Identification of the metalloregulatory element of the plasmid-encoded arsenical resistance operon

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ABSTRACT

The regulatory region of the plasmid-encoded arsenical resistance (*ars*) operon was cloned as a 727-bp *Eco*RI-*Hin*dIII fragment. When cloned into a promoter probe vector this fragment conferred arsenite inducible tetracycline resistance in *Escherichia coli*, indicating that the fragment carried a regulatory gene, the *arsR* gene. A single region corresponding to −35 and −10 promoter recognition sites was identified. The transcriptional start site of the mRNA was determined by primer extension. The sequence has an open reading frame for a potential 13,179 Da polypeptide, termed the ArsR protein. The fragment was cloned into a temperature regulated expression vector. A protein with an apparent molecular mass of about 12 kDa was induced by either temperature or arsenite. This protein was purified and used to produce antibodies specific for the ArsR protein.

INTRODUCTION

The salts of arsenic and antimony are toxic to bacteria. The arsenical resistance (*ars*) operon of resistance plasmid R773 encodes an oxyanion pump, the first member of a new family of ion-translocating ATPases (1−3). In *Escherichia coli* this system catalyzes extrusion of arsenite, antimonite, and arsenate. Resistance results from lowering of the intracellular concentration of these toxic oxyanions (4−6). The nucleotide sequence of the structural genes of the operon has been reported (1). There are three structural genes, and the product of each has each been identified (1,2,7). The *arsA* and *arsB* gene products are sufficient to form a pump for arsenite and antimonite, the (+III) oxidation states of the metals (8), while the ArsC protein is postulated to be a modifier subunit which increases the substrate specificity to include arsenate, the (+V) oxidation state of arsenic (2,8,9).

Oxyanion resistance is inducible in the conjugative R-factor R773 but constitutive in the recombinant plasmid pUM3 (10). In pUM3 expression of the structural genes of the operon is dependent on the tetracycline P1 promoter of pBR322. In this report we describe the cloning of the *ars* operon with an intact regulatory region. Features of the regulatory region were identified, including the transcriptional start site and the product of the fourth gene, *arsR*. The *arsR* gene product, the ArsR protein, was subcloned, overexpressed and purified.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage

The *E. coli* strains and plasmids used in this study are described in Table 1. Cells were grown in LB medium (11). Where required ampicillin (40 μg/ml), kanamycin (40 μmg/ml), tetracycline (35 μg/ml) and arsenite (1 mM) were added to the growth medium. When used as a noninhibitory inducer, arsenite was added to 50 μM. Procedures for manipulating DNA were as described by Maniatis *et al.* (11). Plasmid pWSU1 was constructed from plasmid pUM1, which is inducible for arsenical resistance (10). The 33-kb plasmid pUM1 was digested completely with *Eco*RI and partially with *Hin*dIII. The fragments were ligated into pBR322 which had been digested with both *Eco*RI and *Hin*dIII. Transformed cells were screened for inducible arsenite resistance, resulting in the isolation of the 9.4-kb plasmid pWSU1 (Fig. 1). The restriction map differs from that of pUM3 only by the presence of the 0.73-kb *Eco*RI-*Hin*dIII fragment. For expression studies this fragment was excised from pWSU1 and inserted into the multiple cloning site of plasmid pKK175-6 (12) to create plasmid pWSU2, into pCP40 (13) to create pWSU3, and into plasmid pT7-5 (14) to create plasmid pWSU4. The fragment was also cloned into plasmids pUC18 and pUC19 and phages M13mp8 and M13mp9 (15) for sequencing with *E. coli* strain JM103 used as host. A *Hin*cII-*Hin*dIII digest of the 0.73-kb fragment cloned into M13mp9 was also used for sequencing.

DNA sequencing

The nucleotide sequence was determined by the dideoxy chain termination method of Sanger *et al.* (16) in both M13 and pUC plasmid derivatives using the enzyme Sequenase (United States Biochemicals). The primer for M13 derivatives was the M13 universal primer. In addition the M13 reverse primer was used with pUC18 and pUC19 derivatives. Analysis of the nucleotide sequence was performed using GENEPRO 4.20 (Riverside Scientific, Seattle, WA).

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Table 1. Strains and plasmids

Strain/plasmid /phage	Genotype/Description	Source or Reference
C600	*thr-1,leu-6,thi-1,supE44,lacY1,tonA21,*λ^-	11
HB101	F^-, *hsdS20,recA13,ara-14,proA2,lacY1,galK2, xyl-5,rpsL20,mlt-1,supE44,*λ^-	11
JM103	Δ(lac pro),strA,thi-1,supE,endA,sbcB,hsdR$^-$, proAB,lacI^q,ZDM15,traD36,F′	11
K38	HfrC(λ)	25
pWSU1	pBR322 with the *ars* operon cloned into *Eco*RI-*Hin*dIII digested pBR322 (Ap^r)	This study
pKK175-6	Promoter probe vector with multiple cloning region upstream of the *tet* gene (Ap^r)	12
pWSU2	0.73-kb *Eco*RI-*Hin*dIII fragment of pWSU1 cloned into *Eco*RI-*Hin*dIII digested pKK175-6	This study
pCP40	Expression vector with temperature-sensitive replication and a multiple cloning site downstream of the λ P_L promoter (Ap^r)	13
pWSU3	0.73-kb *Eco*RI-*Hin*dIII fragment cloned into *Eco*RI-*Hin*dIII digested pCP40	This study
p*cI*857	pCP40 compatible plasmid containing the temperature-sensitive *cI*857 gene	13
pGP1.2	T7 phage RNA polymerase gene under control of λp_L promoter; λcIts	14
pT7-5	Multiple cloning site downstream of T7 phage RNA polymerase promoter.	14
pWSU4	0.73-kb *Eco*RI-*Hin*dIII fragment inserted into *Eco*RI-*Hin*dIII digested pT7-5	This study

Transcript mapping

E. coli HB101 cells containing pWSU1 were grown in LB medium supplemented with ampicillin at 37°C to early log phase. Portions were transferred to two flasks prewarmed to 37°C, and sodium arsenite was added to one to a final concentration of 5 mM. The second flask received no inducer. Growth was continued for another 10 min. RNA was extracted from the cultures essentially as described (17).

A synthetic oligonucleotide, 5′-TTCCCGTAGCATCGCCA-GATGA-3′, complementary to the transcribed strand of *arsR* was end labeled using T4 polynucleotide kinase (Bethesda Research Laboratories) and γ-[^{32}P]ATP (6000 Ci/mmol, NEN) as described (11). RNA (10 μg) was annealed with labeled primer (5 fmol) for 20 min at 55°C in a total volume of 10 μl of a buffer consisting of 40 mM PIPES, pH 6.5, 0.4 M NaCl, and 1 mM EDTA. The RNA-DNA hybrids (10 μl) were added to 90 μl of extension mix containing 50 mM Tris-HCl, pH 8.5, 6 mM $MgCl_2$, 0.1 M KCl, 2 mM dithiothreitol, 2.5 μg actinomycin D, 100 units RNasin (Promega), 0.5 mM each of dATP, dCTP, dGTP and dTTP, and 30 units AMV reverse transcriptase (Life Sciences Inc.) and incubated at 42°C for 90 min. Samples (0.1 ml) were precipitated with ethanol and suspended in 10 μl dye mix (95% formamide, 0.04% xylene cyanol, 0.04% bromophenol blue). The cDNA products were heated at 85°C for 5 min, cooled on ice and separated by electrophoresis on 6% polyacrylamide gel containing 7 M urea. Single stranded DNA from an M13mp8 phage with the 0.73-kb *Eco*RI-*Hin*dIII fragment was sequenced using the end-labeled oligonucleotide described above as primer. The sequencing reactions were run in parallel with the products of primer extension for determination of the transcript start site.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Protein samples were prepared by boiling in sodium dodecyl sulfate (SDS) sample buffer (18) for 5 min. SDS polyacrylamide gel electrophoresis (PAGE) was carried out as described by Laemmli (18) on 18% acrylamide gels with 40% urea to increase resolution of low molecular weight proteins. Proteins were visualized with a silver stain (19). The ArsR protein could not be detected by staining with Coomassie blue.

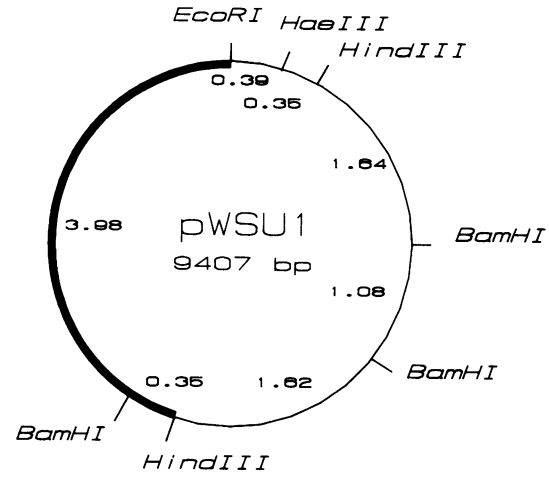

Figure 1. Physical map of plasmid pWSU1. Plasmid pWSU1 was constructed from the 33-kb plasmid pUM1 by complete digestion with *Eco*RI and partial digestion with *Hin*dIII and ligation into plasmid pBR322 digested with both *Eco*RI and *Hin*dIII. Distances between restriction sites are given in kilobase units. The vector is shown as the heavy line, the insert as the light line.

Induction, isolation and purification of the ArsR protein

Cells of *E. coli* strain C600 bearing plasmids p*cI*857 and either pCP40 or pWSU3 were grown overnight at 28°C. In the morning cells were diluted 20-fold into fresh medium at 28°C incubated until the culture attained an optical density at 600 nm of 0.5 (approximately 3 hr). Induction was initiated by quickly raising

the temperature to 39°C. Samples were taken at hourly intervals and analyzed for induction of protein by SDS PAGE.

For the isolation of the ArsR protein, a culture of *E. coli* strain C600 bearing plasmids p*cI*857 and pWSU3 were grown at 28°C to an absorbance of 0.5 at 600 nm in 1 liter of LB medium containing 50 μM sodium arsenite. The culture was induced by raising the temperature to 39°C for 2 hr. The cells were harvested by centrifugation for 30 min at 9000×g and washed with a buffer consisting of 20 mM Tris-HCl, pH 7.4, all at 4°C . The pelleted cells (2 g wet weight) were suspended in 10 ml of the same buffer and lysed by a single passage through a French pressure cells at 20,000 psi. Unbroken cells were removed by centrifugation for 20 min at 12,000×g, and membranes were removed by centrifugation at 260,000×g for 2 h. All lysis and centrifugation steps were performed at 4°C. The cytosol was applied to a 1-ml Whatman P11 phosphocellulose column pre-equilibrated with a buffer of 20 mM potassium phosphate, pH 7.6, washed with 15 ml of the same buffer, and eluted with a 20 ml linear gradient of 0 to 1M NaCl in the same buffer. Column fractions of 1 ml were collected and analyzed by SDS PAGE. Fractions containing the ArsR protein were pooled, lyophylized, and dissolved in 1/5 volume of 0.1 M NH_4HCO_3 buffer, pH 7.8. To remove the minor high molecular weight contaminants, a portion was applied to a Superose 12 column (Pharmacia Chemical Co.) and eluted with the same buffer. All chromatography steps were performed at 4°C. Fractions containing the ArsR protein were pooled, lyophylized, dissolved in HPLC grade water, and used for amino acid analysis. Vapor phase hydrolysis was performed on a Waters 'Pico Tag' work station for 75 min at 150°C. A Waters 'Pico Tag' HPLC system employing pre-column phenylisothiocyanate derivatization was used for amino acid analysis and was performed by the Macromolecular Core Facility of the School of Medicine, Wayne State University.

^{35}S-Methionine labeling of the ArsR protein

The T7 expression system was used as described (14). Plasmid pWSU4 was used to transform strain K38 containing the gene for T7 RNA polymerase under the control of a temperature sensitive repressor on plasmid pGP1-2. To enable identification of the cloned gene product, cells were grown at 30°C in LB medium to an OD_{590} of 0.5 for labeling studies. A portion (0.2 ml) of these cells was washed four times with 5 ml of M-9 medium (11). The pelleted cells were suspended in 1 ml of M-9 medium supplemented with 0.4% glucose, 20 μg/ml thiamine and 19 common amino acids except methionine, each at a concentration of 50 μg/ml. The culture was incubated for 1 hr at 30°C. The T7 RNA polymerase was derepressed by transferring the cells to 42°C degrees for 15 min. Rifampicin (0.2 mg/ml) was added to inhibit *E. coli* RNA polymerase, and the cells incubated for another 10 min at 42°C. The temperature was then shifted to 30°C for 40 min. Aerated cells were labeled with 10 μCi ^{35}S-methionine (1000 Ci/mmol) for 5 min at 30°C, centrifuged and suspended in SDS sample buffer for SDS PAGE.

Immunoblot Procedure.

Samples were prepared by boiling in SDS sample buffer for 5 min and electrophoresed on a 15% polyacrylamide gel. Proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose for 12−16 h at 50 mA. Unoccupied sites on the nitrocellulose filter were blocked using 5% non-fat dried milk in phosphate buffered saline (21 mM KH_2PO_4, 11 mM $Na_2HPO_4 \cdot 7H_2O$, 0.138 M NaCl, 2.5 mM KCl, pH 7.4). The filter was incubated with rabbit anti-ArsR serum (1:2000) for 1 h and washed three times for 10 min each time with 15 ml of the same buffer containing 5% dried milk, all at 37°C. To detect the antigen antibody complex the filter was incubated with goat anti-rabbit IgG (1:5000) to which horse radish peroxidase was conjugated. The filter was incubated with the conjugated second antibody for 1 h at 37°C and washed three times with 15 ml of the same buffer. Hydrogen peroxide (0.03%) and 4-chloro-1-naphthol (3 mg/ml) were added in phosphate buffered saline to produce a color reaction. Polyclonal antibodies were raised in New Zealand rabbits immunized with purified ArsR protein.

Protein determination: The protein content of purified ArsR preparations was estimated from absorption at 280 nm (20). From the predicted amino acid composition the $E^{1\%}_{1\ cm}$ was calculated to be 14.0. Assays based on the procedure of Lowry *et al.* (21) gave much lower values.

RESULTS

Cloning the *ars* regulatory DNA

A 4.3-kb *Hin*dIII fragment was cloned in several steps from the 90-kb R-factor R773 into plasmid pBR322 to produce plasmid pUM3 (10). Although resistance to the toxic oxyanions was inducible by subinhibitory concentrations of arsenite, antimonite, and arsenate in cells bearing either the original R-factor R773 or the 33-kb recombinant plasmid pUM1, resistance was constitutive in cells bearing plasmid pUM3. A 5-kb DNA fragment was isolated from pUM1 following complete *Eco*RI digestion and partial *Hin*dIII digestion. Insertion of the fragment into *Eco*RI-*Hin*dIII digested pBR322 produced pWSU1 (Fig. 1), which exhibited inducible resistance to all three toxic oxyanions.

These results suggest that a 0.73-kb *Eco*RI-*Hin*dIII fragment, the only difference between pUM3 and pWSU1, contains the regulatory DNA of the *ars* operon. The 0.73-kb fragment was cloned into the multiple cloning site of the promoter probe vector pKK175-6, which contains a promoterless tetracycline resistance gene, to create pWSU2. The vector alone did not confer resistance to tetracycline, arsenite, or antimonite. Cells bearing plasmid pWSU2 were sensitive to tetracycline in the absence of arsenite or antimonite (Table 2). When added in sublethal concentrations (50 μM arsenite, 50 μM antimonite, or 1 mM arsenate) the oxyanion inducers of the *ars* operon were inducers of tetracycline resistance in cells bearing pWSU2, demonstrating that the 0.73-kb *Eco*RI-*Hin*dIII fragment is sufficient to confer the metalloregulatory phenotype.

Sequence analysis of the *ars* regulatory DNA

The regulatory DNA was determined to consist of 727 bp (Fig. 2). 73 bp from the *Eco*RI site are sequences common to the −35 and −10 regions of *E. coli* promoters (22). From bp 125−475 there is an open reading frame for a 117 amino acid residue polypeptide with a predicted mass of 13,213 kDa and an isoelectric point of 9.8. This is preceded by a reasonable ribosome binding site (23). From bp 482 to 512 is an inverted repeat capable of forming a stable stem-loop structure. Between the end of the open reading frame and the start of the *arsA* gene, the first structural gene, is a 431 bp untranslated region.

Localization of the 5′ terminus of *ars* mRNA

To identify the 5′ end of *ars* mRNA, primer extension analysis was performed using total RNA prepared from HB101 cells

Table 2. Promoter activity of the *ars* regulatory DNA in a promoter probe vector

Plasmid[1]	Addition[2]				
Antibiotic:	tet	tet	tet	tet	none
Oxyanion:	none	AsO_2^{-1}	SbO_2^{-1}	AsO_4^{-3}	AsO_2^{-1}
	Growth[3]				
pBR322 (vector)	+	+	+	+	−
pWSU1	−	−	−	−	+
pKK175-6 (vector)	−	−	−	−	−
pWSU2	−	+	+	+	−

[1] All plasmids expressed in *E. coli* strain HB101.
[2] Columns 1−4: tetracycline added at 35 μg/ml. Column 2: arsenite added at a subinhibitory concentration of 50 μM. Column 3: antimonite added at a subinhibitory concentration of 50 μM. Column 4: arsenate added at a subinhibitory concentration of 1 mM. Column 5: arsenite added at an inhibitory concentration of 1 mM. Ampicillin was added to all plates at 40 μg/ml.
[3]Growth was determined on LB agar plates and were scored for lack of growth (−) or growth of single colonies (+) after 48 hr at 37°C.

harboring the plasmid pWSU1. An oligonucleotide that hybridized to mRNA sequences within the coding region of the *arsR* gene was used as a primer. A major extension product ended at the T or C residue 16 or 17 nucleotides upstream of the *arsR* initiation codon (Fig. 3). Several minor species were observed within the *arsR* transcript and may be due to incomplete extension of the primer. The initiation site(s) are 6−7 nucleotides downstream of the '−10' region. The data are consistent with the presence of a single promoter involved in the transcription of *ars* mRNA.

Expression and purification of the ArsR protein

The 727-bp regulatory DNA was cloned into the multiple cloning site of pCP40 to create pWSU3. This puts the potential open reading frame under the control of tandem lambda phage pL and *ars* promoters. A polypeptide with an apparent mass of approximately 12 kDa, designated ArsR, was induced by temperature inactivation of the *cI*857 repressor (Fig. 4).

When cells induced at 42°C for more than 3 hr were lysed, nearly all of the ArsR protein was found in the low speed pellet. This indicates that the protein forms inclusion bodies under those conditions. The inclusion bodies could be solubilized with 0.5 M NaCl (24). The solubilized material migrated on SDS PAGE as a broad band in the range of 12 kDa, however the results of amino acid analysis suggested the presence of multiple species. Moreover, substantial amounts of the material precipitated upon removal of the NaCl. In contrast, the ArsR protein remained in the high speed supernant following induction for 2 hr at 39°C. Chromatography of this material on phosphocellulose resulted in a nearly homogeneous preparation of the induced polypeptide (Fig. 5). The minor amounts of higher molecular weight contaminants were removed by Superose 12 chromatography. The results of amino acid analysis of this protein were consistent with the theoretical composition deduced from the nucleotide sequence (Table 3). This protein was used to inoculate rabbits for production of antibodies.

The 0.73-kb *Eco*RI-*Hind*III fragment was cloned into a T7 expression system (14). Expression of the *arsR* gene under control of the T7 RNA polymerase resulted in incorporation of [^{35}S]methionine into a single band (Fig. 6). This band migrated to the same position as the silver stained ArsR protein (data not shown). Antibodies prepared against purified ArsR protein specifically reacted with the [^{35}S]-labeled translation product (Fig. 6). These results demonstrate that the *arsR* gene product is immunologically identical to the ArsR protein.

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   1 GAATTCGAAGTTATCTCACCTAGCTTAAGGTAATAGTGTGATTAATCATATGCGTTTTTG
                    -35                    -10      VV      RBS
  61 GTTATGTGTTGTTTGACTTAATATCAGAGCCGAGATACTTGTTTTGTACAAAGGAGAG
       M  L  Q  L  T  P  L  Q  L  F  K  N  L  S  D  E  T  R  L
 121 GGAAATGTTGCAACTAACACGACTTCAGTTATTTAAAAACGTGTCGGATGAAACGCGTTT
       G  I  V  L  L  L  R  E  M  G  E  L  C  V  C  D  L  C  M  A
 181 GGGTATCGTGTTGTTGCTCAGGGAGATGGGAGAGTTGTGCGTGTGTGATCTTTGCATGGC
       L  D  Q  S  Q  P  K  I  S  R  H  L  A  M  L  R  E  S  G  I
 241 ACTGGATCAATCACAGCCCAAAATATCCCGTCATCTGGCGATGCTACGGGAAAGTGGAAT
       L  L  D  R  K  Q  G  K  W  V  H  Y  R  L  S  P  H  I  P  S
 301 CCTTCTGGATCGTAAACAGGGAAAATGGGTTCACTACCGCTTATCACCGCATATTCGTTC
       W  A  A  Q  I  I  E  Q  A  W  L  S  Q  Q  D  D  V  Q  V  I
 361 ATGGGCTGCCGAGATTATTGAGCAGGCCTGGTTAAGCCAACAGGACGACGTTCAGGTCAT
       A  R  K  L  A  S  V  N  C  S  G  S  S  K  A  V  C  I
 421 CGCACGCAAGCTGGCTTCAGTTAACTGCTCCGGTAGCAGTAAGGCTGTCTGCATCTAAAA
          ---->    ---->|<----    <----
 481 AATTTGCCTGAACATATATGTTTTATCAAATGCGAGGTATTTAAGATGAAAACGTTAATG
 541 GTATTTGACCCGGCGATGTGTTGCAGCACCGGCGTCTGCGGTACAGATGTTGATCAGGCT
 601 CTGGTCGATTTTTCTACAGATGTGCAATGGCTCAAACAATGCGGTGTACAAATTGAGCGT
 661 TTCAATCTTGCGCAACAACCGATGAGCTTTGTACAGAACGAGAAGGTCAAAGCGTTTATT
 721 GAAGCTT
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Figure 2. Nucleotide sequence of the metalloregulatory element of the *ars* operon. The nucleotide sequence of the *Eco*RI-*Hind*III fragment was determined as described under *Materials and Methods*. Underlined regions include the putative promoter, transcriptional start site, ribosome binding site, protein coding region, and inverted repeat.

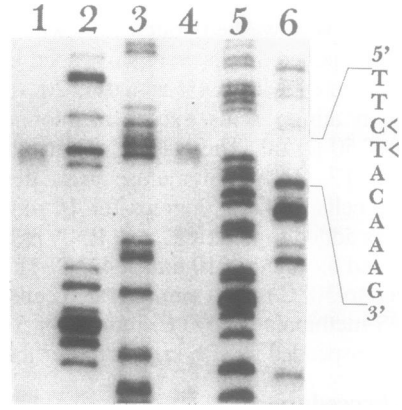

Figure 3. Determination of the transcriptional start site of the *ars* mRNA. The primer and reaction conditions were as described in *Materials and Methods*. *Lanes 1 and 4*: Primer extended with reverse transcriptase using RNA from induced pWSU1. *Lanes 2,3,5 and 6*: G,A,T and C reactions, respectively using the same [^{32}P]-labeled oligonucleotide as primer generated with the Klenow fragment of DNA polymerase. The template was single stranded DNA derived from M13mp8 containing the 727 base pair *Eco*RI-*Hind*III fragment. In the right margin the nucleotide sequence of the coding strand (the reverse complement of the observed sequence) is shown with the *ars* transcript initiation site(s) indicated.

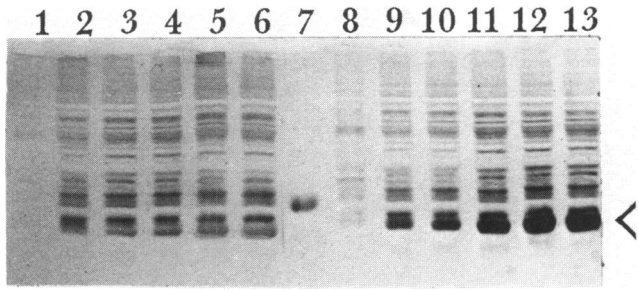

Figure 4. Expression of the *arsR* gene. Cultures of *E. coli* strain C600 bearing plasmids pcI857 and either pCP40 (vector) (*Lanes 1–6*) or pWSU3 (*arsR*$^+$) (*Lanes 8–13*) were grown at 28°C to 0.5 OD_{600nm}. The cultures were then induced by raising the temperature to 42°C. At the indicated times aliquots were centrifuged, suspended in SDS sample buffer in 1/15th the original volume and boiled for 5 min. Portions (5 μl) of each were analyzed for induction of ArsR protein by SDS PAGE on 18% polyacrylamide gels containing 40% urea. *Lanes 1 and 8*: 0 hr; *Lanes 2 and 9*: 1 hr; *Lanes 3 and 10*: 2 hr; *Lanes 4 and 11*: 3 hr; *Lanes 5 and 12*: 4 hr; *Lanes 6 and 13*: 5 hr; *Lane 7*: Cytochrome c (12 kDa). The position of the induced protein is indicated.

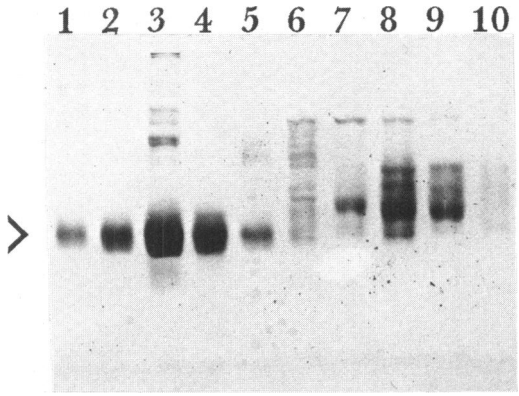

Figure 5. Phosphocellulose chromatography of the ArsR protein. The ArsR protein was isolated and chromatographed on a phosphocellulose column as described in *Materials and Methods*. Portions (15 μl) of column fractions were analyzed by SDS PAGE on an 18% polyacrylamide gel containing 40% urea. Lanes 1–10 represent successive fractions from the gradient of 0–1M NaCl. The ArsR protein (*arrow*) eluted over a salt concentration of 0.1–0.3 M NaCl.

DISCUSSION

The toxicity of the oxyanions of arsenic and antimony have led to the evolution of plasmid-mediated resistances. In gram negative bacteria the mechanism of resistance is ATP-driven anion extrusion catalyzed by a plasmid-encoded oxyanion-translocating ATPase (2,3,6). The arsenical resistance operon of R-factor R773 is metalloregulated, with expression dependent on the levels of arsenic or antimony salts in the medium. The structural genes alone are constitutively expressed when under control of the tetracycline P1 promoter of pBR322 (10). In this report we show that all information necessary for regulation of the operon is contained within a 727-bp *Eco*RI-*Hin*dIII fragment. Within this region are a single promoter region followed by the start site of the mRNA transcript. Only a single mRNA species is transcribed from that promoter, and no internal promoter-like sequences are observed (Owolabi and Rosen, unpublished). The 727-bp fragment produces arsenite inducible tetracycline resistance in a promoter probe vector, and so is sufficient for induction. Within the fragment is an open reading frame preceded by a reasonable ribosome binding site. The putative protein

Table 3. Composition of the ArsR protein[1]

Residue	Experimental[2]	Theoretical[1]	% Total Residues
A	9	8	6.8
C	nd	5	4.3
D	9 (D+N)	6	5.1
E	15 (E+Q)	5	4.3
F	2	1	0.9
G	5	5	4.3
H	2	3	2.6
I	7	8	6.8
K	6	6	5.1
L	19	19	16.2
M	3	4	3.4
N	–	2	1.7
P	4	4	3.4
Q	–	10	8.5
R	9	7	6.0
S	10	11	9.4
T	3	2	1.7
V	8	7	6.0
W	nd	3	2.6
Y	1	1	0.9

[1] Theoretical composition deduced from the nucleotide sequence. Single letter codes are used for each amino acid.
[2] Experimental composition determined by amino acid analysis. Values are normalized using L (leucine) = 19, the theoretical number of leucine residues per ArsR molecule. nd: not determined.

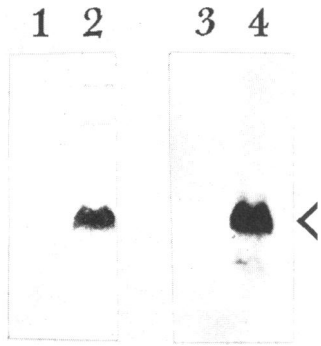

Figure 6. Immunoblotting of [^{35}S]methionine labeled *arsR* gene product. The *arsR* gene was cloned under the control of the rifampicin-resistant T7-RNA polymerase promoter. *In vivo* synthesis of protein was accomplished in the presence of rifampicin and [^{35}S]methionine, allowing specific labeling of the cloned gene products, as described in *Materials and Methods*. Samples from labeled cells of *E. coli* strain K38 bearing plasmids pGP1.2 and either pT7-5 (vector) (*lanes 1 and 3*) or pWSU4 (*arsR*) (*lanes 2 and 4*) were boiled in SDS sample buffer and electrophoresed on a 15% SDS polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose and probed with anti-ArsR serum (*lanes 1 and 2*), following which the blotted nitrocellulose was autoradiographed (*lanes 3 and 4*).

product is a basic 13 kDa polypeptide. When cloned behind either the λ_{pL} or T7 promoters the fragment produces a polypeptide of about 12 kDa which was purified. Although migration on SDS PAGE of this basic protein is slightly faster than expected, the amino acid composition of the polypeptide was consistent with that of the putative *arsR* open reading frame. Antibodies prepared against the protein specifically reacted against the [^{35}S]methionine-labeled polypeptide, demonstrating that the purified protein was indeed a product of the *arsR* gene and is presumably the regulatory protein of the *ars* operon.

The function of ArsR as a regulatory protein has yet to be demonstrated. The *arsR* gene is transcribed from the same promoter as the three structural genes, forming a single transcriptional unit (Owolabi and Rosen, unpublished).

Preliminary results suggest that in the absence of inducer a short transcript corresponding only to the *arsR* message is produced. Of interest is an inverted repeat potentially capable of forming a stable stem-loop structure immediately following the *arsR* reading frame (Fig. 2). One possible model is that this structure acts as a transcriptional terminator in the absence of inducer. In the presence of inducer the ArsR protein serves as an antiterminator to allow transcription of the structural genes.

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