The divergent chromosomal *ars* operon of *Acidithiobacillus ferrooxidans* is regulated by an atypical ArsR protein

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The chromosomal arsenic-resistance (*ars*) operon of *Acidithiobacillus ferrooxidans* is atypical in that it is divergent, with its *arsCR* and *arsBH* genes transcribed in opposite directions. Furthermore, the amino-acid sequence of the putative ArsR-like regulator of the *ars* operon is not conserved in regions that have been shown to be responsible for binding to arsenic. Instead, the ArsR-like protein of *At. ferrooxidans* is related to a group of unstudied ArsR-like proteins that have been found to be associated with chromosomal *ars*-like operons identified during genome-sequencing projects. Using *arsB-lacZ*, *arsR-lacZ* and *arsC-lacZ* fusions, it was shown that the ArsR-like protein of *At. ferrooxidans* is a repressor of the *arsBH* and *arsRC* genes of this organism, and that induction of gene expression took place when either AsIII (arsenite) or AsV (arsenate) were added. Deletion of 19 aa from the C terminus of the 118 aa ArsR protein did not affect the regulation of its activity, but deletion of an additional 28 aa inactivated ArsR. Northern-blot hybridization suggested that on induction of expression, the *arsRC* genes were transcribed in greater quantities than the *arsBH* genes, but that the level of induction was not affected by the form of arsenic added (AsIII or AsV).

Keywords: arsenic resistance, ArsR-like proteins, gene regulation

INTRODUCTION

Acidithiobacillus ferrooxidans has been shown to contain genes for arsenic resistance on its chromosome (Butcher *et al.*, 2000). Arsenic-resistance genes have been found on both the chromosomes and plasmids of various bacteria, and arsenic resistance has been reviewed extensively (Rosen, 1999; Xu *et al.*, 1998; Tsai *et al.*, 1997; Silver & Phung, 1996; Cervantes *et al.*, 1994). These arsenic-resistance genes are usually arranged in an operon, the two most common forms of which contain either three (*arsRBC*) or five (*arsRDABC*) genes. The ArsR and ArsD proteins are *trans*-acting regulators. ArsR is a repressor that controls the basal level of expression in response to the presence of arsenite, whereas ArsD controls the upper level of expression of the operon. ArsA is an ATPase that forms a complex with the ArsB transmembrane protein to pump arsenite out of the cell. Another gene, *arsH*, has been found in *Yersinia enterocolitica* (Neyt *et al.*, 1997); the function

of this gene is unknown, but it was shown to be required for resistance to arsenite and arsenate in *Y. enterocolitica*.

The arsenic-resistance (*ars*) operon from *At. ferrooxidans* was found to consist of two divergent elements, *arsRC* and *arsBH*. The genes within these elements were shown to confer arsenic resistance upon an *Escherichia coli ars* mutant, AW3110 (Butcher *et al.*, 2000). However, *arsH* was not required for arsenic resistance in *E. coli* and it is not yet known whether it is required in *At. ferrooxidans*. The unusual divergent arrangement of the *ars* operon of *At. ferrooxidans* has raised questions about the regulation of its genes. The finding that the *arsB* and *arsC* genes are located on divergent elements may mean that they are independently regulated. The putative ArsR protein from *At. ferrooxidans* only had weak homology to ArsR proteins from well-studied arsenic-resistance operons and did not contain the conserved metal-binding box, ELCVCDL, to which the arsenite inducer binds (Shi *et al.*, 1994, 1996). Therefore, it was unclear whether the putative ArsR protein coded for within the *At. ferrooxidans* operon was able to regulate these genes in response to arsenite.

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Table 1. Bacterial strains, plasmids, primers and probes used in this study

Strain/plasmid/ primer/probe	Description	Reference/source*
Strain		
<i>E. coli</i> W3110	K-12 F ⁻ IN(<i>rrnD</i> – <i>rrnE</i>)	B. Rosen
<i>E. coli</i> AW3110	W3110 Δ <i>ars</i> :: <i>cam</i>	Carlin <i>et al.</i> (1995)
<i>E. coli</i> CSH50I ^a	<i>rspL</i> Δ (<i>lac</i> – <i>pro</i>) (F' <i>traD36 proAB lacI</i> ^a Δ M15)	Smith & Rawlings (1998)
<i>E. coli</i> ACSH50I ^a	CSH50I ^a Δ <i>ars</i> :: <i>cam</i>	This work
<i>At. ferrooxidans</i> ATCC 33020	Wild-type	ATCC
Plasmid		
pBluescript(SK)	Amp ^R ; <i>lacZ'</i> ColE1 replicon	Stratagene
pMC1403	Amp ^R ; promoterless <i>lacZYA</i> operon ColE1 replicon	Casadaban <i>et al.</i> (1983)
pKK223-3	Amp ^R ; P _{<i>tac</i>} ColE1 replicon	Pharmacia Biotech
pGL10	Kan ^R ; RK2/RP4 replicon	A. Toukdarian
pACYC184	Tet ^R Cm ^R ; p15A replicon	Chang <i>et al.</i> (1978)
pTfarsCRBH-ACYC	Tet ^R ; 5.3 kb <i>HindIII</i> – <i>StuI</i> fragment containing <i>ars</i> genes cloned into pBluescript cut with <i>EcoRV</i> – <i>HindIII</i> , which was then cut with <i>EcoRI</i> and cloned into pACYC cut with <i>EcoRI</i>	This study†
pTfarsCRB-ACYC	Tet ^R ; <i>EcoRV</i> – <i>XbaI</i> fragment of pTfars1b, containing the 5' end of <i>arsB</i> and the whole of <i>arsH</i> , was replaced with the <i>EcoRV</i> – <i>XbaI</i> fragment of pBB08, containing only the 5' end of <i>arsH</i> , which was then cloned into pACYC	This study†
pTfarsBH-ACYC	Tet ^R ; <i>KpnI</i> deletion of pTfarsCBH, cut with <i>KpnI</i> , blunted and cut with <i>EcoRI</i> , which was then cloned into pACYC cut with <i>ScaI</i> and <i>EcoRI</i>	This study†
pTfarsB-ACYC	Tet ^R ; <i>PstI</i> deletion of pTfarsBH, which was then cut with <i>KpnI</i> , blunted, cut with <i>BamHI</i> and cloned into pACYC cut with <i>ScaI</i> and <i>BclI</i>	This study†
pTfarsC-ACYC	Tet ^R ; PCR product containing <i>arsC</i> cloned into pBluescript, which was then cut with <i>HindIII</i> , blunted, cut with <i>EcoRI</i> and the fragment containing <i>arsC</i> cloned into pACYC cut with <i>ScaI</i> and <i>EcoRI</i>	This study†
pTacArsR-ACYC	Tet ^R ; PCR product from <i>arsR</i> – <i>eco</i> and <i>arsR</i> – <i>hind</i> primers cloned into pKK223-3 cut with <i>EcoRI</i> and <i>HindIII</i> ; P _{<i>tac</i>} – <i>arsR</i> was then excised with <i>BamHI</i> and <i>ScaI</i> and cloned into pACYC cut with <i>BclI</i> and <i>ScaI</i>	This study
pTacArsH-ACYC	Tet ^R ; PCR product from ARSHF and ARSHR primers cut with <i>EcoRI</i> and <i>HindIII</i> cloned into pKK223-3 cut with the same restriction enzymes; P _{<i>tac</i>} – <i>arsH</i> was then excised with <i>BamHI</i> and <i>ScaI</i> and cloned into pACYC cut with <i>BclI</i> and <i>ScaI</i>	This study
pBlacZ	Amp ^R ; <i>arsB</i> – <i>lacZ</i> fusion, 99 aa of <i>ArsR</i> (see Fig. 1 and text)	This study
pB2lacZ	Amp ^R ; <i>arsB</i> – <i>lacZ</i> fusion, complete <i>ArsR</i> <i>in cis</i> (see Fig. 1 and text)	This study
pB2lacZ-GL10	Kan ^R ; as above but in low-copy-number vector pGL10	This study
pB3lacZ	Amp ^R ; <i>arsB</i> – <i>lacZ</i> fusion, 71 aa of <i>ArsR</i> (see Fig. 1 and text)	This study
pB3lacZ-GL10	Kan ^R ; as above but in low-copy-number vector pGL10	This study
pRlacZ	Amp ^R ; <i>arsR</i> – <i>lacZ</i> fusion (see Fig. 1 and text)	This study
pRlacC-GL10	Kan ^R ; as above but in low-copy-number vector pGL10	This study
pClacZ	Amp ^R ; <i>arsC</i> – <i>lacZ</i> fusion, <i>arsR</i> <i>in cis</i> but no <i>arsR</i> promoter	This study
pClacZ-GL10	Kan ^R ; as above but in low-copy-number vector pGL10	This study
pC2lacZ	Amp ^R ; <i>arsC</i> – <i>lacZ</i> fusion, <i>arsR</i> <i>in cis</i> with <i>arsR</i> promoter	This study
pC2mlacZ-GL10	Kan ^R ; as above but in low-copy-number vector pGL10	This study
Primers		
BBARSB	(<i>Bam</i> HI) 5'-GCGGATCCAGGGTGACGAGAAATATGGC-3'	Butcher <i>et al.</i> (2000)
BBARSC	(<i>Bam</i> HI) 5'-GCGGATCCGGGGTTTTCATCACTGG-3'	Butcher <i>et al.</i> (2000)
RLACZE	(<i>Eco</i> RI) 5'-GGGAATTCCTTCGCGCTGCACGGTGA-3'	This study
RLACZB	(<i>Bam</i> HI) 5'-CGGGATCCATCGTATTATCCACG-3'	This study
BLACZE	(<i>Eco</i> RI) 5'-CGGAATTCACGACAATGTGCCA-3'	This study
arsR-hind	(<i>Hind</i> III) 5'-ACGAAGCTTGGGGTTTTCATCACTGG-3'	This study

Table 1 (continued).

Strain/plasmid/ primer/probe	Description	Reference/source*
arsR-eco	(<i>EcoRI</i>) 5'-GCGAATTCATGGAACCACTACAAGACCC-3'	This study
ARSHF	(<i>EcoRI</i>) 5'-GCGAATTCTGGTGGCTGCCGCTGGCTTG-3'	Butcher <i>et al.</i> (2000)
ARSHR	(<i>HindIII</i>) 5'-GAAAGCTTGCGTACCCCCAACCTCATGCC-3'	Butcher <i>et al.</i> (2000)
Probes for Northern blots		
<i>ars</i>	1.9 kb <i>PstI</i> fragment from pTfarsCRB	This study
<i>arsB</i>	0.85 kb <i>NcoI</i> fragment from pTfarsCRBH	This study
<i>arsR</i>	PCR product using ars-eco and ars-hind primers	This study
<i>arsH</i>	PCR product using the ARSHF and ARSHR primers	This study

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† The first cloning step was carried out by Butcher *et al.* (2000).

Here we describe an investigation into the regulation of the chromosomal *ars* operon of *At. ferrooxidans* and into whether the ArsR-like protein of this organism is a regulator of the *ars* operon in response to arsenite, arsenate and antimonite.

METHODS

Bacterial strains, plasmids, primers and media. The strains, plasmids and primers used in this study are shown in Table 1. *E. coli* strains were grown on Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) at 30 °C. *At. ferrooxidans* strains were grown in tetrathionate medium (Powles *et al.*, 1995) at 30 °C. Ampicillin (100 µg ml⁻¹), chloramphenicol (20 µg ml⁻¹), tetracycline (20 µg ml⁻¹) and kanamycin (30 µg ml⁻¹) were added to the media when required.

DNA techniques and analyses. Plasmid preparations, restriction endonuclease digestions, gel electrophoresis and cloning techniques were carried out using standard protocols (Sambrook *et al.*, 1989). Searches for sequences related to ArsR were performed using the gapped-BLAST program of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/BLAST/>) (Altschul *et al.*, 1997). The PC-based DNAMAN software (version 4.1; Lynnon BioSoft) was used to create the sequence alignments and homology trees.

Construction of the arsenic-sensitive *lac*-negative *E. coli* strain. The generalized transduction method using phage P1_{vir} (Miller, 1972) was used to construct this strain. Phage P1_{vir} was isolated from *E. coli* AW3110 (Carlin *et al.*, 1995) and used to transduce *E. coli* CSH501^a. The transduced cells were selected on chloramphenicol and X-Gal plates. They were then checked for sensitivity to arsenic on plates containing 0.5 mM sodium arsenite. Sensitivity indicated that the $\Delta ars::cam$ from *E. coli* AW3110 had replaced the *ars* genes from *E. coli* CSH501^a, resulting in strain *E. coli* ACSH501^a.

Construction of the promoter-reporter gene (*lacZ*) constructs. The putative promoter regions were amplified by PCR using primer pairs BBARSB/BLACZE (*arsB* promoter) and RLACZB/RLACZE (*arsR* promoter) (Table 1). The resulting PCR products were digested with *Bam*HI and *Eco*RI (the restriction sites of these enzymes were included in the primers; Table 1) and ligated to the promoterless *lacZ* gene of pMC1403, which had been digested with the same

enzymes; this resulted in pB3lacZ and pRlacZ, respectively, which were translational fusions of *arsB* and *arsR* with the *lacZ* gene (Fig. 1). Primers BBARSB/BBARSC were used to amplify a region containing the *arsB* promoter and the intact *arsR* gene (Fig. 2). This PCR product was digested with *Bam*HI and cloned into pMC1403, which had been digested with *Bam*HI. The product could be cloned in either direction. Cloning the insert in one direction resulted in pB2lacZ, in which the *arsB* promoter was fused to *lacZ* of pMC1403 and the intact *arsR* gene was transcribed in the opposite direction (Fig. 1). Cloning the insert in the other direction resulted in *lacZ* being fused in-frame at the start of *arsC*, resulting in pC2lacZ (Fig. 1). In both constructs, the *arsR* promoter and the intact *arsR* gene were also included. The above PCR product was also digested with *Bam*HI and *Sma*I and cloned into pMC1403, which had been digested with the same enzymes. The resulting construct, pBlacZ, contained a translational fusion of *arsB* with *lacZ* and a partly truncated *arsR* gene *in cis* (Fig. 1).

The translational fusions from pB3lacZ, pRlacZ, pB2lacZ and pC2lacZ were cloned into the low-copy-number, RK2-replicon-based vector pGL10. The fusions were obtained by digesting the construct with *Sal*I, blunting this site with T4 DNA polymerase (Roche Molecular Biochemicals) and then digesting with *Eco*RI. The fragment containing the fusion was then cloned into pGL10, which had been digested with *Sma*I and *Eco*RI. The constructs pB3lacZ-GL10, pRlacZ-GL10, pB2lacZ-GL10 and pC2lacZ-GL10 were constructed in this way. The *ars* genes were also cloned into the relatively low-copy-number, ColE1-replicon-compatible vector pACYC184, to enable expression *in trans* with the promoter fusion constructs (Table 1). Correct construction of all *lacZ* fusions was confirmed by DNA sequencing.

β -Galactosidase assays. Overnight cultures were diluted 1:100 into fresh medium containing the appropriate antibiotics. The fresh cultures were grown at 30 °C until an OD₆₀₀ value of between 0.3 and 0.4 was obtained. Twenty-five micromolar sodium arsenite, sodium arsenate or potassium antimonite was then added to the cultures as an inducer and the cultures were grown for a further 1 h, after which the β -galactosidase activities of the cultures were measured using the method of Miller (1972).

Isolation of total mRNA. The yeast total RNA isolation protocol of Liang & Pretorius (1992) was used. RNA was isolated from *E. coli* ACSH501^a strains carrying pTfarsCRBH-

Fig. 1. Arrangement of the *At. ferrooxidans* arsenic-resistance genes (GenBank accession no. AF173880) showing the restriction endonuclease map, ORFs and translational fusions of the putative *ars* promoter regions (P_{arsB} , P_{arsR} and P_{arsC}) to *lacZ*. The *lacZ* gene is shown by the open arrow and the directions of the promoters are shown by the small, solid arrows. The positions of the probes used in the Northern-blot experiments are shown at the top of the figure, with the method used to isolate the probes shown in parentheses.

Fig. 2. Sequence of *arsR* and the 93 bp intergenic region, showing the locations of the primers used to create the *ars-lacZ* fusions (underlined) and the positions of the translational fusions (open arrows). Putative ribosome-binding sites are highlighted (bold), while the broken lines underline putative -10 and -35 sequences for *arsB* that have a good consensus for a σ^{70} promoter. Numbering shown is according to the *At. ferrooxidans* *ars* sequence (GenBank accession no. AF173880).

ACYC, which contains the complete *ars* operon from *At. ferrooxidans*. The cells were grown overnight in LB and then diluted 1:100 into fresh media containing 25 μ M sodium arsenite or 50 μ M sodium arsenate. The cells were grown at 30 $^{\circ}$ C for a further 4 h, prior to RNA being isolated from the cultures. RNA was also isolated from *At. ferrooxidans* ATCC 33020. *At. ferrooxidans* was first grown without arsenic in 800 ml tetrathionate medium. The cells from this culture were resuspended in 1.5 ml acid water and used to inoculate media containing 25 μ M sodium arsenite, 25 μ M sodium arsenate or no arsenic. The resulting cultures were incubated at 30 $^{\circ}$ C overnight and then used for RNA extractions.

Northern-blot analysis. RNA (10 μ g) from each sample was separated on a 1% denaturing agarose/formaldehyde gel. The gel was soaked in 20 $\times$ SSC for 1 h and the RNA was then transferred to a Hybond-N nylon membrane under capillary-blotting in the presence of 20 $\times$ SSC. The required probes (Fig. 1 and Table 1) were labelled with [32 P]dATP using the Random Primed DNA labelling kit (Roche Molecular Biochemicals) and hybridized to the RNA overnight at 60 $^{\circ}$ C in hybridization buffer (7% SDS, 1% BSA, 1 mM EDTA, 0.25 M Na_2HPO_4). After hybridization, the membrane was washed in 1 $\times$ SSC/0.1% SDS and 0.1 $\times$ SSC/0.1% SDS; it was then exposed to X-ray film to detect the bound probe.

RESULTS

Comparison of ArsR sequences

The predicted amino-acid sequence of the putative ArsR protein from *At. ferrooxidans* was compared with sequences in the NCBI database using the BLAST search tool; there were no proteins with known functions that showed strong matches to the putative protein from *At. ferrooxidans*. ArsR from *At. ferrooxidans* was most similar to unstudied proteins discovered during genome-sequencing projects, with the closest match (E-value of

$3e^{-18}$) being to a hypothetical transcriptional regulator from *Sinorhizobium meliloti* (GenBank accession no. NP_385183). Putative regulators that gave the closest matches to the *At. ferrooxidans* ArsR protein were located adjacent to a putative membrane binding protein and often to ArsC and ArsH homologues. An alignment of the *At. ferrooxidans* ArsR sequence with the sequences of its nine most similar proteins (on the basis of the BLAST search results) and other known ArsR proteins was constructed (Fig. 3a). Of the nine closest matches to the ArsR protein of *At. ferrooxidans* from the database, none contained the conserved cysteine residues in its metal-binding domain (ELCVCDL) (Shi *et al.*, 1996). However, there were a number of residues that were conserved throughout all of the proteins studied here, including the known ArsR proteins. These conserved residues were grouped in two regions, one before and one after the metal-binding motif previously identified in the known ArsR proteins. Besides a totally conserved arginine, serine and leucine residue within the ArsR and ArsR-like sequences, the other conserved residues were mainly hydrophobic.

Several observations could be made from the multiple-sequence alignment detailed above. While the sequences of ArsR from *At. ferrooxidans* and its closest matches did not contain the putative metal-binding sequence, they did contain a conserved sequence [GX(L/I)A] that was situated immediately downstream of the region corresponding to the experimentally established metal-binding motif. All proteins that lacked the putative metal-binding sequence had two extra residues before the second helix of the DNA-binding helix–turn–helix motif. Furthermore, all of the atypical ArsR-like proteins contained a cysteine doublet towards their C-terminals.

The sequences of the *At. ferrooxidans* ArsR protein and its nine closest matches clustered as a second subgroup of ArsR regulators upon homology analysis (Fig. 3b). Although all members of this subgroup shared conserved regions among themselves, and with SmtB and other known ArsR regulators, in the absence of a metal-binding domain, it was important to discover whether the atypical *At. ferrooxidans* ArsR protein was able to regulate the arsenic-resistance genes in response to arsenic.

arsBH and arsRC promoter studies

The arsenic-sensitive *E. coli* mutant strain AW3110 was wild-type for *lac*; hence, we constructed an *E. coli* strain (ACSH50I^q) that was both arsenic-sensitive and *lac*-negative. This ensured that there was no host cell background β -galactosidase activity and that there were no *E. coli* chromosomal *ars* genes present that may have interacted with the promoter-fusion constructs. *E. coli* ACSH50I^q was used for all of the work presented here; this strain also contained *lacI*^q on the F' plasmid, ensuring that any genes that were added *in trans* and controlled by P_{tac} were repressed in the absence of IPTG.

To study the expression of the *ars* genes we designed a number of translational gene fusions. The use of reporter genes to study gene expression should ideally be done in a homologous host, with a single gene copy integrated into the chromosome; however, this is not possible with *At. ferrooxidans*. To avoid many integration experiments into a heterologous *E. coli* host, all of the *ars*–reporter gene fusions were transferred into the low-copy-number vector pGL10. Translational fusions were constructed where an intact *arsR* gene was included *in cis* (pB2lacZ-GL10 and pC2lacZ-GL10) and an *arsB*–*lacZ* fusion was constructed in which 100 aa of ArsR were included (pBlacZ) (Fig. 1). An additional *arsB* translational fusion was constructed, in which the promoter region with only 71 aa of ArsR was included (pB3lacZ-GL10). Other fusions are shown in Fig. 1.

Expression of arsBH

When the *arsB*–*lacZ* constructs were expressed in *E. coli* ACSH50I^q in the absence of arsenic, β -galactosidase activities of approximately 19 and 75 units, respectively, were obtained when the complete ArsR protein (pB2lacZ-GL10) and 71 aa of ArsR (pB3lacZ-GL10) were present (Fig. 4a). This indicated that in the absence of arsenic, the presence of an intact ArsR protein repressed the *arsBH* genes by approximately fourfold. With the addition of either 25 μ M sodium arsenite or sodium arsenate this repression was relieved by three- to 3.5-fold provided that intact ArsR was present. When similar experiments were carried out with fusions in a higher-copy-number pMC1403-based vector, the presence of intact ArsR repressed *arsB*–*lacZ* expression by about sevenfold (pB3lacZ vs pB2lacZ) in the absence of an inducer (Fig. 4b). Following the addition of 25 μ M sodium arsenite or sodium arsenate repression was lifted by about fivefold, while the addition of 25 μ M potassium antimonate reduced repression by about threefold (Fig. 4b). An ArsR construct with 18 aa deleted from its C terminus (pBlacZ) gave similar levels of repression and derepression, respectively, in the absence and presence of arsenite, arsenate and antimony (Fig. 4b). This indicated that the product of the *arsR* gene was able to regulate expression from P_{arsB} and that while 100 aa of ArsR was sufficient for regulation of the promoter in response to arsenic and antimonite, the deletion of a further 28 aa inactivated ArsR. This additional deletion may have affected the ability of the *At. ferrooxidans* ArsR protein to dimerize, a feature that is essential for the functioning of the *E. coli* chromosomal ArsR protein and which was lost when more than 27 aa were deleted from the C terminus of this protein (Fig. 3) (Xu & Rosen, 1997).

In trans expression of *arsR* on its own under the control of P_{tac} produced problems. The *tac*–*arsR* construct was toxic and host cells grew poorly at 30 °C with no growth at 37 °C. Although the addition of 2.5 mM IPTG alone reduced expression of the *arsB*–*lacZ* fusion by 20%, regulation of the expression of the fusion by arsenic in the presence of IPTG was the same as in its absence. We are unsure as to the reason for this small reduction in

(a)

SmtB	MTKPVLDGGETVVCQGTHAAIASELQAIPEVAQSLAEFFAVLADPNRHLILSLITA.RSELVGDHACIGVSES..AVSHGIRSRNLR	87
CadC	...MKKKDTCBIFCYDEEKVNRIQGDLTQVDISGVSQILMAIDENFAHITYALCQDEELCVCDTANILGVTTIA..NASHHIRTLYKCG	84
Ec chrom	MSFL.L.PIQLFKILADETRIGIVLLISELQELCVCDLCTALDQSQP..KISRHIALIRESG	58
R773	M..LQLTPLQLFKNISDETRIGIVLLIREMQLCVCDLCTALDQSQP..KISRHIALIRESG	58
Bs skin	MDETSELRLKYEQKFHALADQKRLEIMYELCQKTCVCDLTFEFTVTS..KLSYHLKILLDAN	64
pI258	MSYKELSTILKILSDSRRLHILDILS..CGELCACDILEHFQFSQP..TISHHMKSLVDNE	57
pSX267	MSYKELSTILKVLSDPSRLHILDILS..CGELCACDILEHFQFSQP..TISHHMKSLVDNE	57
pYVe227	MLQPVLQFKILSDETRIGIVMLIRESGEMCVCDLCTALDQSQP..KISRHMAIIREAE	56
P.aeruginosa	MPSPAEVFKLADETRIVRATLLIVDQELCVCELMCALADSQP..KISRHIAQLRSAG	56
NP_486806	MQTPTATVPHFIAAGFHAIISDPIRINVLLELR..QRELVCYCDLCTALDQSQP..KISRHIAQLRSAG	63
Sa ZntR	MSEQYSEINTDTLERVTBIFALGQYNRIHIMELLIS.VSEASVGHISHOLNLSQS..NVSHGLKILKSVH	67
Atf ArsR	MEPLQDPAQIVARLEALASPVRLEIFRILLVEQEPTGLVSGDIAEHLGQPHNGISFHILKNIQHAG	64
NP_354498	MDNIGATAALSAIAQTTRILETFRRLVQHEPEGIPAGEIARLRIHVQNTMSAHIAATLARAG	60
NP_385183	MDTVDIIAAFALAQPTRLDAFRILVKHEPDGLPAGEIARLLNVPHNTMSTHIAQLQKAG	60
NP_103579	MDKATALQALAAIGQETRLHIVFRILLVRAGAKGVPAEHIATRLSTVQNTMSAHILKILQKAG	60
NP_396254	MDERQALTSFAALSQETRLHIVRILLVAGPDGMAAGAAERAKVSFSPNVSEHILKELERAG	60
NP_437556	MEERQARSSFAALSQETRLHIVRILLVAGPVGMAAGAAEAKMGVSFSPNVSEHILKELERTG	60
NP_420316	MLSAISHBGRILAFRILLVQAGPAGVANGELARALNVLPNSISANILNVLSHSC	52
NP_522690	MEETDAIRSLAALAHSLIRILVFRMLVAGPAGLTGGAIAEQLDVPGATLSFHILKELMNAA	60
NP_103576	MKLEQAQQLHALGNPTRLNLYRILLVRAGEAGRPVGHLOESLGIAASTLSFHILHRLILTG	60
NP_217156	MPKSLPVIDISAPVCCAPVAAGPMSDGDALAVALLRALADPARVRLTMSYLFSSPAGEQVSGLIAALSLSDGTVSFHIAQLRKAG	86
SmtB	LVSRRKQGRHVVYQLODHHIVALYQNALDHLQECR.....	122
CadC	VVNRRKRGKLLALYSIGDEHIRQIMMIALAHKKEVKVNV.....	122
Ec chrom	LLLLRRKQCKWVHYRLSPHIPAWAAKIIDEAWRCEQEKVQAIVRNLARQNCSGDSKNICS.....	117
R773	IILRRKQCKWVHYRLSPHIPSWAAQIIIEQAWLSQDDVQVIARKLASVNCSSGSSKAVCI.....	117
Bs skin	LITKETNGTWSYYDLNDEEVNGIISSEHCCIFRKKGBGDCC.....	105
pI258	LVTIRKNGKHWYQINHAILEDIIONLNIINTSNQRCVCKNVKSGDC.....	104
pSX267	LVTIRKNGKHWYQINHEFLDYINQNLDIINTSDQGCACKNMKSGEC.....	104
pYVe227	LVLDRRQCKWVHYRLSPHMPAWAAETITTSWHCCGKMFVSGWIN.QRHHPAEMNRTHSFNHM.....	117
Pa chrom	LLLLRRKQCKWVHYRLINPALPAWIHEVLQVTLRQAPATGCKPTQRPGRHGTAARRRASACCQ.....	118
NP_486806	LLLLRRKQCKWVHYRLINLPQFA.VLEKYITDYQHFGQVLPARCCDNK.....	108
ZntR	LVNSRQGRWMIYSIDDIHVATMLKQATIHANHPKESGL.....	106
Atf ArsR	LVKAKRQGSQRYRAAMPVVR.ALVAYLITENCCHGTDCALSGETRSPSVQEQNGQ.....	118
NP_354498	LVTVCRRGRYIIRADLEGLR.ALTLFLKLCGGGATELCAPLIAELTPCCCQEAAL.....	117
NP_385183	LVKSERQSRSIIRADLDGLRHVVS.FILKTCAGHPDVCAPLVADLTCCSPMDRPARSHRSEGGGMDGEAADRN	137
NP_103579	LVTIRQSRSIIRYTADMTGFR.DLLAYLMEDCCGAPELCQPILOAVTCKC.....	110
NP_396254	LVAHERGRSIITYTANYHCLGA.LIRFILMEDCCAGHPEICQAV.EVAAGGVQTAEAKRVV.....	119
NP_437556	LIKQRHARSIVYSASYDAL.ADLVKFILMEDCCVGHASVRA.SLNEQNPCRVASSTPAEGNGG.....	121
NP_420316	LITQRRESRSIITYTADYDAMTG.LLGFLMEDCCAGAPQICAPLSAIVSGMGACGAAGA.....	109
NP_522690	LVTQERGRHIIYRAAFDHMG.LLGFLITENCQGEACPEFSAAAGCKC.....	107
NP_103576	LVSQERCATTTICTRANYPMNM.LLIGFLADECCADAACNPAAGDAAA.....	106
NP_217156	LVISDRGGMHVFHRVHPEAL.QALCTVLNPNCGA.....	119

(b)

Fig. 3. For legend see facing page.

Fig. 4. (a) Expression of β -galactosidase under the control of the *arsB* (pB3lacZ-GL10) and *arsR* promoters (pRlacZ-GL10) in the presence of 25 μ M sodium arsenite and sodium arsenate. Also shown is the *arsB* promoter fusion with an intact *arsR* gene in *cis* (pB2lacZ-GL10) and the *arsRC-lacZ* fusion (pC2lacZ-GL10). These fusions were cloned into the low-copy-number vector pGL10. Fusions to *arsR* (pRlacZ) and *arsRC* (pC2lacZ) are also shown in a higher-copy-number pMC1403-based vector as expression was very low. (b) *arsB* promoter-lacZ fusions containing 71 aa (pB3lacZ), 100 aa (pB2lacZ) and complete (pB2lacZ) *ArsR* in *cis* induced with 25 μ M sodium arsenite, sodium arsenate or antimonyl tartrate. (c) Various *ars* constructs added *in trans* with the *arsB* promoter-lacZ fusion pB3lacZ-GL10. pTfarsB-ACYC and pTfarsBH-ACYC contain the same truncated yet functional *ArsR* as pB2lacZ. All assays were carried out in triplicate and the bars represent the SD for two or more experiments.

expression caused by IPTG. However, when constructs containing *arsR* expressed from its own promoter, together with other *ars* genes (pTfarsCRBH-ACYC and

pTfarsCRB-ACYC), were added *in trans* with the pB3lacZ-GL10 construct, reporter gene expression was repressed to levels similar to those in pB2lacZ-GL10. This repression could be relieved by the addition of both arsenite and arsenate (Fig. 4c), although the level of expression after induction was not as high as the unrepressed β -galactosidase levels. (Note that pTfarsBH-ACYC and pTfarsB-ACYC contained the same truncated but functional *arsR* gene as pB2lacZ).

Interestingly, when the expression of pB3lacZ-GL10 was compared with the expression of pTfarsBH-ACYC and pTfarsB-ACYC *in trans*, there was a suggestion that *arsH* may play a regulatory role in *arsB* expression. However, attempts to investigate reporter gene expression with *arsH* expressed on its own from a P_{tac} indicated that *ArsH* did not affect *tac-arsR* expression.

Expression of *arsRC*

When the *arsR-lacZ* constructs were expressed in *E. coli* ACSH50I^a in the absence of arsenic in both a low-copy-number (pRlacZ-GL10) and a higher-copy-number vector (pRlacZ), the β -galactosidase activities were 2 and 14 Miller units, respectively (Fig. 4a). These values were more than 15-fold less than the expression levels seen from the *arsB-lacZ* fusion in equivalent vectors. The levels of expression detected from P_{arsR} in the low-copy-number vector pRlacZ-GL10 were so low that further work was carried out only with reporter gene fusions in the higher-copy-number vector pRlacZ. As might be expected, the addition of arsenic to the *arsR-lacZ* fusion did not result in an increase in reporter gene activity, presumably due to the absence of a functional *ArsR* protein (Fig. 4a). Unexpectedly, the addition of combinations of the same *ars* genes, shown in Fig. 4(c), *in trans* did not affect the expression from pRlacZ in the presence or absence of arsenic (not shown). Therefore, it appears that if *ArsR* does autoregulate P_{arsR} , either it is required *in cis* or there are elements downstream of the promoter that are required for induction by arsenic which were not included in pRlacZ.

An *arsC-lacZ* fusion was constructed that included *arsR* and its putative promoter region (pC2lacZ) and a second fusion was constructed that excluded the P_{arsR} region (pClacZ). There was no detectable expression

Fig. 3. (a) Multiple-sequence alignment of a selection of functionally characterized *ArsR* proteins compared with the *ArsR* protein from *At. ferrooxidans* and its nine closest matches, as determined by the BLAST search. The arrows above the alignment indicate the region of the *E. coli* chromosomal *ArsR* that is required for dimerization. Also included are SmtB from *Synechococcus* sp. PCC7942 (GenBank accession no. S31197), ZntR from *Staphylococcus aureus* (AAC32484) and CadC from *S. aureus* pl258 (B32561). Known *ArsR* proteins are labelled as follows: pl258, from plasmid of *Staphylococcus aureus* (GenBank accession no. AAA25636); pSX267, from plasmid of *Staphylococcus xylosus* (AAA27587); Ec chrom, from *E. coli* chromosome (AAC76526); R773, from plasmid of *E. coli* (CAA34168); pYVe227, from plasmid of *Y. enterocolitica* (AAD16860); Bs skin, *Bacillus subtilis* skin element (BAA06967); *P. aeruginosa*, from *Pseudomonas aeruginosa* chromosome (AAC69642). The nine closest matches to *ArsR* from *At. ferrooxidans* are labelled with their GenBank accession numbers (see Fig. 3b). The closed boxes highlight amino acids that are conserved between at least 14 of the 21 sequences or which are found in all members of one of the two subfamilies of *ArsR* proteins. For the comparison, amino acids assumed to be functionally equivalent were I/L/V, R/K and G/A. The metal-binding motif found in the characterized *ArsR* proteins has been highlighted using broken boxes. The alignments were performed using the CLUSTAL W-based optimal alignment tool of DNAMAN. (b) Dendrogram of the proteins detailed in (a) showing the possible grouping of *At. ferrooxidans* *ArsR* in a separate subclass of *ArsR* regulators. Values shown represent the percentage amino-acid identity between the proteins.

Fig. 5. Total mRNA prepared from *E. coli* ASCH501^q(pTfarsCRBH-ACYC) was probed with DNA probes to the *arsB*, *arsR* and *arsH* genes and a probe to *arsR* and *arsB* including the intergenic region (*ars* probe). RNA was isolated from cells grown in the absence (–) or presence of 25 μ M sodium arsenite (AsIII) or 50 μ M sodium arsenate (AsV). The positions and sizes of the transcripts are marked with arrows.

from pClacZ, which suggested that *arsC* does not have a promoter of its own but is transcribed together with *arsR* (results not shown). The *arsC*–*lacZ* fusion pC2lacZ, which contained an intact *arsR* gene, did respond to the addition of arsenic, and expression by this fusion was induced four- and 4.5-fold in the presence of 25 μ M arsenite and arsenate, respectively (Fig. 4a).

Number and size of transcripts

To investigate whether there were two mRNA transcripts for the *ars* operon, and to estimate their sizes and whether they were regulated differently by arsenate and arsenite in *E. coli*, Northern-blot analysis was performed. Total RNA was prepared from *E. coli* ACSH501^q(pTfarsCRBH-ACYC) cells. Probes for the *arsR*, *arsB* and *arsH* genes and a region including the *arsB* and *arsR* genes (*ars* probe) were used (Fig. 1). About 10 μ g of RNA was loaded into the wells of the gel shown in Fig. 5. Attempts to obtain a stronger signal by loading 30 μ g of RNA resulted in an increase in the signal from the degraded transcript smear and blot resolution was not improved. When RNA was isolated from *At. ferrooxidans* strains no transcripts were observed; hence, we believe that the *ars* genes are expressed at low levels in this organism.

Two different patterns of expression were seen in *E. coli* ACSH501^q(pTfarsCRBH-ACYC) depending on whether *arsB*/*arsH* or *arsR* were used as probes (Fig. 5). The size of the transcript detected using either *arsB* or *arsH* as probes was about 2.2 kb, while the *arsRC* transcript was a smear of about 400–950 bp. When the *ars* probe (containing *arsR* and *arsB*) was used, two transcripts of approximately the aforementioned sizes were visible. The calculated sizes of the regions encompassing the *arsBH* and *arsRC* ORFs were 2038 and 847 bp, respectively, which is consistent with the sizes of the transcripts observed. It was also clear that the quantity of both transcripts, but especially that of *arsRC*, increased when cells were grown in the presence of either arsenite or arsenate. No clear differences in the levels of arsenite-induced expression compared to arsenate-induced expression were observed. When a probe

from the tetracycline gene of pACYC184 was used for Northern blotting, a signal (1.2 kb) of about equal intensity was observed in all three lanes, indicating that approximately equal amounts of RNA had been loaded into each lane (results not shown). Therefore, the approximately equal levels of induction observed irrespective of whether arsenite or arsenate was used are in agreement with the results from the reporter gene fusion experiments.

DISCUSSION

The arsenic-resistance genes from *At. ferrooxidans* have an unusual divergent arrangement, with the two major genes, *arsB* (encoding the arsenite membrane pump) and *arsC* (encoding the arsenate reductase), transcribed in opposite directions. A gene showing similarity to the regulatory gene *arsR* was found in the middle of these genes, which was transcribed in the same direction as *arsC*. While members of the family of ArsR regulatory proteins often show low similarity to each other (e.g. the ArsR proteins from staphylococcal plasmids are only approximately 30% identical to those from the *E. coli* chromosome and plasmid R773), those that have been functionally investigated all contain a highly conserved metal-binding domain with an ELCVCDLC consensus sequence. Shi *et al.* (1994, 1996) found that the three cysteine residues of this consensus sequence were able to interact with arsenite. Mutations in the first two cysteine residues resulted in a protein that was able to bind the promoter and repress expression, but it was not able to undergo the conformational change required for its release from the promoter in response to an inducer. The finding that the putative *At. ferrooxidans* ArsR protein and its closest matches are located adjacent to a putative membrane protein (and often to ArsC and ArsH homologues) provides circumstantial evidence that these proteins are *ars* regulators. However, none of the putative ArsR proteins contained the conserved metal-binding motif, with the only cysteine residues being at the C termini of these proteins. Nevertheless, we found that mRNA production was increased in response to the presence of arsenite and arsenate. This indicated that regulation of the *ars* genes occurred at the transcriptional level and we showed, using promoter fusions to a β -galactosidase reporter gene (*lacZ*), that this regulation was controlled by the atypical ArsR protein of *At. ferrooxidans*. This suggests that the other related, but unstudied, ArsR-like proteins discovered during genome-sequencing projects may do the same. However, the inducer–metalloid binding mechanism of the two families of ArsR regulator proteins may be different. For example, the *ars* operon of *E. coli* plasmid R773 was derepressed by arsenite and antimony but not by arsenate (Wu & Rosen, 1993). It is thought that induction by arsenate takes place after its reduction to arsenite, typically by the ArsC reductase. The *At. ferrooxidans* ArsR protein appeared to respond to arsenate in the *E. coli* *ars* deletion mutant containing no ArsC. This suggests that either the ability of arsenate to bind the *At. ferrooxidans* ArsR is different, or some

other mechanism exists for the reduction of sufficient arsenate to arsenite, which then serves as the inducer. In a study on ZntR from the chromosome of *S. aureus* (Singh *et al.*, 1999), it was reported that this protein was able to bind to the promoter of the zinc-resistance operon and that this binding was inhibited in the presence of zinc, even though ZntR did not contain any cysteine residues (Fig. 3a) – this protein is related to the ArsR family of regulatory proteins (Fig. 3b).

The ArsR regulators are part of the ArsR family of metalloregulatory proteins, which also includes CadC (the regulator of the Cd^{2+} -efflux ATPase) and SmtB (the regulator of the metallothionein SmtA protein in response to the presence of zinc). In this study, we found that although ArsR from *At. ferrooxidans* does not contain the conserved metal-binding domain, there are many other strongly conserved areas within its sequence and the sequences of SmtB, other known ArsR proteins and its closest matches from the BLAST search. The crystal structure of SmtB has been determined and has been shown to have a winged helix–turn–helix (HTH) structure (Cook *et al.*, 1998). The secondary structure of SmtB consists of four α -helices and two β -sheets, with the β -sheets having been shown to form a hairpin structure. The conserved Gly-X-X motif (where X represents a large hydrophobic residue) at the end of the DNA-binding HTH domain is found in many of the proteins shown in Fig. 3(a). Cook *et al.* (1998) believe that these hydrophobic residues are important for anchoring the β -sheet that follows. Secondary-structure predictions indicate that many of these general features appear to be present in the second family of ArsR-like regulators. However, the absence of the established metal-binding motif and the observation that there are two extra amino-acid residues before the conserved hydrophobic region corresponding to the second helix of the HTH domain argue against a detailed extrapolation from the secondary structure of SmtB to the ArsR protein of *At. ferrooxidans*.

We have shown that at least two RNA transcripts, one corresponding to *arsBH* and the other corresponding to *arsRC*, were induced in the presence of arsenite and arsenate. Both Northern hybridization and reporter gene studies indicated that the level of expression from P_{arsB} was low. Although expression was increased following induction, the levels of expression remained low. While this could be due to inefficient expression in *E. coli*, these low levels of expression were also observed when fusions to the *ars* genes from the *Bacillus subtilis* *skin* element were constructed and integrated into the chromosome (Sato & Kobayashi, 1998). The reason for such low levels of expression could be due to the toxic nature of the *ars* genes. Overexpression of the *E. coli* ArsB protein from a P_{tac} has been reported to be toxic to cells (e.g. Cai & DuBow, 1996). Despite the low levels of expression, induction of expression by arsenite, arsenate and antimonite was observed.

The relative levels of expression from the *arsRC* promoter gave apparently conflicting results, depending

on whether Northern hybridization or reporter gene studies were used. Although the quantity of *arsRC* transcript seen on the Northern blot was higher than for *arsBH*, the level of *arsRC-lacZ* expression was much lower than for the *arsB-lacZ* fusion. The observation that the *arsRC* mRNA transcript was a smear may indicate that this mRNA has a higher turnover rate than *arsBH* mRNA. If much of the degradation takes place from the 5' end, this may be the reason for the lower level of reporter gene expression. Because reporter gene expression from the *arsRC* promoter was so low (less than 10 Miller units), we worked with a higher-copy-number vector and obtained results that were consistent with those observed in the low-copy-number vector. Expression from an *arsRC* promoter fused at the start of *arsR* was unresponsive to the presence of arsenic, presumably due to the absence of *arsR*. The addition of an *arsR* gene *in trans* did not induce expression, possibly because sequences downstream of the point of fusion were required or because the *At. ferrooxidans* *arsR* gene is not autoregulated. This inability to demonstrate autoregulation may indicate a difference between the two families of ArsR proteins and needs to be investigated further. However, when a translational fusion to *arsC* (which included *arsR* and the *arsRC* promoter) was investigated, expression was induced in the presence of both arsenite and arsenate. This indicated that *arsR* was required for regulation of this promoter. As an *arsC-lacZ* fusion containing the region upstream of *arsC* but not including the *arsR* promoter (pClacZ) showed no β -galactosidase activity, it appears that *arsR* and *arsC* are expressed as a single transcript. Therefore, although the two *ars* transcripts of the *At. ferrooxidans* *ars* operon were transcribed at different levels, both were regulated by arsenite and arsenate.

We found that the last 19 aa of the C terminus of *At. ferrooxidans* ArsR were not required for either repression or induction of the *arsBH* promoter by arsenite, arsenate or antimony. However, when a further 28 aa of ArsR *in cis* with the promoter fusion were deleted (leaving only 71 aa), expression from the promoter was constitutive. Similarly, it was shown that *E. coli* chromosomal ArsR– β -lactamase chimeras were constitutive when fused to regions upstream of the 79th amino acid, but were inducible when β -lactamase was fused downstream of the 92nd amino acid (Xu *et al.*, 1996). These positions align with amino acids 85 and 98 of the *At. ferrooxidans* ArsR. An interesting observation is that the double cysteines conserved in *At. ferrooxidans* ArsR and its closest relatives were retained in the functional truncated ArsR. Further experiments are required to show whether these residues are involved in regulation.

We were unable to find a clear role for ArsH in the regulation of the *At. ferrooxidans* *ars* genes. While the addition of constructs containing *arsH in trans* with the *arsB* promoter fusions appeared to result in increased repression of the promoter and lower levels of induction, especially when arsenate was the inducer, these differences were small (Fig. 4b). We were unable to add the

tac-regulated *arsH* gene back *in trans*, as we found that IPTG on its own decreased expression from the *At. ferrooxidans* *arsB* promoter. It is possible that the role that ArsH plays is only important in *At. ferrooxidans* and not in *E. coli*.

This is the first functional study of an ArsR regulator that is able to respond to arsenic, but which does not contain the conserved metal-binding motif. We propose that the ArsR protein from *At. ferrooxidans* is the first reported member of a subclass of ArsR regulators that may have a different method of binding the inducer to other ArsR regulators.

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