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 pL258 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.
oxygen 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 oxygenases, 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 nonenteric 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 oxygenases, 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::Δ(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::lacAB chromosomal transcriptional gene fusion (Cai & Dubow, 1996), and is thus genotypically arsB and hypersensitive to arsenic oxygenases (Cai & Dubow, 1997). P. aeruginosa PA01, 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. Rampal (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’) 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 pTJ140 (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 PA01 cells was performed according to Farinha & Krepsinski (1990). Transformation of pTJ140 and pKAS300 into PA01 was performed by electroporation. a-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 PA01 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 PA01 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 Scal–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 ColEl origin for replication in E. coli. The recombinant plasmid from E. coli, called pKAS300, was used to transform P. aeruginosa strain PA01.

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 (Epigen Centre 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 PA01 strain with or without the cloned P. aeruginosa chromosomal ars operon homologue was also determined. Since P. aeruginosa PA01 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 PA01 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 10⁻⁴ 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 × 10⁶ c.p.m. labelled probe for 16–18 h at 39 °C. The probe was isolated from pJC801 as a 1:14 kb BamHI–Sp6I 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 PA01 and dot-blotting analysis. Cultures of P. aeruginosa strain PA01 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

<table>
<thead>
<tr>
<th><em>ars</em> gene</th>
<th>ArsR</th>
<th>ArsB</th>
<th>ArsC</th>
</tr>
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<tr>
<td><em>E. coli</em></td>
<td>69.3 (84.9)</td>
<td>69.9 (85.5)</td>
<td>10.6 (27.6)</td>
</tr>
<tr>
<td>R773</td>
<td>43.6 (59.8)</td>
<td>69.3 (85.6)</td>
<td>12.8 (24.1)</td>
</tr>
<tr>
<td>IncN R46</td>
<td>43.6 (57.3)</td>
<td>69.9 (85.5)</td>
<td>12.1 (23.6)</td>
</tr>
<tr>
<td>Tn2505</td>
<td>42.7 (56.4)</td>
<td>68.9 (83.3)</td>
<td>13.5 (29.1)</td>
</tr>
<tr>
<td>pJC801</td>
<td>28.8 (49.0)</td>
<td>51.7 (72.7)</td>
<td>29.0 (45.8)</td>
</tr>
<tr>
<td>pSX267</td>
<td>27.9 (46.2)</td>
<td>51.4 (72.6)</td>
<td>29.0 (45.8)</td>
</tr>
</tbody>
</table>

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, 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.
Pseudomonas aeruginosa chromosomal ars operon

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. Cellulor 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).

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).

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 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 arsenite resistance in the E. coli arsB mutant as well as in P. aeruginosa PAO1, it did not show any detectable enhancement of arsenite 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
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 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 μg 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. 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>As(III)</th>
<th>As(V)</th>
<th>Sb(III)</th>
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<tbody>
<tr>
<td>P. aeruginosa</td>
<td>200</td>
<td>800</td>
<td>75</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>200</td>
<td>&gt;2000</td>
<td>&lt;25</td>
</tr>
<tr>
<td>P. diminuta</td>
<td>75</td>
<td>&gt;2000</td>
<td>&lt;25</td>
</tr>
<tr>
<td>P. stutzeri</td>
<td>75</td>
<td>&gt;2000</td>
<td>50</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>300</td>
<td>&gt;2000</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 2. MICs of arsenic and antimony oxyanions (μg ml$^{-1}$)
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/ammonium-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 pL258 *ars* operon requires thioredoxin and thioredoxin reductase for proper function (Ji & 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 *ArsR* 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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