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# An *Escherichia coli* Chromosomal *ars* Operon Homolog Is Functional in Arsenic Detoxification and Is Conserved in Gram-Negative Bacteria

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**Arsenic is a known toxic metalloid, whose trivalent and pentavalent ions can inhibit many biochemical processes. Operons which encode arsenic resistance have been found in multicopy plasmids from both gram-positive and gram-negative bacteria. The resistance mechanism is encoded from a single operon which typically consists of an arsenite ion-inducible repressor that regulates expression of an arsenate reductase and inner membrane-associated arsenite export system. Using a *lacZ* transcriptional gene fusion library, we have identified an *Escherichia coli* operon whose expression is induced by cellular exposure to sodium arsenite at concentrations as low as 5 μg/liter. This chromosomal operon was cloned, sequenced, and found to consist of three cistrons which we named *arsR*, *arsB*, and *arsC* because of their strong homology to plasmid-borne *ars* operons. Mutants in the chromosomal *ars* operon were found to be approximately 10- to 100-fold more sensitive to sodium arsenate and arsenite exposure than wild-type *E. coli*, while wild-type *E. coli* that contained the operon cloned on a ColE1-based plasmid was found to be at least 2- to 10-fold more resistant to sodium arsenate and arsenite. Moreover, Southern blotting and high-stringency hybridization of this operon with chromosomal DNAs from a number of bacterial species showed homologous sequences among members of the family *Enterobacteriaceae*, and hybridization was detectable even in *Pseudomonas aeruginosa*. These results suggest that the chromosomal *ars* operon may be the evolutionary precursor of the plasmid-borne operon, as a multicopy plasmid location would allow the operon to be amplified and its products to confer increased resistance to this toxic metalloid.**

Arsenic is a metalloid found in the environment, and it exists commonly in trivalent and pentavalent ionic forms (24). Its toxic properties are well-known and have been exploited in the production of antimicrobial agents, such as the first specific antibiotic (Salvorsan 606) and the African sleeping sickness drug Melarsen, in addition to the commonly used wood preservative chromated copper arsenate (5, 45). Because of increasing environmental concentrations as a result of industrialization, perhaps it is not surprising that plasmid-located genes which confer resistance to arsenic have been isolated from bacteria (21, 35). These arsenic resistance determinants (*ars*), isolated from both gram-positive and gram-negative bacterial species, have been found to be very homologous and generally consist of either three or five genes that have been organized into a single transcriptional unit (38). In the well-studied *ars*-containing plasmid R773, isolated from *Escherichia coli* (7, 34), the operon consists of five genes that are controlled from a single promoter located upstream of the first cistron (*arsR*). These cistrons, *arsRDABC* (in that order), encode an arsenic-inducible repressor (*arsR*) (46), a negative regulatory protein that controls the upper level of transcription (*arsD*) (48), an ATPase plus membrane-located arsenite efflux pump (*arsA* and *arsB*, respectively) (20, 29, 35, 42), and an arsenate reductase (*arsC*) (18). In the well-studied *ars*-containing plasmids isolated from *Staphylococcus* species (plasmids pI258 and pSX267), the *arsR*, *arsB*, and *arsC* cistrons are conserved, while the *arsD* and *arsA* cistrons are absent (17, 30). In this case, the ArsB protein is believed to use the cell's membrane potential to drive the efflux of intracellular arsenite ions (18). The origin of these homologous plasmid-borne arsenic resistance determinants has not yet been defined.

Given the ubiquitous presence of arsenic, we sought to determine if bacteria contain chromosomally located genes whose expression is induced at elevated arsenic ion concentrations and which aid cells in detoxification of episodic increases in extracellular arsenic (39). We have previously reported the presence of aluminum (12)- and nickel (14)-inducible genes in *E. coli* by screening a library of 3,000 single-copy *Vibrio harveyi* luciferase gene fusion chromosomal insertion clones (13) for changes in light emission upon addition of these metals. Using a collection of *lacZ* chromosomal gene fusions prepared with MudI (6), we report here the identification of an arsenic-inducible operon in the chromosome of *E. coli* located at 77.5 min. The cloning and sequencing of this operon revealed that it can encode proteins that are highly homologous to plasmid-encoded *ars* determinants and that its expression is inducible at arsenic ion concentrations just above the environmental background (9, 39). We also show that this operon is present in the chromosomes of a wide variety of gram-negative bacterial species and that it is a functionally important determinant in detoxification of arsenic ions in *E. coli.*

## MATERIALS AND METHODS

**Bacterial strains and phages.** The following bacterial strains used were all derivatives of *E. coli* K-12: *E. coli* 40 (Δ*pro-lac rpsL trp*), *E. coli* BU5029 (a *recA* mutant derivative of strain 40), and those described (including sources) by Autexier and DuBow (1). Phages MudI and P1*vir* were kind gifts of M. Casadaban (University of Chicago) and R. Stewart (McGill University), respectively.

**DNA manipulations.** All restriction endonuclease hydrolyses and DNA ligations were performed as described by Tolias and DuBow (43). DNA sequencing

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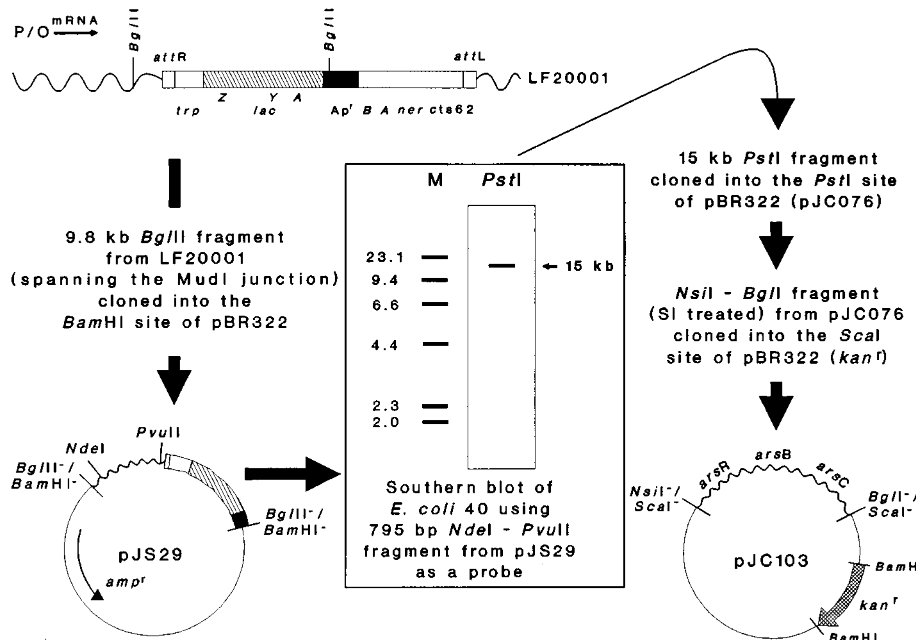


FIG. 1. Strategy used to clone the chromosomal arsenic-inducible gene. See Materials and Methods for details. Displayed at the top of the figure is a schematic drawing (not drawn to scale) of the MudI insertion in the chromosome. Mu DNA (▤), the *bla* gene (■), the *lac* operon (▧), *trp*-derived sequences (□), and *E. coli* chromosomal DNA (wavy line) are indicated. The direction of transcription from the putative arsenic-inducible promoter-operator region (P/O) is also shown. The construction of pJS29 and pJC103 is outlined. Lane M in the Southern blot, the positions of λ DNA fragments generated after cleavage with *Hin*dIII. The 2,973-bp *Nsi*I-*Bgl*I fragment from pJC103 was subcloned and sequenced.

of both strands (see Fig. 2A) was performed by the dideoxy DNA sequencing method with single-stranded DNAs from cloned fragments in plasmids pUC118 and pUC119 (44) by using the Sequenase version 2.0 kit from United States Biochemicals. Southern blotting and hybridization, as well as isolation of total cellular DNA, were performed according to the method of Autexier and DuBow (1), while P1 transduction was done according to the method of Miller (25). DNA was isolated from stationary-phase cells grown in Luria-Bertani (LB) (25) broth (*E. coli* 40 and *Pseudomonas aeruginosa* PA01) or nutrient broth (Difco Laboratories) (*Shigella sonnei*, *Citrobacter freundii*, *Enterobacter cloacae*, *Salmonella arizonae*, *Erwinia carotovora*, and *Klebsiella pneumoniae*) at 37°C, except for *E. coli* 40, *P. aeruginosa* PA01, and *S. arizonae*, which were grown at 32°C. For Southern blotting, 10 μg of total cellular DNA was digested with the appropriate restriction enzyme and blotted to nylon (Hybond-N; Amersham) membranes following electrophoresis through 0.75% agarose gels (31). Membranes were probed with $2 \times 10^8$ to $4 \times 10^8$ cpm of an $\alpha$-$^{32}$P-labelled 1,188-bp *Eco*RV (bp 1664 to 2852 [see Fig. 2A]) or a 587-bp *Nde*I-*Eco*RV (bp 1077 to 1664 [see Fig. 2A]) DNA fragment (prepared by the random priming method [31]) per ml under high-stringency conditions (1). After being washed, membranes were exposed to Agfa Curix RPI X-ray film.

**Construction of strains LF20001 and LF20018.** *lacZ* fusions to chromosomal genes were constructed by infecting *E. coli* 40 with MudI (*amp lac*) bacteriophages as described by Casadaban and Cohen (6). The resultant clones were picked to a master plate and replicated on LB agar plates that contained ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Research Organics, Inc.) plus increasing concentrations of sodium arsenate (0.1 to 10 μg/ml). One clone, which became blue when it was grown in the presence of sodium arsenate and remained white in its absence, was named LF20001 and isolated for further study. *E. coli* LF20018 was constructed by P1*vir* transduction (25) of *E. coli* LF20001 into *E. coli* 40 and selection on LB plates that contained ampicillin. The resultant Ap$^r$ clone (*E. coli* LF20018) was tested for arsenic induction of β-galactosidase, and the location of the MudI prophage was determined by Southern blotting and hybridization, with the *lacZ* gene as the probe.

**Isolation of the arsenic-inducible operon.** To isolate the proximal portion of the arsenate-inducible operon, a *lac* operon-Mu *attR*-*E. coli* DNA fragment was cloned from strain LF20001 via isolation of total cellular DNA (13), cleavage with *Bgl*II, ligation into *Bam*HI-cleaved pBR322 DNA, and transformation into *E. coli* BU5029. One colony, which developed a blue color on LB agar with ampicillin and X-Gal (because of amplification of the *lac* operon), was selected, and its plasmid was designated pJS29 (Fig. 1). The cloned chromosomal DNA adjacent to the right end of the MudI insertion was isolated and used as a probe to identify a 15-kb *Pst*I fragment from the chromosome of *E. coli* 40. The 15-kb fragment was cloned into pBR322 (digested with *Pst*I) to yield plasmid pJC076 (Fig. 1) by standard procedures (31). A 3-kb *Nsi*I-*Bgl*I fragment that encompassed the site of MudI insertion in strain LF20001 was subcloned (plasmid pJC103) and completely sequenced.

**β-Galactosidase assays.** β-Galactosidase assays were performed as described by Miller (25) by the chloroform-sodium dodecyl sulfate cell lysis procedure. Cells were grown to an $A_{550}$ of 0.4 in LB broth at 32°C and exposed to various arsenic and antimony compounds, and aliquots were removed for β-galactosidase assays after 30 min.

**Arsenic sensitivity tests.** The sensitivities of *E. coli* strains to trivalent and pentavalent arsenic ions were determined by preparing petri plates that contained LB agar and various concentrations of sodium arsenate and sodium arsenite. Overnight cultures of *E. coli* strains grown in LB broth were diluted in fresh LB broth and grown at 32°C to an $A_{550}$ of approximately 0.4. Then, cells were diluted $10^5$-fold in LB broth, and 0.1 ml of these dilutions was spread (in triplicate) on different agar plates. Petri dishes were incubated at 32°C overnight, and then colonies were counted.

**Nucleotide sequence accession number.** The nucleotide sequence reported here has been submitted to the EMBL database, and it has been assigned accession number X80057.

## RESULTS

**Discovery of a chromosomal *ars* operon homolog.** A collection of *lacZ* transcriptional gene fusions was prepared by using *E. coli* 40 and the MudI bacteriophage (6). In order to identify any gene whose transcription is specifically affected by arsenic salts, this collection of clones was replicated on petri dishes in the absence and presence of various concentrations of sodium arsenate and the β-galactosidase indicator substrate X-Gal. A single clone which formed blue colonies on petri plates that contained sodium arsenate and white colonies in its absence was identified. This clone, designated strain LF20001, was isolated for further study. A P1*vir* transductant of the MudI prophage region was also prepared in *E. coli* 40 and designated *E. coli* LF20018. The DNA adjacent to the MudI prophage was mapped by Southern blotting (with the *lac* operon as the probe), cloned, and used as a probe to map and isolate the DNA sequences that flank the MudI insertion site from the chromosome of *E. coli* 40 (Fig. 1). By hybridization of the

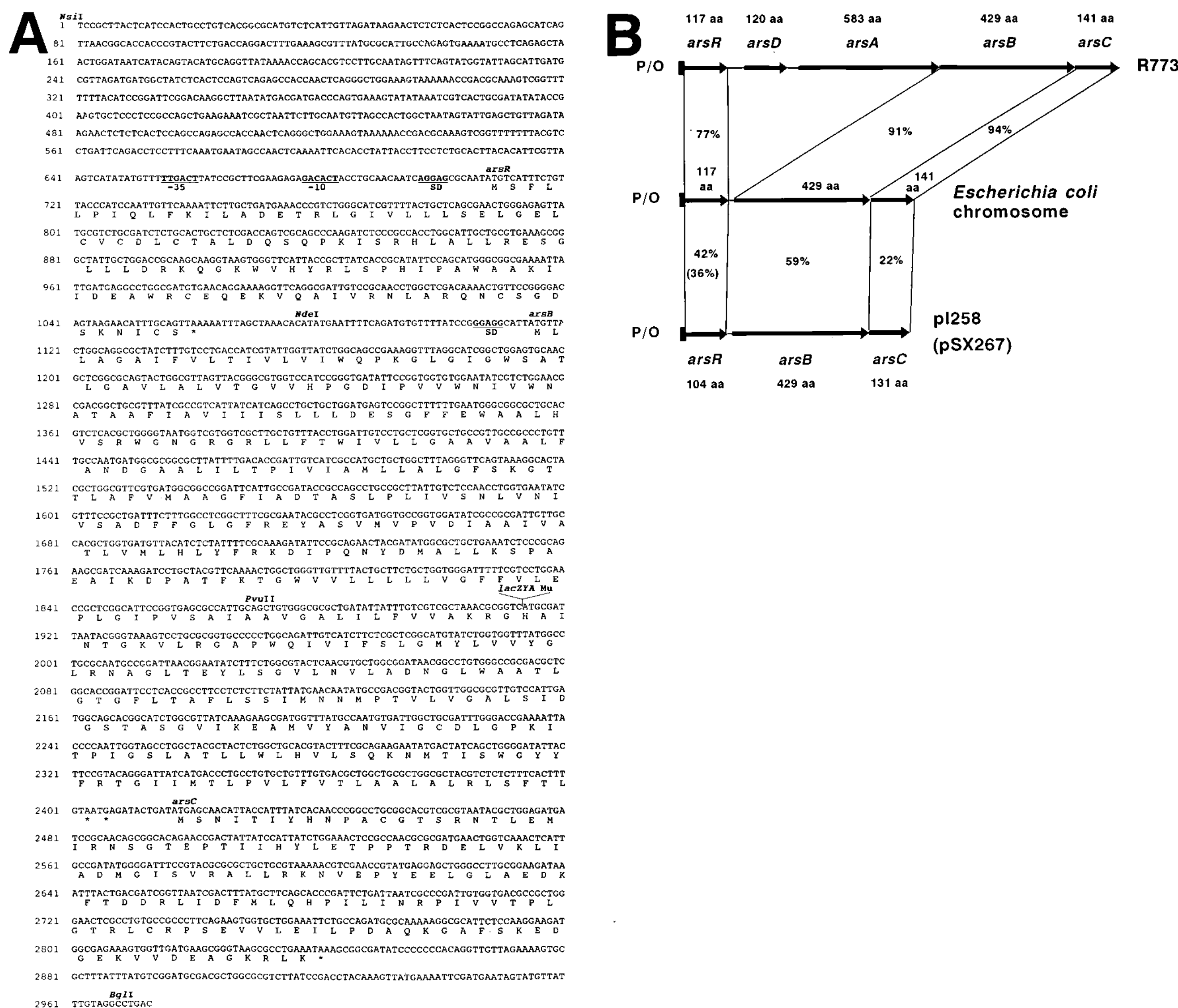


FIG. 2. (A) Nucleotide sequence of DNA cloned in pJC103 that contained the *arsRBC* genes. The predicted amino acid sequences of gene products are shown below the DNA sequence. Stop codons are marked by asterisks. Shine-Dalgarno (SD) sequences are printed in bold and underlined. The putative promoter sequences for $\sigma^{70}$ RNA polymerase are printed in bold, underlined, and identified as −10 and −35. The location and orientation of the MudI insertion, as determined by DNA sequencing from LF20001, as well as several restriction enzyme sites are also shown. (B) Homologies among the arsenic resistance determinants of *E. coli* plasmid R773, *Staphylococcus* plasmids pI258 and pSX267, and the chromosomal *ars* operon of *E. coli*. The promoter-operator (P/O) regions are indicated. The sizes of putative gene products (in amino acids [aa]) are shown above or below genes. The numbers between lines are percentages of similarity among the ArsR, ArsB, and ArsC proteins.

chromosomal DNA (12) in plasmid pJC103 to the Kohara et al. phage set (22), it was determined that the arsenate-inducible gene was located at 77.5 min on the *E. coli* genetic map. A total of 2.973 kb of DNA was sequenced (EMBL accession number X80057) (Fig. 2A) from plasmid pJC103 and used to scan databases with the University of Wisconsin Genetics Computer Group sequence analysis software package. It was found that this region of the chromosome is highly homologous to the arsenic-inducible *ars* operons of plasmid isolates from *E. coli* (7, 32, 48), *Staphylococcus aureus* (17), and *Staphylococcus xylosus* (30) (Fig. 2B); thus, because of its homology and arsenate inducibility, it was designated *ars*. This chromosomal *ars* operon was found to consist of three cistrons, which we have named *arsR*, *arsB*, and *arsC* because of strong homology to the plasmid-borne *ars* operons. The *arsR* cistron is 77.0% homologous (at the protein level) to the same cistron in the *ars* operon isolated from plasmid R773 of *E. coli*, while the *arsB* and *arsC* cistrons are 90.7 and 94.3% homologous, respectively, with this operon. Weaker, though still significantly homologous, are the plasmid-encoded ArsR, ArsB, and ArsC proteins of both *S. aureus* and *S. xylosus* (Fig. 2B). The location of the MudI prophage in *E. coli* LF20001 was found to be in the *arsB* gene, 799 bp downstream from the ATG start codon. Transcription of the inserted promoterless *lac* operon would occur from an upstream promoter (Fig. 2A), presumably located in a position similar to that of the *ars* operon in plasmid R773 (47).

**Effects of various arsenic and antimony compounds on *ars* gene expression.** The plasmid-borne *ars* operons are inducible by various toxic arsenic and antimony compounds (38). In order to measure induction of expression of the chromosomal *ars* operon by these compounds, *E. coli* LF20001 was grown

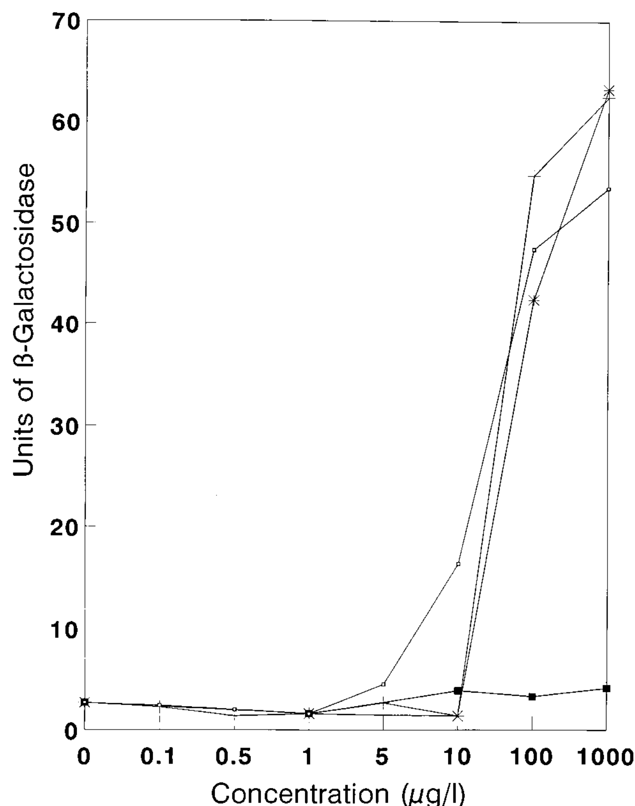


FIG. 3. β-Galactosidase levels in *E. coli* LF20001 cells at 30 min after exposure to arsenic and antimony compounds. Cells were grown to an $A_{550}$ of 0.4 and exposed to the indicated final concentrations of sodium arsenate (+), sodium arsenite (□), cacodylic acid (■), and antimony oxide (✻), and the amounts of β-galactosidase produced after 30 min were measured as described in Materials and Methods.

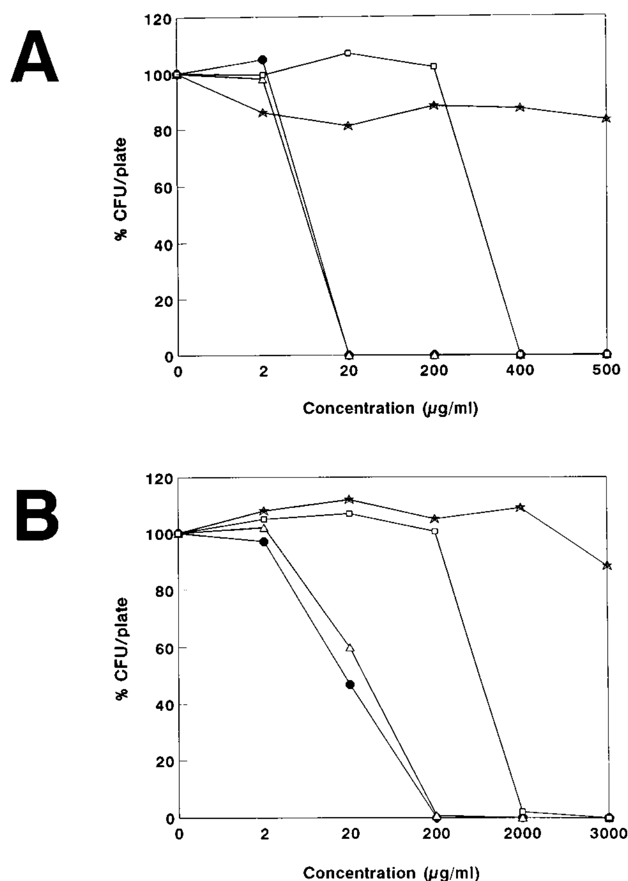


FIG. 4. Arsenic sensitivity tests on solid media. Log-phase cultures of *E. coli* 40 (□), LF20001 (△), LF20018 (●), and LF20020 [*E. coli* 40(pJC103)] (★) were diluted and spread on petri dishes that contained the indicated concentrations of sodium arsenite (A) and sodium arsenate (B), as described in Materials and Methods. Colonies were counted after 18 h and expressed as the average percentage of CFU per plate, compared with those obtained in the absence of added arsenic compounds.

in LB broth and exposed to various compounds, and β-galactosidase activity was quantified. Maximal levels of β-galactosidase activity were reached at approximately 60 min after exposure of strain LF20001 to sodium arsenate at final concentrations that ranged from 1 to 10 μg/ml (data not shown). When strain LF20001 was exposed to increasing concentrations of sodium arsenite, induction of gene expression was detectable at 30 min postexposure and 5 μg/liter, with maximal induction observed at 1 μg/ml (Fig. 3). Sodium arsenate, the pentavalent (and less toxic) form of arsenic, did not induce *ars* operon expression at 30 min postexposure and 5 μg/liter. However, higher concentrations (100 and 1,000 μg/liter) were able to induce expression of the *arsB*::*lacZ* fusion. Antimony (as antimony oxide), located just below arsenic in the periodic table, was also found to induce *ars* operon expression, as it does for plasmid-borne *ars* operons (34). However, cacodylic acid, a relatively nontoxic pesticide which contains arsenic in an organic formulation (3), was unable to induce expression of the *arsB*::*lacZ* fusion, even when added at arsenic concentrations as high as 1 μg/ml (Fig. 3). The observed threshold and concentration-dependent induction of the chromosomal *arsB*::*lacZ* fusion are very similar to those observed for plasmid-borne *ars* operons (8). No induction of the *arsB*::*lacZ* fusion was observed with other (Pb, Zn, and Cu) metal ions at concentrations that ranged from 0.01 to 10 μg/ml, nor was β-galactosidase activity from wild-type *E. coli* induced or affected by arsenic and antimony compounds at the concentrations used here (data not shown).

**A functional role for the *ars* operon in protection from arsenic toxicity.** In order to determine if the chromosomal *ars* operon plays a functional role in arsenic detoxification, clones with (*E. coli* 40) and without (*E. coli* LF20001 and LF20018) a functional *ars* operon were tested in identical genetic backgrounds for their growth in arsenic-containing LB media. Increasing concentrations of sodium arsenite or sodium arsenate were added to LB agar on petri dishes, and the colony-forming capacities of various strains were examined after overnight growth. The 50% lethal concentrations of sodium arsenate and sodium arsenite for *E. coli* 40 were found to be between 200 and 2,000 μg/ml (Fig. 4), and similar results were obtained for *E. coli* MG1655 (data not shown). Disruption of the chromosomal *ars* operon by MudI insertion was found to increase the sensitivity of *E. coli* 40 to sodium arsenite (Fig. 4A) and sodium arsenate (Fig. 4B) by approximately 10- to 100-fold (Fig. 4A). However, when the complete *ars* operon, cloned on a multicopy plasmid (pJC103), was introduced into wild-type *E. coli* 40, resistance to sodium arsenite (Fig. 4A) and sodium arsenate (Fig. 4B) increased by at least 3- to 10-fold, though the absolute levels of resistance were somewhat lower than those observed for *E. coli* that contained the *arsRDABC* operon of plasmid R773 (34). The ability of *E. coli arsB* mutant strains (LF20001 and LF20018) to survive at arsenic levels which

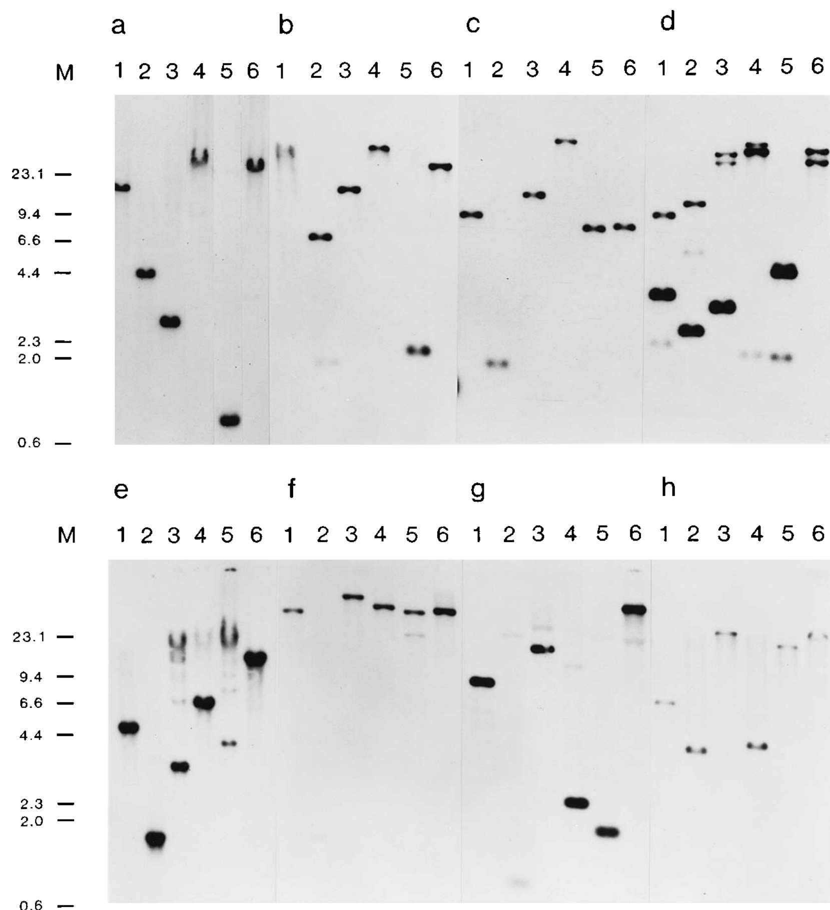


FIG. 5. Southern hybridization analyses of chromosomal DNAs from various bacterial species to detect sequences homologous to those of the *E. coli* chromosomal *arsRBC* genes. DNA was digested with *Pst*I (lanes 1), *Eco*RV (lanes 2), *Bgl*II (lanes 3), *Bam*HI (lanes 4), *Pvu*II (lanes 5), and *Hin*dIII (lanes 6). (a) *E. coli* 40; (b) *S. sonnei*; (c) *C. freundii*; (d) *E. cloacae*; (e) *S. arizonae*; (f) *E. carotovora*; (g) *K. pneumoniae*; (h) *P. aeruginosa*. Lanes M, markers (in kilobases) are λ DNA digested with *Hin*dIII. (a and b) Hybridized with an *Nde*I-*Eco*RV *arsB* probe; (c to h) probed with an *Eco*RV fragment that contained the *arsB* and *arsC* genes. See Materials and Methods for details.

induce *ars* operon expression (0.01 to 1 μg/ml) may be due to other cellular detoxification mechanisms, such as those provided by glutathione and thioredoxin (11, 15, 16).

**Sequences homologous to the chromosomal *ars* operon are highly conserved.** Because of the high degree of homology between the protein products of the *E. coli* chromosomal *ars* operon and those found on plasmids from both gram-negative and gram-positive bacteria, we sought to determine if the chromosomal operon was conserved at the DNA level (and thus, possibly the progenitor of plasmid-based arsenic resistance determinants). DNA was isolated from a number of plasmid-free, gram-negative bacterial species (1), hydrolyzed with restriction enzymes, and Southern blotted after agarose gel electrophoresis. After hybridization with an *E. coli* *ars*-specific probe, sequences that were homologous to the *E. coli* chromosomal *ars* operon were found in all of the enterobacterial species examined. Moreover, homologous sequences to the *ars* operon were detected in the nonenterobacterial species *P. aeruginosa* (Fig. 5). This high degree of evolutionary conservation at the DNA level strongly reinforces notions that the chromosomal *ars* operon is functionally important and that its chromosomal presence is not of recent origin.

## DISCUSSION

We have discovered a functional, arsenic-inducible operon in the chromosome of *E. coli*, with homologous sequences detectable in many other gram-negative bacterial species. This operon displays strong homology, both in protein sequence and genetic organization, with plasmid-borne arsenic detoxification operons. During the later stages of this work, continued sequencing of the *E. coli* genome also uncovered this chromosomal *ars* operon homolog, though no functional studies were performed (40). The names *arsE*, *arsF*, and *arsG* were given to these three homologous cistrons, but *arsR*, *arsB*, and *arsC* more accurately reflect their evolutionary relatedness and probable function(s). Thus, it is likely that the chromosomal *ars* operon is organized as a single transcription unit that is regulated by the arsenic- and antimony-inducible ArsR repressor. Moreover, the structural genes of the chromosomal *ars* operon appear to encode an arsenate reductase (*arsC*) and an arsenite-specific efflux system (*arsB*). The apparent strong evolutionary conservation of chromosomal *ars* determinants also suggests that this operon may be the progenitor of plasmid-borne *ars* operons. The origins of many plasmid-borne resistance determinants have not yet been elucidated. However, it is known that β-lactamases are also highly conserved, whether their lo-

cations are chromosomal or on plasmids (26, 33). In addition, hemolysins show similarly strong evolutionary conservation (2, 10). More recently, however, a chromosomal homolog of a plasmid-borne copper resistance operon has been found in *Pseudomonas syringae* (23). To our knowledge, its evolutionary conservation has not been determined.

It has been proposed that the structure of the plasmid-borne, ATP-driven arsenic efflux pump, made up of the ArsA and ArsB proteins, may be structurally related to the multiple drug resistance ATP-driven efflux pump found amplified in mammalian cancer cells (36, 37, 49). During chemotherapy of cancer patients, cells become resistant to anti-cancer chemotherapeutic agents by amplification of the number of copies of the multiple drug resistance gene and thus overexpression of the multiple drug resistance pump (28). In analogous fashion, amplification of the chromosomal *ars* operon, by its presence on multicopy plasmids, should allow increased resistance to cellular exposure to toxic arsenic salts. In this regard, we found that the presence of this operon in pBR322 (pJC103), under its own regulation, conferred at least a 3- to 10-fold increase in the resistance of *E. coli* to arsenate or arsenite exposure.

A *lacZ* fusion to the chromosomal *ars* operon was found to be induced by arsenic compounds at concentrations that reflected their relative toxicities (arsenate < arsenite). Moreover, antimony oxide also induced expression of the *arsB*::*lacZ* fusion. These results are consistent with those observed for the R773 *ars* operon of *E. coli* (47). We also observed a threshold of *ars* operon expression; induction was not observable at a concentration of less than 1 μg of arsenite per liter. A similar threshold effect on induction has been shown for the *mer* operon by its inducer, mercury (27). In addition, cacodylate did not induce the *arsB*::*lacZ* fusion, even at elevated concentrations. Thus, the induction of *ars* gene expression appears to reflect the relative toxicities of arsenic and antimony compounds. The LF20001 (*lacZYA* inserted in *arsB*) clone may therefore prove useful in determining the potential cytotoxicities of arsenic compounds (for enteric bacteria), as the assay for β-galactosidase is both rapid and quantitative (25).

In contrast to the *E. coli* plasmid-borne *ars* operon, the chromosomal *ars* operon contains neither the *arsD* nor *arsA* cistron. Low-stringency Southern blotting (41) and hybridization with cloned *arsR*, *arsB*, and *arsC* cistrons from plasmid R773 enabled these cistrons to be detected in the chromosome of *E. coli*, and the pattern of fragments was consistent with restriction enzyme mapping of the chromosomal *ars* region (data not shown). However, no signal was detected with the *arsD* and *arsA* cistrons as probes, even after long exposure times. These results imply that the *arsD* and *arsA* cistrons are not present at another location on the *E. coli* chromosome. Whether (and from where) plasmid-borne *ars* operons have gained the *arsD* and *arsA* cistrons or the *E. coli ars* operon has lost them is not clear at present. In this regard, it is interesting that the chromosomal *ars* operon organization more closely resembles that of gram-positive bacteria in lacking *arsD* and *arsA*, even though it is more homologous to that of gram-negative bacterial species.

The lack of an *arsA*-encoded ATPase subunit in the chromosomal *ars* operon is striking. Nonetheless, the ArsB subunit may function as an arsenite-specific efflux system which uses membrane potential instead of ATP hydrolysis by the *arsA* cistron. This mechanism has been observed for the plasmid-borne ArsB protein from *Staphylococcus* spp. (4) and in tetracycline efflux for the Tn*10*-derived tetracycline resistance determinant (19). Determinations of the structure(s) and function(s) of other chromosomal *ars* operons and the origin(s) of the *arsD* and *arsA* genes are currently in progress.

## ACKNOWLEDGMENTS

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