A chromosomal *ars* operon homologue of *Pseudomonas aeruginosa* confers increased resistance to arsenic and antimony in *Escherichia coli*

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Operons encoding homologous arsenic-resistance determinants (*ars*) have been discovered in bacterial plasmids from Gram-positive and Gram-negative organisms, as well as in the *Escherichia coli* chromosome. However, evidence for this arsenic-resistance determinant in the medically and environmentally important bacterial species *Pseudomonas aeruginosa* is conflicting. Here the identification of a *P. aeruginosa* chromosomal *ars* operon homologue via cloning and complementation of an *E. coli* *ars* mutant is reported. The *P. aeruginosa* chromosomal *ars* operon contains three potential ORFs encoding proteins with significant sequence similarity to those encoded by the *arsR*, *arsB* and *arsC* genes of the plasmid-based and *E. coli* chromosomal *ars* operons. The cloned *P. aeruginosa* chromosomal *ars* operon confers augmented resistance to arsenic and antimony oxyanions in an *E. coli* *arsB* mutant and in wild-type *P. aeruginosa*. Expression of the operon was induced by arsenite at the mRNA level. DNA sequences homologous with this operon were detected in some, but not all, species of the genus *Pseudomonas*, suggesting that its conservation follows their taxonomic-based evolution.

Keywords: arsenic resistance, *Pseudomonas*, chromosomal *ars* operon, evolutionary conservation

INTRODUCTION

Plasmid-based arsenic resistance in bacteria, encoded by the *ars* operons, has been known for many years (Kaur & Rosen, 1992). Five genes, transcribed as a single polycistronic mRNA in the order *arsRDABC*, have been found in *Escherichia coli* plasmids R773 and R46 (Owolabi & Rosen, 1990; Bruhn *et al.*, 1996). These *ars* operons confer resistance to arsenic and antimony oxyanions via an ATP-dependent efflux mechanism (Mobley & Rosen, 1982). The first two genes, *arsR* and *arsD*, of the R773 *ars* operon encode two inducer-dependent *trans*-acting repressors that control the basal and upper levels of operon expression, respectively (San

Francisco *et al.*, 1990; Wu & Rosen, 1991, 1993a, b; Chen & Rosen, 1997). True inducers for expression of the *ars* operon are trivalent, but not pentavalent, arsenic and antimony oxyanions (Wu & Rosen, 1993b; Chen & Rosen, 1997). The *arsABC* genes encode the structural components of the arsenic pump (Chen *et al.*, 1986). The ArsA protein is an arsenite- and antimonite-stimulated ATPase (Rosen *et al.*, 1988) which forms a complex with the membrane-bound ArsB protein (Tisa & Rosen, 1990; Wu *et al.*, 1992), and actively exports arsenite and antimonite ions upon hydrolysis of ATP (Kaur & Rosen, 1994a, b; Li *et al.*, 1996). Interestingly, the ArsB protein of the R773 *ars* operon actively exports arsenite ions even in the absence of the ArsA ATPase (Dey & Rosen, 1995), suggesting that both ATP and membrane potential can stimulate the function of the ArsB membrane pump. Consistent with this, the *ars* operons in the Gram-positive staphylococcal plasmids pI258 and pSX267 contain only *arsR*, *arsB* and *arsC* genes, lacking the *arsA* and *arsD* coding sequences. Nonetheless, these *ars* operons can actively export arsenite and antimony

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Abbreviation: SD, Shine–Dalgarno sequence.

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oxyanions using membrane potential, rather than cellular ATP, as an energy source (Ji & Silver, 1992b; Rosenstein *et al.*, 1992; Bröer *et al.*, 1993). The ArsC protein is a small cytosolic protein that acts to reduce arsenate to arsenite (Gladysheva *et al.*, 1994; Oden *et al.*, 1994), which can then be extruded by the ArsAB complex.

The recently discovered *E. coli* chromosomal *ars* operon also contains three ORFs, *arsRBC*, structurally resembling the staphylococcal plasmid *ars* operons (Sofia *et al.*, 1994; Diorio *et al.*, 1995), and is functional in conferring increased resistance to arsenic (Carlin *et al.*, 1995; Diorio *et al.*, 1995). Moreover, an *arsH* gene is required for arsenic resistance in a *Yersinia enterocolitica* plasmid-located *ars* operon, although the function of the *arsH* product has not yet been elucidated (Neyt *et al.*, 1997). In addition to resistance to arsenic and antimony oxyanions, the plasmid R773 *ars* operon can confer increased resistance to tellurite (Turner *et al.*, 1992), a result not observed with the *E. coli* chromosomal *ars* operon (Cai & DuBow, 1996), though whether this was due to the inability of tellurite to induce *ars* operon expression has yet to be determined. Nonetheless, significant sequence similarity between the *E. coli* chromosomal and the plasmid-encoded *ars* operons (Diorio *et al.*, 1995) suggests a common ancestor. Since the *E. coli* chromosomal *ars* operon is the only functionally characterized chromosomally located *ars* operon in bacteria to date, it is important to identify and characterize others to better understand the evolutionary relationship among *ars* operons.

We have previously observed (via Southern blotting) that sequences hybridizing with the *E. coli* chromosomal *ars* operon are present in the chromosomes of many Gram-negative bacterial species, including the non-enteric bacterium *Pseudomonas aeruginosa* (Diorio *et al.*, 1995). Although it has been reported that pUM310 of *P. aeruginosa* contains a genetic determinant that confers resistance to arsenic and antimony ions (Cervante & Chávez, 1992), the evidence for a chromosomal *ars* operon in members of the genus *Pseudomonas* has been controversial (Carlin *et al.*, 1995; Diorio *et al.*, 1995). The aim of this study was to determine if an *ars* operon homologue exists in the chromosome of *P. aeruginosa*. Here we report the isolation, DNA sequencing, and *E. coli* *ars* operon complementation of a chromosomal *ars* operon of *P. aeruginosa*. It consists of three ORFs encoding protein products similar in sequence to those encoded by the *arsR*, *arsB* and *arsC* genes of known *ars* operons. The *P. aeruginosa* *ars* operon is functional in conferring increased resistance to arsenic and antimony oxyanions, as determined by complementation of an *E. coli* *arsB* mutant. Moreover, expression of this operon, as measured by RNA dot blots, is induced by sodium arsenite. Sequence analyses revealed that the *P. aeruginosa* chromosomal *ars* operon is evolutionarily related to plasmid-based *ars* operons from both Gram-negative and Gram-positive bacteria, and is conserved in the chromosomes of some, but not all, *Pseudomonas* species.

METHODS

Bacterial strains and plasmids. *E. coli* strain 40 [$F^- \Delta(\text{pro-lac})$ *rpsL trp*] is an *E. coli* K-12 strain in which we isolated and sequenced the chromosomal *ars* operon (Diorio *et al.*, 1995). *E. coli* strain LF20012 is derived from *E. coli* strain 40, but contains an *arsB::luxAB* chromosomal transcriptional gene fusion (Cai & DuBow, 1996), and is thus genotypically *arsB* and hypersensitive to arsenic oxyanions (Cai & DuBow, 1997). *P. aeruginosa* PAO1, *Pseudomonas fluorescens*, *Pseudomonas stutzeri*, *Pseudomonas diminuta* and *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) are all plasmid-free wild-type strains obtained from Dr G. Hegeman (Indiana University, USA). *P. aeruginosa* wild-type strain PAK was from Dr R. Ramphal (University of Florida, USA). *E. coli* cultures were grown in Luria-Bertani (LB) medium (Miller, 1972) at 37 °C. Cultures of *Pseudomonas* and *Burkholderia* species were grown initially in Brain Heart Infusion (BHI; Difco) broth and then in LB broth at 32 °C, except *P. fluorescens*, which was grown at 25 °C. pJC801 (Ap^r) is a pBR322 derivative that contains the cloned *P. aeruginosa* chromosomal *ars* operon as a 3.4 kb *EcoRV* fragment. The same restriction fragment was also cloned into pTJS140 (Darzins & Casadaban, 1989), yielding pKAS300 (described in the following sections). pUC119 and pUC118 (Vieira & Messing, 1987; Sambrook *et al.*, 1989) were used as vectors for subcloning and sequencing the *P. aeruginosa* chromosomal *ars* operon. Ampicillin and piperacillin were used at a final concentration of 40 µg ml⁻¹ and 50 µg ml⁻¹, respectively, when required.

Cloning and sequencing of the *P. aeruginosa* chromosomal *ars* operon. All restriction enzymes and T4 DNA ligase used for cloning purposes were purchased from New England Biolabs and used following the supplier's instructions. Isolation of plasmid and genomic DNA, preparation of competent *E. coli* cells, and DNA transformation into *E. coli* cells were performed as described by Sambrook *et al.* (1989). Preparation of competent PAO1 cells was performed according to Farinha & Kropinski (1990). Transformation of pTJS140 and pKAS300 into PAO1 was performed by electroporation. α -³²P-labelling of DNA probes for Southern and RNA dot-blotting analyses was performed using the random priming method (Sambrook *et al.*, 1989) with random hexanucleotide primers (Regional DNA Synthesis Laboratory, University of Calgary, Canada). Radioisotopes were purchased from Amersham Life Science.

To clone the *P. aeruginosa* strain PAO1 chromosomal *ars* operon homologue, restriction fragments approximately 3.4 kb in size, previously shown to hybridize to the *E. coli* chromosomal *ars* operon (Diorio *et al.*, 1995), were purified from a 0.8% agarose gel of *EcoRV*-cleaved chromosomal DNA of *P. aeruginosa* strain PAO1 using the GeneClean II kit (Bio101), and ligated to *EcoRV*-linearized pBR322. The ligation mixture was transformed into *E. coli* strain JM105 (Yanisch-Perron *et al.*, 1985). Recombinant transformants were selected for ampicillin resistance and screened for tetracycline sensitivity. To identify the plasmids containing the putative *P. aeruginosa* chromosomal *ars* operon homologue, clones from the recombinant transformants were lysed by the 'cracking' procedure (Barnes, 1977), subjected to 0.8% agarose gel electrophoresis, transferred to a Hybond-N nylon membrane (Amersham Life Science) and hybridized (Diorio *et al.*, 1995) to a ³²P-labelled *E. coli* chromosomal *arsB* probe, isolated as a 658 bp *ScaI*-*PvuII* restriction fragment from pJC701 (Cai & DuBow, 1996). Following autoradiography, plasmid DNA was isolated from recombinant clones with enhanced hybridization signals (Sambrook *et al.*, 1989),

and used to transform *E. coli* LF20012 (*arsB* mutant). The transformants were screened for enhanced resistance to sodium arsenite (when compared with *E. coli* LF20012 containing pBR322) by replica plating them on LB agar containing increasing concentrations of sodium arsenite (0, 20, 100, 200, 400 and 800 µg As ml⁻¹). One of the clones showing enhanced arsenic resistance was isolated, and its recombinant plasmid, containing a 3.4 kb *EcoRV* *P. aeruginosa* chromosomal DNA fragment, was named pJC801. The DNA fragment cloned in pJC801 was subcloned in the shuttle vector pTJS140, which contains an RK2 replicon that allows replication in *P. aeruginosa* and a ColE1 origin for replication in *E. coli*. The recombinant plasmid from *E. coli*, called pKAS300, was used to transform *P. aeruginosa* strain PAO1.

Fragments of the *P. aeruginosa* chromosomal DNA in pJC801 were further subcloned into pUC119 or pUC118 (Sambrook *et al.*, 1989). Double- or single-stranded DNA templates for sequencing analysis were prepared using the QIAprep Spin Mini Prep kit (Qiagen), or following the method of Vieira & Messing (1987). DNA sequencing of both strands of these subclones was performed manually by the dideoxy DNA sequencing method using the ISOTHERM sequencing kit (Epicentre Technologies), and by automatic sequencing (Core Facilities for Protein/DNA Chemistry, Biochemistry Department, Queen's University, Kingston, Ontario, Canada).

Complementation assays in an *E. coli* *arsB* strain and determination of the arsenic-resistance phenotype and MICs in wild-type *E. coli* and *P. aeruginosa*. The plasmid pJC801 (and the parental plasmid pBR322) were used to transform the *E. coli* *arsB* mutant strain LF20012 (*arsB::luxAB*) (Cai & DuBow, 1996), as well as its parent, *E. coli* 40. The resistance profile to arsenic and antimony salts of all four transformants was determined by their ability to grow in LB broth containing increasing amounts of arsenic or antimony oxyanions as follows. Overnight cultures of each sample were diluted 100-fold in LB broth containing the appropriate antibiotics and increasing concentrations of sodium arsenite, sodium arsenate and potassium antimony tartrate hemihydrate. The cultures were incubated for 6 h at 37 °C, and the OD₆₀₀ of each sample (in triplicate) was determined. The resistance profile of each strain to arsenic or antimony oxyanions was expressed as the percentage OD₆₀₀ compared with the control culture with no added arsenic or antimony oxyanions calculated (Cai & DuBow, 1997) as a function of the elemental concentrations of arsenic or antimony in the compounds used.

The resistance phenotype of the *P. aeruginosa* PAO1 strain with or without the cloned *P. aeruginosa* chromosomal *ars* operon homologue was also determined. Since *P. aeruginosa* PAO1 is normally somewhat mucoid, cell growth cannot be measured accurately by its turbidity in liquid medium (not shown). Therefore, sensitivity of these strains to arsenic/antimony oxyanions was measured by dilution (to 100-fold) of an overnight culture of *P. aeruginosa* PAO1 into LB broth, growing to an OD₆₀₀ of 0.2 in a 32 °C air shaker. Aliquots of diluted exponentially growing cells (100 µl of the 10⁻⁶ dilution per plate) were then plated on LB agar containing increasing concentrations of arsenic and antimony salts. The c.f.u. of each strain were determined after 24 h growth of the cells at 32 °C. Each assay was performed in triplicate. The mean number of c.f.u. of each strain in the absence of added chemicals was defined as 100% survival. The percentage survival of each strain in the presence of specific concentrations of added chemicals was expressed as its mean c.f.u. value with the oxyanions present, divided by its c.f.u. value in the absence of added chemicals.

The MICs of each oxyanion for the wild-type *Pseudomonas* strains were measured after diluting the overnight culture (1:100) in LB broth containing increasing concentrations of arsenite, arsenate and antimonite, and determined as the minimum concentrations that resulted in no increase in cell density (OD₆₀₀) after 6 h incubation in an air shaker.

Southern blotting analysis. Genomic DNAs (10 µg) were cleaved with *EcoRV* and *PstI*, fractionated by 0.8% agarose gel electrophoresis and transferred to a Hybond-N membrane following denaturing and neutralization (Sambrook *et al.*, 1989). The subsequent prehybridization and hybridization with a ³²P-radiolabelled probe were performed as previously described (Autexier & DuBow, 1992; Diorio *et al.*, 1995). Briefly, the membrane was incubated with prehybridization solution [5× SSC, 5× Denhardt's solution (Sambrook *et al.*, 1989), 50 µg calf thymus DNA ml⁻¹, 50% (v/v) deionized formamide] for 2 h at 40 °C, and then with hybridization solution [5× SSC, 1× Denhardt's solution, 50 µg calf thymus DNA ml⁻¹, 50% (v/v) deionized formamide, 0.3% (w/v) SDS, 25 µM ATP and 1 mM EDTA, pH 8.0] containing 2–4 × 10⁵ c.p.m. labelled probe for 16–18 h at 39 °C. The probe was isolated from pJC801 as a 1.14 kb *Bam*HI–*Sph*I fragment containing the second half of *arsR* and the first two-thirds of *arsB*. After hybridization, the membrane was washed (in a solution containing 0.2%, w/v, SDS and 0.5× SSC) three times for 30 min at room temperature, and exposed to Kodak XAR-5 film (Eastman Kodak), using Dupont Cronex intensifying screens.

Preparation of total cellular RNA of *P. aeruginosa* PAO1 and dot-blotting analysis. Cultures of *P. aeruginosa* strain PAO1 were grown in LB broth at 32 °C in a shaking incubator until mid-exponential phase (OD₆₀₀ = 0.5–0.6), and then sodium arsenite was added to a final concentration of 0.1 µg As ml⁻¹. Choice of this concentration was based on our previous observations for the *E. coli* *ars* operon (Cai & DuBow, 1996). Samples were removed at 0, 15, 30 and 60 min after arsenic addition and kept on ice until all samples were ready. Total cellular RNA was isolated using the RNaid Plus kit (Bio101), following the manufacturer's instructions. The RNA concentrations of each sample were spectrophotometrically determined using a UV-1201 spectrophotometer (Shimadzu Scientific Instruments). Different amounts (5, 2.5 and 1 µg per slot) of total cellular RNA from arsenite-exposed and unexposed *P. aeruginosa* were loaded onto a Hybond-N nylon membrane (Amersham Life Science) in a Bio-Rad dot-blot apparatus as described by Sambrook *et al.* (1989). The RNA was fixed to the membrane by microwave exposure for 2.5 min and hybridized to the same probe as used for Southern blotting. Prehybridization and hybridization reactions were performed as described by Cai & DuBow (1996), and the membrane was washed four times for 15 min at 65 °C in a solution containing 40 mM Na₂PO₄, pH 7.2, 1 mM EDTA, 1% (w/v) SDS, and exposed to Kodak XAR-5 film.

RESULTS

Cloning of an *ars* operon homologue of *P. aeruginosa*

Based upon our previous observation that an approximately 3.4 kb *EcoRV* DNA fragment of *P. aeruginosa* contained sequences homologous to the *E. coli* chromosomal *ars* operon, and thus a potential chromosomal *ars* operon homologue (Diorio *et al.*, 1995), *EcoRV* fragments of this size were isolated and cloned in pBR322. *E. coli* transformants were screened to detect sequences

Table 1. Percentage identity (similarity) of *P. aeruginosa* ArsR, B and C polypeptides to amino acid sequences deduced from known *ars* genes

<i>ars</i> gene	ArsR	ArsB	ArsC
<i>E. coli</i>	44.4 (55.5)	69.3 (84.9)	10.6 (27.6)
R773	43.6 (59.8)	69.3 (85.6)	12.8 (24.1)
IncN R46	43.6 (57.3)	69.9 (85.5)	12.1 (23.6)
Tn2505	42.7 (56.4)	68.9 (83.3)	13.5 (29.1)
pI258	28.8 (49.0)	51.7 (72.7)	29.0 (45.8)
pSX267	27.9 (46.2)	51.4 (72.6)	29.0 (45.8)

hybridizing with an *E. coli* *arsB* probe. Four putative clones were confirmed by a complementation assay, and were isolated due to their ability, in transformants of an *E. coli* *arsB* mutant host, to confer high levels of resistance to arsenite (i.e. the ability to allow growth on solid media containing 400 µg As ml⁻¹ as sodium arsenite; not shown). Based upon restriction enzyme mapping, these four plasmids, designated pJC801 through pJC804, contained an identical DNA insert (data not shown). Therefore, pJC801 was chosen for further studies.

DNA and predicted amino acid sequence analyses

Subcloning and DNA sequencing analyses showed that the cloned *P. aeruginosa* PAO1 chromosomal DNA fragment contained three ORFs encoding proteins with significant sequence similarities to the ArsR, ArsB and ArsC polypeptides of the *E. coli* chromosomal and plasmid-encoded *ars* operons. Comparison of the amino acid sequences of the *P. aeruginosa* *ars* operon with other known *ars* operons, using the method of Myers & Miller (1988), revealed striking similarities, summarized in Table 1. In the case of ArsR, the amino acid sequence identity between the *P. aeruginosa* chromosomal *ars* operon and other *ars* operons ranged from 28 to 44% (Table 1). In particular, the putative metal-binding box (ELCVCDL) and the DNA-binding helix–turn–helix motif identified in the ArsR proteins of other *ars* operons and known to be critical in arsenic-dependent regulation of operon expression, were also conserved in the ArsR sequence of the *P. aeruginosa* chromosomal *ars* operon (Fig. 1). These results suggest that the *P. aeruginosa* chromosomal *ars* operon is a new member of the arsenic efflux system family, and may be regulated in a manner similar to that used by other known *ars* operons. The highest sequence identity, ranging from 51 to 70%, was found between the ArsB protein of the *P. aeruginosa* chromosomal *ars* operon and that of other known *ars* operons (Table 1). Multiple alignments (Higgins & Sharp, 1988, 1989) of the *P. aeruginosa* ArsB sequence with the ArsB proteins of the *ars* operons listed in Table 1 revealed an overall amino acid identity (similarity) of 45.3% (78.8%) (data not shown). Hydrophobicity analyses, using the method of Eisenberg *et al.* (1984), indicate that the *arsB* gene product is a hydrophobic protein with 12 putative membrane-associated helices,

Fig. 1. Multiple alignments of amino acid sequences of the ArsR proteins from various *ars* operons using the CLUSTAL computer program (PC/Gen software, IntelliGenetics). Identical amino acid residues are indicated in hatched areas; similar amino acid residues are indicated in dotted areas. The potential metal-binding and DNA-binding motifs are marked.

consistent with its potential function as a membrane transporter and with the structures of other known ArsB proteins. It is interesting to note that the ArsR and ArsB proteins of the *P. aeruginosa* chromosomal *ars* operon show close similarity to homologues from the *ars* operons of Gram-negative bacteria. By contrast, the ArsC protein is more similar to the ArsC proteins of Gram-positive *ars* operons (Table 1). Analysis of the codon usage of the *P. aeruginosa* *ars* operon indicated that the *P. aeruginosa* *ars* genes preferentially contain a cytosine at the third nucleotide of their amino acid codon position when compared with those of the *E. coli* chromosomal *ars* operon, consistent with the codon usage of many other chromosomal genes of *P. aeruginosa* (West & Iglewski, 1988).

The cloned *P. aeruginosa* chromosomal *ars* operon confers increased arsenite and antimonite resistance in both *E. coli* and *P. aeruginosa*

To examine its role in arsenic resistance, the cloned *P. aeruginosa* chromosomal *ars* operon was introduced into *E. coli* and *P. aeruginosa* PAO1. In *E. coli*, pJC801 was used to transform the *arsB* mutant strain LF20012

Fig. 2. Growth of plasmid-containing *E. coli* strains in the presence of increasing amounts of arsenic and antimony oxyanions. Overnight cultures were diluted 100-fold in LB broth containing the appropriate antibiotics and increasing amounts of arsenic or antimony salts. Cellular growth was measured 6 h after addition of chemicals and expressed as percentage OD₆₀₀ versus elemental concentrations of arsenic (antimony) added to the growth media (see Methods). Standard deviations are represented by error bars. ▲, *E. coli* 40(pBR322); ◆, *E. coli* 40(pJC801); ●, LF20012(pBR322); ■, LF20012(pJC801).

(Cai & DuBow, 1996), and the parental strain 40 (*ars*⁺). As controls, both *E. coli* strains were transformed with pBR322. Cultures from these four strains were analysed for their ability to grow on increasing concentrations of arsenite, arsenate and antimonite ions (Fig. 2). Introduction of pJC801 into *E. coli* conferred an increase in arsenite resistance in both *ars*⁺ and *arsB* strains in a manner similar to that observed when the *E. coli* chromosomal *ars* operon is cloned in a multicopy plasmid (Diorio *et al.*, 1995) (Fig. 2a). A similar increase in antimonite resistance was also observed (Fig. 2c). *P. aeruginosa* strain PAO1, containing the cloned chromosomal *ars* operon on pKAS300, also showed increased resistance to all three oxyanions when compared to the strain containing the vector plasmid only (Fig. 3). These

Fig. 3. Resistance profile of *P. aeruginosa* strains containing pKAS300 or pTJS140 to arsenite (a), arsenate (b) and antimonite (c). A 10⁻⁶ dilution of an exponentially growing culture of each strain was spread (100 μl per plate) onto LB agar plates containing 0, 20, 50, 100, 200, 500 and 1000 μg arsenic or antimony ml⁻¹ as sodium arsenite, sodium arsenate or potassium antimony tartrate, respectively. The percentage survival of each sample was determined as described in Methods. Standard deviations are represented by error bars. ■, PAO1(pTJS140); ◆, PAO1(pKAS300).

results suggest that the *P. aeruginosa* *ars* operon not only increased resistance to all three oxyanions in *P. aeruginosa* when cloned in a high-copy-number plasmid, but also complemented an *E. coli* *arsB* mutant and augmented wild-type *E. coli* resistance to arsenite and antimonite ions. Although the cloned *P. aeruginosa* *ars* operon increased arsenate resistance in the *E. coli* *arsB* mutant as well as in *P. aeruginosa* PAO1, it did not show any detectable enhancement of arsenate resistance in wild-type *E. coli* (Fig. 2b).

Transcription of the *P. aeruginosa* chromosomal *ars* operon is inducible by sodium arsenite

To determine if arsenic-regulated expression of the *P. aeruginosa* chromosomal *ars* operon occurs at the

Fig. 4. (a) Dot-blot hybridization of total cellular RNA to a ^{32}P -labelled *P. aeruginosa* chromosomal *ars* operon probe (see Methods). Lanes: 1, RNA from unexposed cultures; 2–4, RNAs from cells incubated with sodium arsenite (at a final concentration of $0.1\ \mu\text{g As ml}^{-1}$) for 15, 30 and 60 min, respectively. Samples in rows A, B and C are loaded with 5, 2.5 and $1\ \mu\text{g RNA per slot}$, respectively. (b) Southern hybridization of genomic DNA from various bacterial species to a ^{32}P -labelled *P. aeruginosa* chromosomal *ars* operon (see Methods). The numbers on top indicate different bacterial strains: 1, *B. cepacia*; 2, *P. diminuta*; 3, *P. fluorescens*; 4, *P. stutzeri*; 5, *P. aeruginosa* PAK; 6, *P. aeruginosa* PAO1. The letters on top indicate the restriction enzymes used to digest the genomic DNAs: P, *Pst*I; E, *Eco*RV. The numbers on the left side are the positions migrated by DNA size markers during electrophoresis.

transcriptional level, equal amounts of total cellular RNA, isolated from sodium arsenite-exposed (for 15, 30 and 60 min, respectively) and unexposed cells, were loaded onto a nylon membrane, fixed, dried and hybridized to a ^{32}P -labelled DNA fragment containing

Table 2. MICs of arsenic and antimony oxyanions ($\mu\text{g ml}^{-1}$)

Strain	As(III)	As(V)	Sb(III)
<i>P. aeruginosa</i>	200	800	75
<i>P. fluorescens</i>	200	> 2000	< 25
<i>P. diminuta</i>	75	> 2000	< 25
<i>P. stutzeri</i>	75	> 2000	50
<i>B. cepacia</i>	300	> 2000	50

the *P. aeruginosa* chromosomal *ars* genes. The autoradiograph (Fig. 4a) showed a dramatic increase in hybridization in the arsenite-exposed RNA samples when compared to the unexposed sample, suggesting that transcription of the *P. aeruginosa* chromosomal *ars* operon is inducible by arsenic oxyanions. Quantification analysis using the Image Quant program (Molecular Dynamics) revealed at least a 15-fold increase in *ars*-specific RNA upon addition of $0.1\ \mu\text{g As ml}^{-1}$ (as sodium arsenite) for 15 min, indicating that *ars* mRNA is rapidly induced by arsenite. This level of increase is in a similar range with that of the *E. coli* *ars* operon, as determined by an *arsB::lacZ* gene fusion expression assay (Diorio *et al.*, 1995). Moreover, a decrease in the intensity of the *ars*-specific RNA was observed at 60 min post-exposure (Fig. 4a), consistent with previous observations for the *E. coli* chromosomal and the plasmid R773 *ars* operons (Cai & DuBow, 1996; Owolabi & Rosen, 1990).

The *P. aeruginosa* *ars* operon is conserved in the chromosomes of other *Pseudomonas* species

Southern blotting analysis of chromosomal DNAs from other *Pseudomonas* species using a ^{32}P -labelled probe containing part of the *P. aeruginosa* chromosomal *ars* operon (Fig. 4b) detected hybridizing sequences in the chromosomes of *P. aeruginosa* strain PAK, another major wild-type strain of *P. aeruginosa* (Minamishima *et al.*, 1968; Takeya & Amako, 1966), and of *P. fluorescens*. However, no hybridizing sequences were found in the chromosomal DNAs of *P. stutzeri*, *P. diminuta* and *B. cepacia* under the conditions used, suggesting that the *P. aeruginosa* chromosomal *ars* operon is conserved, at the DNA level, in some, but not all, *Pseudomonas* species.

To determine whether the conservation of the *ars* operon is related to the intrinsic level of resistance to these toxic oxyanions, the MICs of arsenic and antimony oxyanions for each of the plasmid-free wild-type *Pseudomonas* species were determined (Table 2). The intrinsic resistance to arsenite appears to be related to the presence of the *ars* operon in the *Pseudomonas* species examined, as *P. aeruginosa* and *P. fluorescens* were found to have higher MICs than the two species (*P. diminuta* and *P. stutzeri*) that do not have any detectable *ars*-homologous sequences. However, the conservation of the *ars* operon does not directly correlate with cellular resistance to arsenate and antimonite oxyanions. Moreover, *B.*

cepacia showed the highest resistance to arsenite whilst displaying no detectable homology to the *ars* operon. These observations support the existence of other mechanisms against arsenic toxicity (Huang *et al.*, 1993; Huckle *et al.*, 1993).

DISCUSSION

The *P. aeruginosa* chromosomal *ars* operon is the second functionally characterized chromosomally located *ars* operon of bacteria identified so far. The significant sequence similarities shared by its three ORFs with *arsR*, *arsB* and *arsC* of other known *ars* operons, and its ability to complement an *E. coli* *ars* mutant, confirmed its molecular identity as a member of the *ars* family. Multiple alignments of the amino acid sequences of the putative *P. aeruginosa* ArsR with the ArsR proteins of other known *ars* operons, using the CLUSTAL program (Higgins & Sharp, 1988, 1989), revealed the conservation of the putative metal-binding motif and DNA-binding helix–turn–helix motif. This suggests that the *P. aeruginosa* chromosomal *ars* operon may be regulated in a similar manner to other known *ars* operons, and we found that expression of the operon was arsenic/antimony-inducible at the transcriptional level. The fold induction is in a similar range to that of the *E. coli* *ars* operon. Among the three *ars* polypeptides, the putative ArsB protein of the *P. aeruginosa* chromosomal *ars* operon is the most conserved, consistent with what has been observed with other known *ars* operons (Silver *et al.*, 1993; Diorio *et al.*, 1995; Silver, 1996). Both the ArsR and ArsB proteins share greater homology with their respective proteins from Gram-negative *ars* operons, which is not unexpected since *P. aeruginosa* is a Gram-negative bacterial species. Surprisingly, the putative ArsC protein of the *P. aeruginosa* chromosomal *ars* operon shares greater homology with the ArsC polypeptides of Gram-positive *ars* operons than with those of *ars* operons from Gram-negative bacteria.

A functional analysis of the *P. aeruginosa* chromosomal *ars* operon in *P. aeruginosa* and *E. coli* suggests that, like its *E. coli* counterpart, it can provide enhanced resistance to arsenic and antimony oxyanions in both bacterial species when cloned in a multicopy plasmid (Carlin *et al.*, 1995; Diorio *et al.*, 1995). However, when the *P. aeruginosa* chromosomal *ars* operon was introduced into wild-type *E. coli*, only increased resistance to arsenite and antimonite was observed, and no detectable increase was found in arsenate resistance. This is in contrast to our observation that the *E. coli* chromosomal *ars* operon cloned in a multicopy plasmid and introduced into wild-type *E. coli* increased resistance to both arsenite and arsenate ions (Diorio *et al.*, 1995). The lack of increased arsenate resistance could be due to several reasons. First, the MIC assay suggests that the intrinsic level of arsenate resistance in *P. aeruginosa* PAO1 is much lower than other *Pseudomonas* strains and species tested. Secondly, previous studies have shown that the Gram-positive ArsC protein of the *Staphylococcus aureus* plasmid p1258 *ars* operon requires thioredoxin and thioredoxin reductase for proper function (Ji &

Fig. 5. Sequence and prediction of a potential mRNA secondary structure in the intergenic region between *arsB* and *arsC* of the *P. aeruginosa* chromosomal *ars* operon. The termination codon UGA of the *arsB* gene is underlined. The putative ribosome-binding sequence (SD) is marked with triangles. The translation initiation codon AUG of the *arsC* gene is marked with circles. The free energy of formation of the indicated secondary structure is $-29.3 \text{ kcal mol}^{-1}$ (-123 kJ mol^{-1}), as estimated by the Hibio DNASIS program (Hitachi Software Engineering).

Silver, 1992a; Ji *et al.*, 1994), whereas the activity of the Gram-negative ArsC protein from the *E. coli* plasmid R773 *ars* operon requires glutathione and glutathione reductase (Oden *et al.*, 1994). As the ArsC protein is required for resistance to arsenate oxyanions, the physiological differences between *E. coli* and *P. aeruginosa* may contribute to suboptimal functioning of the *P. aeruginosa* ArsC protein in the heterologous cells. In addition, the closer relationship of ArsC to Gram-positive *ars* operons may result in the *arsC* gene of *P. aeruginosa* not being well expressed in *E. coli*. Analysis of the DNA sequence of the *P. aeruginosa* chromosomal *ars* operon revealed a larger intergenic region between the *arsB* and *arsC* genes (26 bp) than that found in other known *ars* operons (e.g. 12 bp in the *E. coli* chromosomal and plasmid R773 *ars* operons). It is possible that this large intergenic region may negatively affect expression of the *arsC* gene in *E. coli*, as previous studies have shown an optimal aligned spacing of 5 nt between the Shine–Dalgarno (SD) sequence and the translation-initiation codon for ribosomes to efficiently translate mRNAs in *E. coli* (Chen *et al.*, 1994). Secondary structures involving the SD sequence and internal complementary sequences inhibit translation initiation (Chang *et al.*, 1995), and predictions of the *P. aeruginosa* chromosomal *ars* mRNA in the intergenic region between the *arsB* and *arsC* cistrons show a potential secondary structure (Fig. 5) involving both the putative SD sequence and the initiation codon of ArsC. Formation of this secondary structure in the *ars* mRNA could hinder the binding of ribosomes to the SD sequence and inhibit the initiation of translation. None-

theless, in an *ars* mutant background, a low level of ArsC expression may still lead to significant reduction of arsenate to arsenite, and result in the observed higher level of arsenate resistance in *E. coli* LF20012(pJC801) than in strain LF20012(pBR322). This low-level expression (or activity) of ArsC from pJC801 may have been obscured by the fully induced endogenous *E. coli* chromosomal *arsC* gene, leading to no observable increase of arsenate resistance in wild-type *E. coli*.

It has been shown that both the *E. coli* chromosomal and plasmid R773 *ars* operons are transcribed upon arsenic exposure, and that the transcripts are processed within 1 h (Cai & DuBow, 1996; Owolabi & Rosen, 1990). Evidence also indicated that overexpression of the *E. coli* ArsR or ArsBC proteins is toxic to the cells (Cai & DuBow, 1996). Therefore, a control on the upper level of *ars* mRNA (provided, where present, by the ArsD protein) may protect the cells from *ars*-polypeptide-induced toxicity. In the present study, the *P. aeruginosa* chromosomal *ars* operon is found to be transcribed upon cellular exposure to subinhibitory levels of arsenite ions, and the level of *ars*-specific mRNA decreases with prolonged induction time, consistent with what has been observed for other *ars* operons, and a potential mechanism to repress further expression of the *ars*-encoded proteins. Southern blotting revealed *ars*-homologous sequences in *P. aeruginosa* strain PAK and in *P. fluorescens*, but not in *P. stutzeri*, *P. diminuta* and *B. cepacia*, a species formerly classified as *P. cepacia*. The latter three species are more distantly related to *P. aeruginosa* than *P. fluorescens*. It is interesting to note that this conservation pattern was not directly reflected in the intrinsic level of resistance to these toxic oxyanions (i.e. MICs) in these bacterial species, suggesting the existence of other cellular mechanisms involved in arsenic resistance in these micro-organisms. Further genomic sequencing and identification of chromosomal *ars* operons in other bacteria will shed light on the evolution of this highly conserved and important bacterial operon. Moreover, recent results have shown this type of arsenic resistance mechanism in mammals (Wang & Rossman, 1993; Wang *et al.*, 1994, 1996), supporting the notion that the protective function of *ars* operons has been strongly conserved in the course of evolution.

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