 200 μ M each dNTP, and 1 U *Taq*I polymerase. Reactions were carried out in a Hybaid Sprint thermocycler, with an initial denaturation at 94 °C for 60 s, followed by 25

cycles of denaturation (30 s at 94 °C), an annealing step of 30 s, and a variable elongation step at 72 °C. Annealing temperatures and elongation times were altered as required.

Arsenic resistance assays. To test for growth of *At. caldus* in the presence of arsenite, cells were cultured in tetrathionate medium containing 0, 20 and 30 mM arsenite. Actively growing cultures were diluted 100-fold into fresh medium, incubated for a 20 day period, and the cell density was determined by OD₆₀₀ measurement. Growth in the presence of arsenate was not tested, as the phosphate concentration in the growth medium contributes to apparent arsenate resistance (Silver *et al.*, 1981). Assays performed in *E. coli* ACSH501^q containing plasmids were carried out in LB medium containing appropriate antibiotics and various concentrations of sodium arsenite. Growth assays to determine the resistance to arsenate were performed in low-phosphate medium (Oden *et al.*, 1994) supplemented with 2 mM K₂HPO₄. Overnight cultures were diluted 100-fold into fresh medium, incubated at 37 °C for 5 h, and the OD₆₀₀ was determined. The incubation time used corresponded to the middle of the exponential growth phase of controls under the same conditions. In all cases, the resistance was expressed as the percentage OD₆₀₀ compared with that of the control culture with no added arsenic.

Construction of the promoter–*lacZ* fusions. The promoter regions for *arsR* and *arsB* were amplified by PCR using the primer pairs ChArsRLacZF/ChArsRLacZR for *arsR*, and ChArsBLacZF/ChArsBLacZR for *arsB* (Table 2). The PCR products were digested with *Bam*HI/*Eco*RI, and ligated to the promoterless *lacZ* reporter gene of pMC1403. Fusions were confirmed by DNA sequencing.

β -Galactosidase assays. Overnight cultures were diluted 1:200 into fresh medium containing the appropriate antibiotics, 0.4 mM IPTG, sodium arsenate or sodium arsenite (25 μ M), when indicated, and were incubated at 30 °C to OD₆₀₀ 0.5. The β -galactosidase activity was measured using the method of Miller (1972).

RNA analysis and RT-PCR. Total RNA was isolated, as described by Trindade *et al.* (2003), from 50 ml mid-exponential-phase cultures of *E. coli* ACSH501^q carrying various plasmids, grown in LB medium containing 0.1 mM arsenate, 0.1 mM arsenite or no

Table 2. Primers used in this study for cloning and reverse transcription

Restriction endonuclease sites incorporated into primers are shown in bold type.

Primer	Sequence
ChArsBLacZF (<i>Eco</i> RI)	5'-CGGAATTCTCGTGTCTCCACCAGTAGG-3'
ChArsBLacZR (<i>Bam</i> HI)	5'-CCGGATCCAAGATGGCCAGGGCAAGC-3'
ChArsRLacZF (<i>Eco</i> RI)	5'-CGGAATTCAAGTCGAGCCGCAACAGG-3'
ChArsRLacZR (<i>Bam</i> HI)	5'-CCGGATCCCGATCGCTGTGCTCTTC-3'
ChArsRF (<i>Eco</i> RI)	5'-CGAATTCGCCGTAAGTCTTGACGAG-3'
ChArsRR (<i>Hind</i> III)	5'-CCCAAGCTTCCAGGATGGAACGACAGG-3'
ArsR-RTFw	5'-CGAAGAGCACAGCGATCG-3'
ArsC-RTRev	5'-CCAGGATGGAACGACAGG-3'
ORF1-RTRev	5'-GATCGCGCAGCCAGAGTT-3'
ArsC-RTFw	5'-CGGAAATCGAGGCCGCTT-3'
ORF5-RTRev	5'-GTTTGGCAGGGATTGCGG-3'
ArsB-RTFw	5'-ACCGTTCTGGCGGCGATT-3'
ORF6-RTRev	5'-ATCTTCTGGGTGCTGCC-3'
ORT5-RTFw	5'-CGGGGAAGATCCTAGGTG-3'

For RT-PCR, the First Strand cDNA Synthesis kit (AMV; Roche) was used for cDNA synthesis and product detection. The protocol of the manufacturer was used for the reverse transcriptase reaction. The PCR was performed as described above, using 2 of the 20 μ l (total volume) of the reverse transcriptase reaction, and the extension times were altered as required for the different primer pairs. Primer combinations used for cDNA synthesis are shown in Fig. 1. To detect DNA contamination in the mRNA extracts, reactions were performed with each primer pair without any AMV reverse transcriptase.

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The partial *Sau3A* gene bank of *At. caldus* strain #6 containing 8–12 kb inserts was screened for arsenite and arsenate resistance in the *E. coli ars* deletion strain ACSH501^q. Plasmids were isolated from colonies that were grown in the presence of 0.5 mM arsenite and 1.0 mM arsenate. These plasmids were retransformed into *E. coli* ACSH501^q to confirm their ability to confer arsenic resistance. Using colony hybridization, a number of clones were selected that tested positive for the *arsBC* genes (*At. ferrooxidans arsB* and *arsC* probes), and negative for the *arsDA* genes (Tn*AtcArs arsDA* probe). Plasmids were prepared from these clones, and based on restriction endonuclease digestion patterns, it was confirmed that they differed from the 12 kb arsenic resistance transposon Tn*AtcArs* (Tuffin *et al.*, 2004, 2005) previously isolated from *At. caldus*. Several of these plasmids were mapped in detail, and two, p*Atcars4* and p*Atcars6* containing 9.7 and 8.6 kb inserts, respectively, were chosen for further study (Fig. 1). Confirmation that the insert originated from *At. caldus* was obtained by Southern hybridization. An internal 1.3 kb partial *NcoI* fragment (Fig. 1) was labelled and used to probe the chromosomal DNA of *At. caldus* strain #6 digested with *StuI*, *BamHI*, *NcoI* and *Sall*. Hybridization signals for 0.4 and 0.7 kb *BamHI* fragments, a 4.7 kb *StuI* fragment, 0.4 and 0.85 kb *NcoI* fragments, and a 9.3 kb *Sall* fragment were obtained for both the chromosomal DNA of *At. caldus* strain #6 and plasmid insert (data not shown). This indicates that the insert DNA originated from *At. caldus* strain #6, that it was present in a single copy, and that no rearrangements in

the region over which restriction endonuclease sites extended had occurred during cloning.

Sequence analysis of the *At. caldus* chromosomal *ars* genes

The entire insert of pAtcars4 was sequenced in both directions, and 10 ORFs were identified (Fig. 1). Three genes were found whose products were clearly related to previously identified *arsC*, *arsR* and *arsB* gene products. Unlike most *ars* operons, in which the *arsR* and *arsC* genes are transcribed together with the *arsB* gene, the *At. caldus* chromosomal *arsRC* and *arsB* genes were divergently transcribed in a manner previously found for only one other bacterium, *At. ferrooxidans* (Butcher *et al.*, 2000). Alignments with other proteins in the NCBI database revealed that *At. caldus* chromosomal *ArsB*, *ArsC* and *ArsR* were most closely related to those of the *At. ferrooxidans* *ars* operon (85, 78 and 74% amino acid sequence identity, respectively). The structure of the *At. caldus* chromosomal *ars* operon differed substantially from that of the TnAtcArs transposon isolated from the same bacterium, which contained *ars* genes *R*, *C*, *D1*, *A1*, *D2*, *A2*, ORF7, ORF8 and *B*, all transcribed in the same direction. The amino acid sequences of *At. caldus* *ArsB*, *ArsC* and *ArsR* chromosomal gene products and the equivalent proteins from the TnAtcArs were substantially different, with 60, 72 and 45% amino acid sequence identity, respectively. In addition to the *ars* genes, seven other ORFs were identified using the Glimmer 2 program. The predicted product of ORF1 had high amino acid sequence identity (76%) to a hypothetical protein from *Polaromonas naphthalenivorus*, and was transcribed in the same direction as *arsRC*, with only 10 bp between the stop codon of *ArsC* and the predicted start codon of ORF1. Likewise, ORFs 5 and 6 were transcribed in the same direction as *arsB*, with 30 bp between the stop codon of *ArsB* and the predicted start codon of ORF5, and 35 bp between ORFs 5 and 6. Although the predicted ORF5 product was most closely related to a hypothetical protein from *Legionella pneumophila*, the two proteins were of very different sizes (130 and 402 aa, respectively), and the region of high identity (60%) was only 41 aa in size. The predicted amino acid sequence of ORF6 was 43% identical to that of a hypothetical protein of *Alkalilimnicola ehrlichei* of equivalent size. ORFs 7–10 appeared to form an operon consisting of a transcriptional regulator and three other genes (Fig. 1), but as deletion of this region did not have an effect on arsenic resistance, they were not studied further.

At. caldus *arsCRB* confers arsenic resistance to *E. coli* that is not enhanced by co-transcribed ORFs 1 and 5

Because of the location and direction of transcription of ORFs 1, 5 and 6, we tested whether they contributed to arsenic resistance in *E. coli*. Constructs pAtcars4, pAtcars6, pAtcarsCRB5, pAtcarsCRB, pAtcarsRB, pAtcarsRB5 and pEcoBlunt (vector control) were transformed into the *E. coli*

arsenic mutant ACSH50I^q, and transformants were tested for their ability to grow in LB medium plus 0.25 mM arsenite and 0.5 mM arsenate. The chromosomal *ars* operon of *At. caldus* (pAtcars4) conferred marked resistance to the *E. coli* *ars* mutant ACSH50I^q in both arsenate and arsenite compared to the negative control pEcoBlunt (Fig. 2A, B). Constructs pAtcars6, pAtcarsRB and pAtcarsRB5 conferred lower resistance to arsenate than did constructs containing *arsC*, as a functional *arsC* is required for the conversion of arsenate to arsenite. There were no consistent differences in arsenic resistance in cells harbouring only *arsCRB* and ORF5 (pAtcarsCRB5) or *arsCRB* (pAtcarsCRB) compared with pAtcars4, suggesting that neither ORF1 nor ORFs 6–10 are required for arsenic resistance in *E. coli* ACSH50I^q.

In spite of an inability to detect an alteration in resistance to arsenite or arsenate by ORFs 1, 5 or 6 in *E. coli*, we carried out RT-PCR experiments to determine whether co-transcription occurred between ORF1 and *arsC*, or ORF5 (and ORF6) and *arsB*. RNA was isolated from *E. coli* ACSH50I^q, transformed with pAtcars4, grown in the

Fig. 2. Growth of the *E. coli* *ars* mutant ACSH50I^q containing subclones of the *At. caldus* chromosomal arsenic operon, in the presence of 0.25 mM arsenite (a) and 0.5 mM arsenate (b). ■, pAtcars4; ●, pAtcars6; ▲, pAtcarsCRB5; △, pAtcarsCRB; ○, pAtcarsRB5; ◆, pAtcarsRB; □, pEcoBlunt. Each data point represents the results of three assays of three independent experiments. Error bars indicate SD.

presence or absence of arsenite and arsenate. When using the ORF1-RT_Rev/ArsC-RT_Fw primer combination (Fig. 1), a 522 bp fragment was obtained from the RNA samples extracted from *E. coli* ACSH50I^q grown in the presence or absence of arsenite and arsenate (data not shown). This indicates that ORF1 was co-transcribed with *arsC*. When using the ArsC-RT_Rev/ArsR-RT_Fw primer combination, a 423 bp fragment was obtained, indicating that *arsC* was co-transcribed with *arsR* (data not shown). A 451 bp product was obtained when primers ArsB-RT_Fw/ORF5-RT_Rev were used, indicating that *arsB* and ORF5 were co-transcribed (data not shown). However, no 473 bp product was obtained using primers ORF5-RT_Fw/ORF6-RT_Rev, indicating that co-transcription of ORF5 and ORF6 did not occur (data not shown). No products were obtained in the absence of AMV reverse transcriptase, indicating that the mRNA was DNA free. This suggests that ORF1 and ORF5 were co-transcribed with *arsRC* and *arsB*, respectively, even though no changes in resistance to arsenate or arsenite could be detected in *E. coli* when ORF1 or ORF5 were deleted. Similarly, RNA was extracted from *At. caldus* strain #6 grown in the presence or absence of arsenite, and ORF1–*arsRC* and ORF5–*arsB* co-transcription was confirmed. The products of ORF1 and ORF5 may play a role in arsenic resistance in the natural host, but in the absence of an effective genetic system for *At. caldus*, this has not been tested.

Regulation of divergent *arsR* and *arsB* of *At. caldus* and cross-regulation by *TnAtcArs*

Using the promoterless *lacZ* gene on pMC1403, we constructed in-frame translational *arsR*–*lacZ* (pChArsRLacZ) and *arsB*–*lacZ* (pChArsBLacZ) fusions to serve as reporter constructs for measuring the transcription of the bi-directional *At. caldus* chromosomal *ars* operon. When transformed into the *E. coli* *ars* mutant ACSH50I^q, pChArsRLacZ and pChArsBLacZ gave β -galactosidase activity averaging 12 and 48 miller units, respectively (Fig. 3A, B). This suggests that transcription of the cloned genes from the promoter region was stronger in the direction of *arsB* than that of *arsRC*. The effect of the *arsR* gene product on the expression of the *arsR*–*lacZ* and *arsB*–*lacZ* fusions was determined by cloning the *arsR* gene behind a *tac* promoter in the compatible vector ptacGL (*tac* promoter of pKK223.3 cloned into pGL10). This construct (ptacChArsR) was placed *in trans* with fusions pChArsRLacZ and pChArsBLacZ in the *E. coli* *ars* mutant ACSH50I^q. To determine the level of arsenic required for induction of the two reporter gene constructs, β -galactosidase assays were performed without arsenic, or with 5, 25 or 100 μ M arsenate or arsenite added (data not shown). Addition of 25 μ M of either arsenite or arsenate was generally sufficient for full induction, while at 100 μ M, cell growth was negatively affected, probably due to the absence of a functional *ars* operon. No significant difference in induction between arsenite and arsenate was observed. In low-phosphate medium, arsenate was able to induce the *ars*

Fig. 3. Expression of β -galactosidase under the control of (a) the chromosomal *arsB* promoter (pChArsBLacZ), (b) the chromosomal *arsR* promoter (pChArsRLacZ) and (c) the transposon *arsR* promoter (pTnArsRLacZ) in *E. coli* ACSH50I^q, in the presence and absence of 25 μ M arsenite and arsenate. The following plasmids were added *in trans*: ptacGL control (black bars), ptacChArsR (stippled bars) and ptacTnArsR (grey bars). All assays were carried out in triplicate and error bars represent the SD of three experiments.

operon more efficiently at lower concentrations (5 μ M) than it did in LB medium (data not shown). It is known that the transport of arsenate into bacterial cells is via some of the phosphate transport systems (Sanders *et al.*, 1997), and in low-phosphate medium, arsenate is likely to be taken up more effectively, due to reduced competition by phosphate.

When the *arsB-lacZ* fusion (pChArsBLacZ) was expressed with *arsR* (ptacChArsR) *in trans*, in the presence of 0.4 mM IPTG and in the absence of arsenite or arsenate, expression was reduced to $\sim 10\%$ of that of the fully induced operon. In contrast, the *arsR-lacZ* fusion was regulated less effectively by ArsR, with expression being reduced to $\sim 50\%$ of fully induced levels in the absence of arsenic (Fig. 3A, B).

As described earlier, the amino acid sequences of the *At. caldus* chromosomal ArsR and the TnAtcArs ArsR shared only 45% sequence identity, and highly arsenic-resistant strains of *At. caldus* have both arsenic resistance systems. We determined how efficiently the heterologous TnAtcArs ArsR was able to regulate the *arsR-lacZ* (pChArsRLacZ) and *arsB-lacZ* (pChArsBLacZ) fusions in *E. coli*. The more strongly expressed *arsB-lacZ* fusion was repressed approximately 8–12-fold by its own ArsR in the absence of arsenite or arsenate, whereas it was not repressed in the presence of the heterologous ArsR (Fig. 3A). The more weakly expressed *arsR-lacZ* fusion was repressed approximately twofold by its own ArsR, but not by the heterologous ArsR (Fig. 3B).

TnAtcArs is an operon transcribed in one direction from *arsR* (Tuffin *et al.*, 2005), and a TnAtcArs *arsR-lacZ* fusion was available (pTnArsRLacZ) from that study, which enabled us to carry out the reverse experiment. The transposon *arsR-lacZ* fusion was expressed at a very much higher level (> 1200 units) than either of the chromosomal *ars* promoters (Fig. 3C), and was more effectively repressed by its own ArsR regulator (fourfold) than by the heterologous chromosomal ArsR (twofold).

Combined chromosomal *ars* and TnAtcArs arsenic resistance in *E. coli* and *At. caldus*

Highly resistant *At. caldus* strains have both chromosomal and transposon-located (TnAtcArs) *ars* genes in the same cell; therefore, we tested which system conferred the greater arsenic resistance, and whether placement of both *ars* systems in *E. coli* resulted in increased resistance to arsenic. The TnAtcArs genes were cloned into the low-copy-number vector pGL10 to produce pTnArs1GL, which is compatible with pEcoR252 used to construct pAtcarsCRB5. pTnArs1GL and pAtcarsCRB5 were transformed into the *E. coli* *ars* mutant ACSH50I^q, individually and together, and growth was assayed in the presence or absence of 1.5 mM arsenate, with antibiotic selection for plasmids (data not shown). In the absence of arsenate, growth of cells containing individual plasmids or the combination of plasmids was approximately the same, suggesting that the presence and

selection of two types of plasmid in one cell did not inhibit cell growth. Growth in the presence of arsenate indicated that *E. coli* containing the transposon *ars* genes (pTnArs1GL) was more resistant than the strain containing the chromosomal *ars* genes (pAtcarsCRB5), in spite of the transposon genes being on a lower copy number vector. Arsenate resistance in *E. coli* cells containing both *ars* systems was intermediate between that in cells containing the two individual *ars* systems, although the small reduction in resistance was within the error associated with the assay.

To test what change in arsenic resistance occurred when *At. caldus* gained a transposon-located *ars* system, we developed a conjugation system for *At. caldus*, as no genetic system for this bacterium has been reported. During experiments to demonstrate that TnAtcArs is transpositionally active in *E. coli*, a broad-host-range plasmid pSa, into which TnAtcArs had been transposed (pTn2), and that can be conjugated between strains of *E. coli*, has been isolated (Tuffin *et al.*, 2005). *E. coli* HB101 cells containing either pTn2 or pSa were mated with *At. caldus* C-SH12 cells lacking the TnAtcArs system. After mating, *At. caldus* cells capable of growth in sodium thiosulphate liquid medium (without yeast extract) plus 100 μ g kanamycin ml⁻¹ were isolated from matings with *E. coli* containing either pTn2 or pSa. After subculturing in fresh sodium thiosulphate medium, *At. caldus* total DNA was extracted, and the TnAtcArs *arsA* gene was PCR-amplified using transposon *arsA* primers (RNAarsAF/RNAarsAR; Tuffin *et al.*, 2005). The predicted 600 bp product was observed when using *At. caldus* C-SH12 (pTn2) total DNA as a template, but not with *At. caldus* C-SH12 (pSa) total DNA (data not shown). To confirm that the transconjugants were *At. caldus* and not some other thiosulphate-oxidizing bacterium, the 16S rRNA genes were amplified and gave the predicted diagnostic bands when digested with *Bam*HI and *Stu*I (Rawlings *et al.*, 1999). To further confirm the presence of TnAtcArs in *At. caldus* C-SH12, Southern hybridization experiments were carried out using total DNA isolated from *At. caldus* strains C-SH12, C-SH12 (pTn2) and C-SH12 (pSa) (Fig. 4). When the *arsD* gene of TnAtcArs was used as a probe, only DNA from C-SH12 (pTn2) gave a positive hybridization signal, whereas, with pSa as a probe, DNA from both C-SH12 (pTn2) and C-SH12 (pSa) gave a positive signal.

We tested whether the newly constructed *At. caldus* C-SH12 (pTn2) exhibited higher resistance to arsenic than did strain C-SH12 (pSa). *At. caldus* C-SH12 (pTn2) and C-SH12 (pSa) were grown in thiosulphate medium containing 0, 20, 30, 40 and 50 mM arsenite. After 8 days, *At. caldus* C-SH12 (pTn2) cells containing the transposon arsenic resistance genes grew in 30 mM arsenite, while the *At. caldus* C-SH12 (pSa) cells harbouring only the chromosomal *ars* genes grew weakly in 20 mM arsenite but not in higher concentrations (data not shown). After 19 days, *At. caldus* C-SH12 (pSa) grew in 20 mM arsenite but poorly, or not at all, in ≥ 30 mM arsenite (Fig. 5), while *At. caldus* C-SH12 (pTn2) grew at all concentrations tested. These results indicate that

Fig. 4. Southern hybridization of total DNA from *At. caldus* strains C-SH12, C-SH12-pTn2, pTn2 (positive control), C-SH12-pSa, and conjugative plasmid pSa digested with *KspI* and probed with (a) transposon *arsD* and (b) pSa. The additional 5 kb fragment represents a *KspI* fragment stretching from a *KspI* site in TnAtcArs to a *KspI* site in pSa.

the addition of the transposon *ars* operon of *At. caldus* strain #6 to *At. caldus* C-SH12 dramatically increased its resistance to arsenite.

DISCUSSION

All six strains of *At. caldus* examined, including strains isolated from the UK, South Africa and Australia, have a set of arsenic resistance genes similar or identical to those described in this manuscript (de Groot *et al.*, 2003; Tuffin *et al.*, 2005). These *ars* genes and their predicted proteins were most similar to those of *At. ferrooxidans*, a mesophilic iron- and sulphur-oxidizing bacterium. The gene order and number of *ars* genes varies between *ars* operons, with two of the most commonly encountered sets of *ars* genes being the *arsRBC* genes, such as those found on the chromosome of *E. coli* (Carlin *et al.*, 1995), and the *arsRDABC* genes, such as those found on plasmid R773 (Chen *et al.*, 1986). Furthermore, transcription is usually unidirectional. The *ars* operons of both of these bacteria are unusual in that they are bi-directional, with the *arsB* gene (arsenite efflux pump) transcribed in the opposite direction to that of the *arsR* (regulator) and *arsC* (arsenate reductase) genes. This raises the possibility that transcription in the two directions might be regulated differently. Reporter gene studies in *E. coli* indicated that the level of transcription in the direction of the *At. caldus arsB* was four- to fivefold higher and subject to greater repression by ArsR than that in the direction of *arsRC*. However, there was no detectable difference in the ability of arsenite or arsenate to derepress the transcription of the *ars* genes in either direction, a finding that is similar to that of ArsR from *At. ferrooxidans* (Butcher & Rawlings, 2002). The amino acid sequences of the two ArsR repressors are 74% identical, and both belong to a family of ArsR regulators that lack a consensus arsenite binding sequence (ELCVCDL) found in the more extensively studied ArsR family (Shi *et al.*, 1994).

In spite of having bi-directional transcription and related *arsRC* and *arsB* genes, the *At. caldus* and *At. ferrooxidans ars* operons have some substantial differences. Unlike the *At. ferrooxidans ars* operon, no *arsH* was located downstream of the *At. caldus arsB*, and the flanking ORFs were different. Although ORF1 and ORF5 of the *At. caldus* operon were found to be transcribed together with the *ars* genes in both *E. coli* and *At. caldus*, no difference in arsenic resistance in *E. coli* was found when these genes were deleted. A similar inability to detect a difference is found when the *arsH* of *At. ferrooxidans* is deleted (Butcher *et al.*, 2000). Recently, an increase in sensitivity to low levels of arsenite and, to a lesser extent, arsenate has been reported when the *arsH* of

Fig. 5. Growth of *At. caldus* C-SH12-pTn2 (▲), and C-SH12-pSa (■) after 19 days in the presence of various concentrations of arsenite. Cell densities were determined (OD₆₀₀) and represented as a percentage of growth in the absence of arsenite. Each data point represents duplicate results of at least two experiments. Error bars indicate SD.

Sinorhizobium meliloti is deleted (Yang *et al.*, 2005). Co-transcription of ORF1 and ORF5 indicates that they are associated with arsenic resistance, although their roles remain uncertain.

Strains of *At. caldus* from bacterial consortia that have been adapted to grow in the presence of high concentrations of arsenic have at least one additional set of arsenic resistance genes located on Tn*AtcArs* (de Groot *et al.*, 2003; Tuffin *et al.*, 2005). The chromosomal and transposon *ars* operons are quite different, and although the ArsR regulators belong to the same family, they share only 45 % amino acid sequence identity. We investigated how the two systems were likely to interact when both were present in the same bacterium. Promoter fusion studies in *E. coli* indicated that each ArsR was a more effective repressor of the promoter of its own operon (both directions in the case of the chromosomal *ars*) than was the heterologous ArsR; however, although the chromosomal ArsR was able to partly regulate the transposon *ars* expression, the transposon ArsR was unable to regulate the chromosomal *ars* operon. The very much higher levels of reporter gene expression from the transposon *ars* operon (Fig. 3), and much less effective repression, might explain the higher levels of resistance conferred by the transposon *ars* operon in *E. coli*.

The conjugation of pSa from *E. coli* to *At. caldus* was attempted with *At. caldus* strains BC13 and KU (data not shown), in addition to *At. caldus* C-SH12. For some, as-yet-unknown reason, only the latter conjugation was successful. Furthermore, *At. caldus* C-SH12 formed indistinct colonies on solid selection medium so that it was not possible to calculate a conjugation frequency. No conjugation or any other genetic system has been established for *At. caldus*; therefore, experiments are currently in progress using this limited, but repeatable, successful plasmid transfer to optimize a conjugation system for *At. caldus*, using a range of conjugative plasmids.

Nevertheless, of more importance for our study was the demonstration that the transfer of Tn*AtcArs* to *At. caldus* C-SH12, which previously contained only the chromosomal *ars* genes, resulted in a marked increase in resistance to arsenite. The increase in resistance was possibly even greater than shown, but arsenite precipitation occurred during extended aeration in the medium, at concentrations above 50 mM. No *At. caldus* strain lacking the chromosomal *ars* genes has been found, and we could therefore not determine the relative contributions of the two *ars* systems to arsenic resistance. However, the experiment clearly demonstrates the advantage to be gained by acquisition of Tn*AtcArs* when *At. caldus* is required to grow in high concentrations of arsenic.

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