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Construction of a Chimeric ArsA-ArsB Protein for Overexpression of the Oxyanion-translocating ATPase*

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Resistance to toxic oxyanions of arsenic and antimony in *Escherichia coli* is conferred by the conjugative R-factor R773, which encodes an ATP-driven anion extrusion pump. The *ars* operon is composed of three structural genes, *arsA*, *arsB*, and *arsC*. Although transcribed as a single unit, the three genes are differentially expressed as a result of translational differences, such that the ArsA and ArsC proteins are produced in high amounts relative to the amount of ArsB protein made. Consequently, biochemical characterization of the ArsB protein, which is an integral membrane protein containing the anion-conducting pathway, has been limited, precluding studies of the mechanism of this oxyanion pump. To overexpress the *arsB* gene, a series of changes were made. First, the second codon, an infrequently used leucine codon, was changed to a more frequently utilized codon. Second, a GC-rich stem-loop (ΔG = −17 kcal/mol) between the third and twelfth codons was destabilized by changing several of the bases of the base-paired region. Third, the re-engineered *arsB* gene was fused 3′ in frame to the first 1458 base pairs of the *arsA* gene to encode a 914-residue chimeric protein (486 residues of the ArsA protein plus 428 residues of the mutated ArsB protein) containing the entire re-engineered ArsB sequence except for the initiating methionine. The ArsA-ArsB chimera has been overexpressed at ~15–20% of the total membrane proteins. Cells producing the chimeric ArsA-ArsB protein with an *arsA* gene in *trans* excluded $^{73}AsO_2^-$ from cells, demonstrating that the chimera can function as a component of the oxyanion-translocating ATPase.

The arsenical resistance (*ars*) operon of the conjugative R-factor R733 of *Escherichia coli* provides resistance to oxyanions of arsenic and antimony (1). Resistance derives from the action of an operon-encoded ATP-dependent pump that extrudes the toxic oxyanions from the cells (2). Thus, the intracellular concentration of the oxyanions never rises to toxic levels, hence resistance.

The *ars* operon has been cloned as a single 5068-bp[1] fragment from the original 90-kilobase R-factor into small multicopy plasmids (3). Analysis of the nucleotide sequence reveals five genes, two regulatory (4)[2] and three that encode the proteins that effect resistance to arsenite (antimonite) and arsenate (2). Of the five, the *arsA* and *arsB* genes encode the subunits of the pump. The ArsA protein is physiologically part of a complex with the ArsB protein in the inner membrane of *E. coli* (5). When overexpressed from multicopy plasmids, the ArsA protein is found predominantly as a soluble protein in the cytosol and is produced to ~10% of the total cell proteins (6). The purified ArsA protein exhibits arsenite (antimonite)-stimulated ATPase activity (6, 7). The extremely hydrophobic ArsB protein, located in the inner membrane of *E. coli*, serves as the membrane anchor for the catalytic ArsA subunit and has been postulated to be the portion of the pump that conducts anions (2, 5).

Previous attempts to express the ArsB protein in amounts sufficient for biochemical analysis by increasing plasmid copy number or the rate of transcription from various promoters have been unsuccessful. The *arsB* gene is transcribed as the middle reading frame of a polycistronic mRNA, with the *arsA* reading frame upstream and the *arsC* reading frame downstream. However, the amounts of ArsB protein synthesized are only a fraction of the ArsA and ArsC proteins. This differential translation correlates with degradation of the mRNA. It was found from Northern analysis that the operon was transcribed initially as a full-length 4400-nucleotide RNA (8). The transcript was rapidly degraded to two species, a 2700-nucleotide RNA containing the *arsR* and *arsA* sequences and a 500-nucleotide RNA containing the *arsC* sequence. The original transcript was cleaved within the *arsB* sequence. The half-life of the 4400-nucleotide transcript was <4 min, whereas the half-lives of the 2700- and 500-nucleotide byproducts were ~10 min. Analysis of the *arsB* translational initiation region (TIR) indicated possible formation a stable hairpin beginning with the third codon of the *arsB* coding sequence. In addition, the second codon is not the most frequently utilized leucine codon (9). Either or both factors could produce pausing of the ribosome at the start of the *arsB* sequence. The downstream RNA would be relatively unpopulated with ribosomes and thus susceptible to endonuclease attack. The combination of inefficient translational initiation of mRNA and a rapid loss of the *arsB* message could be responsible for the low production of the ArsB protein. This might be a general mechanism by which cells prevent overexpression of membrane proteins.

Determination of the mechanism of the arsenical pump requires knowledge of the structure and function of the ArsB protein. For biochemical studies, the protein must be produced in substantial amounts by controlled expression of the *arsB* gene. In this study, we report the functional overexpression of a re-engineered ArsB protein as a chimera with the ArsA

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[1] The abbreviations used are: bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TIR, translational initiation region.

[2] J. Wu and B. P. Rosen, unpublished results.

protein. In addition, we describe insights on difficulties and solutions for the overexpression of membrane proteins in *E. coli* cells.

EXPERIMENTAL PROCEDURES

Materials—All restriction enzymes and nucleic acid-modifying enzymes were obtained from Bethesda Research Laboratories. Oligonucleotides were synthesized in the Macromolecular Core Facility of Wayne State University, School of Medicine. All other chemicals were obtained from commercial sources.

Strains, Plasmids, and Phage—*E. coli* strains, plasmids, and phage used in this study are described in Table I. *E. coli* strains SURE, SK5671, and SG20043 were used to minimize degradation of DNA, mRNA, and proteins, respectively. *E. coli* strain HB101 was mainly used as host cell for gene cloning and DNA preparation.

DNA Manipulations—The conditions for plasmid isolation, DNA restriction endonuclease analysis, ligation, transformation, and sequencing have all been described (10).

Media and Growth Conditions—Cells were grown either in LB medium (11) or, for *E. coli* strains TG1 and JM109, in a basal salts medium (10). Cells were grown at 37 °C, unless otherwise noted. Antibiotics were added as required at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 25 μg/ml; chloramphenicol, 35 μg/ml; and kanamycin, 45 μg/ml. Sodium arsenite was added at the indicated concentrations to test for arsenical resistance. For cloning with plasmid pUC19 as a vector in *E. coli* strain JM109, 50 μg/ml 5-

TABLE I
Strains, plasmids, and phage

Strain/plasmid/phage	Genotype/description[a]	Ref.
SURE	*e14⁻(mcrA), endA1, thi, relA1, gryA96, sbcC, recB, recJ, umaC::Tn5 (Km^r), uvrC*	Stratagene
SG1	K12, *lac-proAB', supE, hsd-5, thi,* F'(*traD36, proAB, lac1^q, ΔlacZ-M15'*)	Amersham Corp.
SK5671	*ams-1, pnp-7, thyA715*	44
JM107	*endA1, gyrA96, thi, hsdR17, supE44, relA1, lac-proAB',* F'(*traD36, proAB, lac1^q, ΔlacZ-M15'*)	39
SG20043	*lon-100, cps-3::Tn10, proCYC221*	48
HB101	*supE44, hsd20(r_B^-, m_B^-), recA13, ara-14, proA2, lacY1, galK2, rpsL20*	10
pACYC184	Tc^r, Cm^r, low-copy number vector	46
pJLA503	Ap^r, temperature-inducible expression vector with $\lambda p_L/\lambda p_R$ promoters and *atpE* leader	15
pJLA504	Ap^r, pJLA503 with different linker	15
pJBS633	Km^r, Tc^r, *blaM* fusion vector	45
pUM3	*arsABC* genes of R773 cloned into *Hin*dIII site of pBR322, As^r	3
pWSU1	Complete *ars* operon cloned into *Eco*RI and *Hin*dIII sites of pBR322, As^r	12
pBM100	224-bp *Sph*I-*Csp*45I fragment containing mutated *arsB1* gene without *arsB* TIR cloned into *Sph*I-*Csp*45I-digested pBR322, Ap^r, As^s	This study
pBM101	*Sph*I-*Bst*XI fragment containing first 39 bp of *arsB1* from pBM100 subcloned into *Sph*I-*Bst*XI-digested pUM3 to replace *arsA* gene and same portion of *arsB* gene, Ap^r, As^s	This study
pBM200	2492-bp *Sph*I-*Eco*RI fragment containing multiply mutated *arsB2* gene cloned into *Sph*I-*Eco*RI-digested pBR322, Ap^r, As^s	This study
pBM202	*Sph*I-*Bst*XI fragment from pBM200 containing first 39 bp of multiply mutated *arsB2* gene inserted into *Sph*I-*Bst*XI-digested pUM3, Ap^r, As^s	This study
pB100	2492-bp *Sph*I-*Eco*RI fragment containing *arsB1* gene of pBM101 cloned into *Sph*I-*Eco*RI-digested pJLA504, Ap^r, As^s	This study
pB200	2492-bp *Sph*I-*Eco*RI fragment containing *arsB2* gene of pBM202 cloned into *Sph*I-*Eco*RI-digested pJLA504, Ap^r, As^s	This study
pB300	Oligonucleotide encoding sequence for 9-amino acid HA1 sequence (YPYDVPDYA) of influenza virus linked to 5'-end of *arsB2* gene in pJLA503, Ap^r, As^s	This study
pBL10	2858-bp *Eco*RI-*Ssp*I fragment encoding first 51 residues of wild-type *arsB* gene fused with *blaM* gene, Km^r, Ap^r (arsenical-inducible)	This study
pBL20	1790-bp *Ssp*I fragment containing first 51 residues of *arsB1* gene from plasmid pB100 fused with *blaM* gene, Km^r, Ap^r (temperature-inducible)	This study
pBL30	1790-bp *Ssp*I fragment containing first 51 residues of *arsB2* gene from plasmid pB200 fused with *blaM* gene, Km^r, Ap^r (temperature-inducible)	This study
pBL40	2513-bp *Eco*RI-*Ssp*I fragment from plasmid pJUN4 cloned into *Eco*RI-*Pvu*II-digested pJBS633 to create ternary fusion gene *arsA-arsB2-blaM*, Km^r, Ap^r (arsenical inducible)	This study
pArsA	2408-bp *Hin*dIII fragment containing *arsA* gene cloned into *Hin*dIII-digested pACYC184, Cm^r	This study
pBC100	681-bp *Pst*I fragment deleted from *arsA* gene of plasmid pWSU1 (*arsBC*).	This study
pHIN19	pUC19 with deletion of sequence AGCT from multiple cloning site, Ap^r	This study
pHIN100	2492-bp *Sph*I-*Eco*RI fragment containing *arsB1* gene from plasmid pB100 cloned into inserted *Sph*I-*Eco*RI-digested pHIN19, Ap^r	This study
pHIN200	2492-bp *Sph*I-*Eco*RI fragment containing *arsB2* gene from plasmid pB200 cloned into inserted *Sph*I-*Eco*RI-digested pHIN19, Ap^r	This study
pJUN4	2498-bp *Sph*I (blunt-ended)-*Sal*I fragment containing *arsB2* gene from plasmid pB200 cloned into plasmid pWSU1 that had been digested with *Bam*HI, blunt-ended, and digested with *Bam*HI to create *arsAB2* fusion gene, Ap^r	This study
M13 mCM6-d6-37	M13 phage carrying wild-type *ars* genes with Bal-31 deletion of *arsA* gene	4

[a] Tc, tetracycline; Cm, chloramphenicol; Ap, ampicillin; Km, kanamycin; As, oxyanions of arsenic and antimony. Superscripts s and r indicate sensitivity and resistance, respectively.

bromo-4-chloro-3-indolyl β-D-galactoside and 45 μg/ml isopropyl-1-thio-β-D-galactopyranoside were added to solid media.

Mutagenesis by Polymerase Chain Reaction—For creation of mutated *arsB* genes, the targeted DNA fragments from positions −10 to 282 of the *arsB* gene were enzymatically synthesized by polymerase chain reaction (PCR) (13). Phage M13mCM6-d6-37 carrying the wild-type *arsB* gene was used as the template DNA (4). Oligonucleotides PCR10 and PCR20 (Table II), which hybridize to the opposite strands around the initiation region of the *arsB* gene, were used as the primers to amplify the flanked region. Oligonucleotide PCR20 was designed to create an *Sph*I site at the start of the *arsB* gene and to change the second codon to the most frequently utilized leucine codon (CUG) (9). The *arsB* gene containing the mutations introduced with oligonucleotide PCR20 is designed *arsB1*. The 240-bp products of PCR were digested with restriction endonucleases *Sph*I and *Csp*45I. The resulting 224-bp *Sph*I-*Csp*45I fragment was purified from a 3% agarose gel and ligated into *Sph*I- and *Csp*45I-linearized plasmid pUM3. The junction fragments were further amplified by PCR using oligonucleotides PCR10 and PCR30 as primers. Oligonucleotide PCR30 (Table II) was designed to add additional mutations to the *arsB1* gene to destabilize the predicted secondary structure in the *arsB* mRNA. The additional mutations were created in the GC-rich stem-loop between the third and twelfth codons of the *arsB* gene. The multiply mutated *arsB* gene is designated *arsB2*. For PCR, the series of cycles of template denaturation at 95 °C for 1 min, primer annealing at 50 °C for 1 min, and extension by Taq polymerase at 74 °C for 1 min was automatically performed using a TwinBlock PCR system (Ericomp, Inc.).

DNA Sequencing—All mutations were verified by dideoxy sequencing (14). The two differently mutated 224-bp *Sph*I-*Csp*45I fragments from the PCR reactions were cloned into plasmid pUM3. The resulting plasmids, pBM100 (*arsB1*) and pBM200 (*arsB2*), were digested with *Sph*I and *Eco*RI and ligated into plasmid pJLA504 (15) which had been linearized with the same enzymes. The double-stranded DNAs from the resulting plasmids, pB100 (*arsB1*) and pB200 (*arsB2*), were directly sequenced using a Sequenase reagent kit (version 2, U. S. Biochemical Corp.).

Construction of Recombinant pJUN4 Plasmid—A 135-bp *Bst*XI-*Sal*I fragment bearing the multiply mutated region of the *arsB2* gene from pBM200 was purified and ligated into plasmid pUM3 partially digested with *Sal*I and *Bst*XI, replacing the corresponding portion of the wild-type *arsB* gene with the mutated gene. The resulting plasmid, pBM202, was digested with *Sph*I, blunt-ended with S1 nuclease, and then digested with endonuclease *Sal*I. The resulting 2498-bp blunt-ended *Sal*I fragment, which contained the entire mutated *arsB* gene except for the initiating ATG, was ligated into plasmid pWSU1 which had been digested with *Sal*I, blunt-ended with S1 nuclease, and further digested with *Bam*HI. The resulting plasmid, pJUN4, contained the *arsB2* gene fused with the first 1458 bp of the *arsA* gene. The resulting $arsAB_2$ gene fusion would utilize the efficient TIR of the *arsA* gene.

Construction of lacZ-arsB Fusions—Plasmids pB100 and pB200, containing the mutated *arsB1* and *arsB2* genes, were digested with *Sph*I and *Eco*RI. The 2492-bp *Sph*I-*Eco*RI fragments were subcloned into plasmid pHIN19. Each mutated *arsB* gene was fused in frame to the first 12 bp of the *lacZ* gene, under control of the *lac* promoter. The resulting plasmids, pHIN100 (*arsB1*) and pHIN200 (*arsB2*), were designed to use the *lacZ* translational initiation region for translation of the *arsB* gene.

Construction of HA1-arsB Fusion—Complementary oligonucleotides HA100 and HA200, containing the corresponding sequence of hemagglutinin of the influenza virus (16), were annealed by heat treatment at 90 °C for 2 min and then cooled at 4 °C for at least 30 min. The annealed oligonucleotides were separated and purified from a 7% polyacrylamide gel and ligated into plasmid pUM3 at the *Nde*I and *Kpn*I sites. The resulting 753-bp *Sph*I fragment was ligated into plasmid pBM202 at the *Sph*I site. The 2525-bp *Nde*I-*Eco*RI fragment from the resulting plasmid was then ligated into *Nde*I-*Eco*RI-digested plasmid pJLA503, creating plasmid pB300. This would allow expression of the mutated *arsB2* gene fused with the HA1 epitope in plasmid pB300 under the control of tandem λp_L and λp_R promoters and would utilize the efficient *atpE* TIR (15).

Construction of arsB-bla Fusions—The 2858-bp *Eco*RI-*Ssp*I fragments from plasmid pWSU1, containing the *ars* promoter region and the first 153 bp of the wild-type *arsB* gene, were ligated into *Eco*RI-*Pvu*II-digested pJBS633 to create an in-frame *arsB-bla* fusion gene. In the same way, the 1790-bp *Ssp*I fragments from plasmids pB100 and pB200, containing the first 153 bp of the mutated *arsB* genes under the tandem λp_{LR} and λp_R promoters and *atpE* leader from pB100 and pB200, were ligated into the *Pvu*II site of pJSB633 to create *blaM* fusions plasmids pBL20 and pBL30, respectively. Transcription was regulated from the temperature-sensitive cI857 repressor on the same plasmid.

A ternary fusion (17) was constructed to put the *blaM* reporter gene at the 3′-end of the binary *arsAB2* fusion described above. Ternary fusion plasmid pBL40 (*arsA-arsB2-blaM*) was constructed from plasmid pJUN4 and contained the first 1458 bp of the *arsA* gene fused to the first 150 bp of the *arsB2* gene, in turn fused to the coding region of the mature *blaM* gene.

Polyacrylamide Gel Electrophoresis and Immunological Blotting—

TABLE II
PCR oligonucleotides

Oligonucleotides	Length	Sequence (5′ → 3′)[a]	Comments[b]
	nt[c]		
PCR10	21	CAGCAGGCGGCCACGACCGTT	Complementary to nt 261–282 of *arsB* coding sequence
PCR20	27	AGGAGGTTGCATGCTGCTGGCAGGAGC	Corresponds to nt-10 to +17 of *arsB* nucleotide sequence
PCR30	42	ATGGTGCATGCTGCTGGCGGGCGCCATTTTTATTCTGACCAT	nt 1–7 are complementary to sequence in pBR322 *tet* gene; nt 8–42 correspond to nt 1–35 of *arsB* coding sequence
HA100	43	TATGTACCCATACGATGTTCCGGATTACGCTGGCATGCGGTAC	nt 1–4 generate *Nde*I site and initiation codon; nt 5–31 contain coding sequence for HA1 epitope; nt 32–34 contain glycine codon and serve as linker; nt 35–43 serve as linker for *Sph*I and *Kpn*I sites for cloning of *arsB2* gene
HA200	37	CGCATGCCAGCGTAATCCGGAACATCGTATGGGTACA	Complementary to HA100

[a] The nucleotides that are different from the wild-type *ars* sequence are underlined.
[b] The A of the initiating codon ATG of the *arsB* gene is designated as +1.
[c] nt, nucleotides.

Samples were prepared by heating to 90 °C in SDS sample buffer for 2 min. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described (18). Immunoblotting was performed by electrophoretic transfer of the proteins to nitrocellulose (0.2 μm) for 18–24 h at 25 mV at 4 °C. Unoccupied sites on the nitrocellulose filter were blocked using 5% nonfat 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 antisera for 1 h and washed three times for 10 min each time with 15 ml of phosphate-buffered saline containing 5% dried milk, all at 37 °C. To detect the antigen-antibody complex, the filter was incubated with goat anti-rabbit IgG (1:3000) to which horseradish 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 phosphate-buffered saline. Hydrogen peroxide (0.03%) and 4-chloro-1-naphthol (3 mg/ml) were added in phosphate-buffered saline to produce a color reaction.

Membrane Binding of ArsA Protein—Partially purified ArsA protein was added to membrane vesicles (1 mg of membrane protein) in an assay mixture containing 25 mM Tris, pH 7.5, and 0.2 M potassium chloride in a total volume of 0.2 ml. The assay mixture was incubated at room temperature for 20 min. To remove unbound ArsA protein, the mixture was diluted 10-fold with a solution of 10 mM Tris, pH 8.0, 20% glycerol, and 0.2 M potassium chloride; pelleted by centrifugation at 100,000 × *g* for 1 h; and washed once in the same buffer. The membranes were suspended in 0.1 ml of SDS sample buffer and incubated at 90 °C for 5 min. Samples (10–20 μl) were analyzed by SDS-PAGE on 10% acrylamide gels. Five concentrations of purified ArsA protein were used to construct a standard curve. The proteins were immunoblotted using anti-ArsA serum (1:1000). The intensities of the color reactions on the nitrocellulose were analyzed by scanning the images with a Hewlett-Packard Scanjet Plus scanner and quantifying with a densitometric program (Stratagene). The standard curves generated with purified ArsA were used to calculate the amounts of the ArsA protein in the gels. The standard curve was linear to 150 ng of ArsA protein.

Alkaline Fractionation—Alkaline fractionation of membranes containing the overexpressed chimeric ArsAB2 protein was performed by a modification of the method of Ito and Akiyama (19). Cultures of *E. coli* HB101 bearing plasmid pJUN4 were induced at an A_{550} of 0.45 with 0.1 mM sodium arsenite for 10–120 min as indicated in the legend to Fig. 4. After induction, the cultures were each diluted to an A_{550} of 0.45 to give a constant number of cells for analysis. The cells from 20 ml of each culture were pelleted; suspended in 0.1 ml of a solution of 10 mM sodium phosphate buffer, pH 7.8, containing 1 mg/ml lysozyme, 5 mM EDTA, and 7 mM β-mercaptoethanol; and incubated for 10 min on ice. Cycles of quick freezing and thawing were repeated until the suspension clarified. The suspension was mixed with 0.1 volume of cold 0.1 N Na_2CO_3, mixed, and immediately centrifuged at 10,000 × *g* at 4 °C for 10 min. The supernatant solution was neutralized with 0.1 volume of 0.5 N phosphoric acid. Equivalent amounts of the supernatant solutions and pelleted materials (~30% of the starting material/lane) were analyzed by polyacrylamide gel electrophoresis (Fig. 4).

Separation of Inner and Outer Membranes—Cultures of *E. coli* HB101 (pJUN4) were induced with 0.1 mM sodium arsenite for 10 min. The cells were pelleted and gently suspended in an ice-cold buffer consisting of 10 mM Tris-HCl, pH 8.0, containing 0.75 M sucrose. Lysozyme was added to 0.1 mg/ml, and the suspension was incubated for 30 min at 4 °C. The suspension was then diluted with 2 volumes of 5 mM disodium EDTA, pH 7.5, and lysed by a single passage through a French pressure cell at 20,000 p.s.i. The lysate was centrifuged at 10,000 × *g* for 20 min. The supernatant solution was further centrifuged at 100,000 × *g* for 1 h to isolate total membranes. Total membranes were separated into inner and outer membrane fractions by suspension in an unbuffered 25% (w/w) sucrose solution containing 5 mM disodium EDTA, pH 7.5 and layered onto a continuous sucrose gradient (25–45%) that had been prepared by five cycles of rapid freezing and thawing. Following centrifugation at 110,000 × *g* for 16 h in a swinging bucket rotor, two bands were obtained. The upper and lower bands represented the cytoplasmic and outer membrane fractions, respectively (20). When the chimeric ArsAB2 protein was expressed at high levels, the upper band of the inner membrane was large and diffuse.

Detection of Inclusion Bodies—Inclusion bodies formed by overexpression of the *arsAB2* gene were isolated by a modification of the procedures of Marston (21) and of Kleid *et al.* (22). Cells of *E. coli* HB101 (pJUN4) were harvested after 60 min of induction with 0.1 mM sodium arsenite. The cells were converted to spheroplasts by suspension in 0.1 volume of a buffer consisting of 10 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl, 1 mM disodium EDTA, and 1 mg/ml lysozyme for 30 min at 23 °C. The spheroplasts were lysed with 0.2% sodium deoxycholate, and DNA was digested with 10 μg/ml DNase I. The inclusion bodies were isolated by centrifugation at 10,000 × *g* for 10 min and analyzed by polyacrylamide gel electrophoresis and immunoblotting.

Uptake of $^{73}AsO_2^-$—Accumulation of $^{73}AsO_2^-$ in the cells was performed as described previously (23). All procedures were performed at room temperature. Stationary phase cells from 200 ml of culture medium were pelleted and suspended in 1 ml of a triethanolamine-buffered minimal medium (24) containing 10 mM glucose, 5 mM KCl, 1 mM sodium arsenite, and 1.25 μCi of $^{73}AsO_2^-$. Portions of the suspension were filtered through nitrocellulose filters (0.45-μm pore size; Whatman) at intervals. The filters were washed with 5 ml of the same buffer, dried, and quantified by liquid scintillation counting.

RESULTS

Attempts at Expression of Re-engineered arsB Genes—The wild-type *arsB* gene of R-factor R733 was subcloned, mutated, and fused with the *arsA* gene as outlined in Fig. 1. To permit subcloning of the *arsB* coding sequence, a *Sph*I site (GCATGC) was created at the initiating codon by PCR. In the same process, the second codon, UUA for leucine, was changed to the more frequently utilized codon CUG (9). Using the newly created unique *Sph*I site, the mutated *arsB1* gene

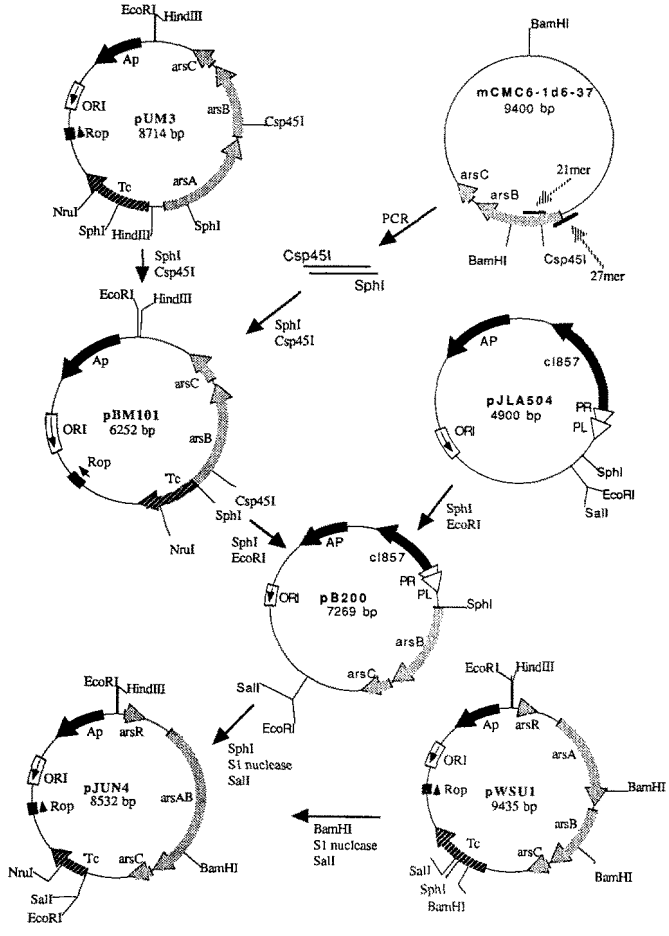

FIG. 1. **Schematic representation of cloning strategy employed for construction of plasmids for overexpression of ArsB protein.** The restriction sites required for cloning are shown. The 4.3-kilobase *Hin*dIII fragment containing the wild-type *arsB* gene was originally isolated from R-factor R773 as described under "Experimental Procedures." The *black segments* illustrate the oligonucleotides used for mutation of the *arsB* gene (for sequences, see Table II). *Ap*, ampicillin; *Tc*, tetracycline.

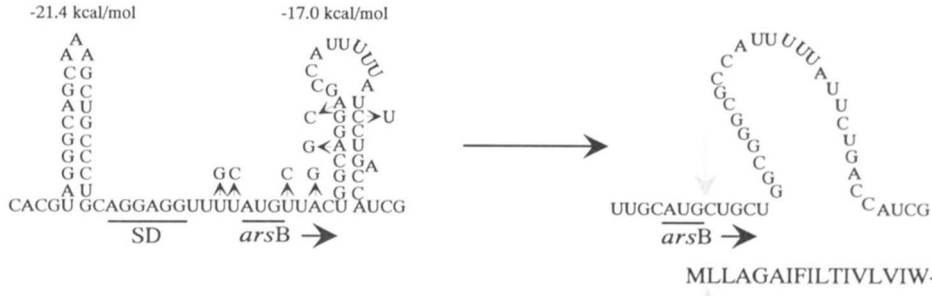

FIG. 2. **Translational initiation region of *arsB* gene.** The primary and proposed secondary structures are shown. The putative ribosome-binding sequence (Shine-Dalgarno (*SD*)) and the AUG initiation codon of the *arsB* gene are *underlined*. The thermodynamic stabilities are calculated according to Tinoco *et al.* (47) and are expressed as the free energy of the secondary hairpin structures. The mutational substitution of bases surrounding the AUG codon are indicated by the *arrows*, as are the base pairs forming the stem-loop. The NH_2-terminal amino acid sequence of the *arsB* gene product is given below the mRNA sequence. The site in *arsB* that was fused to the upstream *arsA* gene is indicated (*arrows*).

was cloned into expression vector pJLA504 under control of the strong λp_L and λp_R promoters and the efficient *atpE* leader, creating plasmid pB100. In this plasmid, transcription from the λ promoters is regulated by the temperature-sensitive cI857 repressor. Plasmid pJLA504 has been used successfully for high level expression of a number of genes following a temperature shift to 42 °C (15). Plasmid pB100 (*arsB1*), with a wild-type *arsA* gene expressed in *trans* from the compatible plasmid pArsA, exhibited arsenite resistance; but the level of resistance was variable, and the plasmid was unstable. The *arsB1* gene was further mutated by PCR to destabilize the GC-rich stem-loop between the third and twelfth codons of the *arsB* gene (Fig. 2). It should be noted that none of the mutations in the *arsB1* or *arsB2* genes changed the amino acid sequence of the ArsB protein. Cells expressing the *arsB2* gene from plasmid pB200 with *arsA* in *trans* showed the same properties as cells with pB100. In neither case could a band corresponding to the ArsB protein be detected on silver- or Coomassie Blue-stained SDS gels of induced cells (data not shown).

The strong λp_R and λp_L promoters, like p_{trp}, are leaky and produce plasmid instability due to the high levels of transcription (25). Likewise, when the mutated *arsB1* and *arsB2* genes were put under the control of the *lac* promoter (plasmids pHIN100 (*arsB1*) and pHIN200 (*arsB2*)), no ArsB protein was observed on SDS-PAGE of induced cells (data not shown).

Gene fusions using a 5′-sequence for a 9-amino acid peptide corresponding to an epitope of hemagglutinin (HA1) of the influenza virus have been useful for identification of membrane proteins (16). A linker composed of the complementary oligonucleotides HA100 and HA200, encoding the HA1 epitope, was fused 5′ to the *arsB2* gene in plasmid pB300. The resulting *HA1-arsB* fusion gene conferred arsenite resistance. However, the chimeric protein was not synthesized in large enough quantities for detection by immunoblotting using a monoclonal antibody against the 9-amino acid epitope, and a band corresponding to the chimera could not be detected by SDS-PAGE (data not shown).

Expression of Functional arsAB2 Gene—With the same rationale as described above, the *arsB2* gene was fused 3′ in frame with the first 1458 bp of the *arsA* gene. The fusion gene would encode a 914-residue polypeptide, with 486 residues of the ArsA protein and 428 residues of the ArsB protein. The polypeptide would contain the entire ArsB sequence except for the initiating methionine. The fusion gene was regulated by the endogenous *ars* promoter. When induced with 0.1 mM sodium arsenite, the chimeric protein was produced at ~15–20% of the total membrane protein (Fig. 3). The fusion protein migrated at a position corresponding to a mass of approximately 80 kDa on SDS-7% polyacrylamide gels and reacted with polyclonal antibodies against the ArsA protein. This is somewhat lower than the predicted mass of 97,976 Da, but the native ArsB protein migrates faster than expected on the same gels (26). Anomalous migration of membrane proteins has been observed with other membrane proteins and could be due to binding of large amounts of SDS.

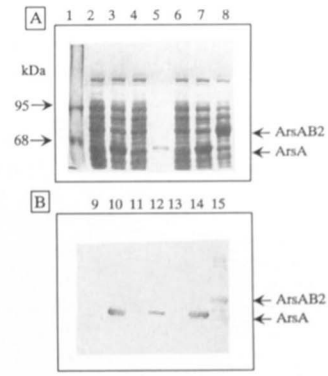

FIG. 3. **Overexpression of chimeric *arsAB* gene product.** *E. coli* HB101 cells carrying plasmid pJUN4, which contains the chimeric *arsAB2* gene, were grown as described under "Experimental Procedures." When the culture reached an A_{550} of 0.5, sodium arsenite (0.1 mM) was added, and the culture was incubated for an additional 60 min. *A*, cells were lysed in SDS sample buffer. Equal amounts of protein (75 μg) were separated by SDS-PAGE and stained with Coomassie Blue. *Lane 1*, molecular mass standards; *lanes 2* and *6*, cells with vector (pBR322); *lanes 3* and *7*, cells with pUM3 (*arsABC*); *lanes 4* and *8*, cells with pJUN4 (*arsAB2*); *lane 5*, purified ArsA protein; *lanes 2–4*, uninduced; *lanes 6–8*, induced. *B*, immunoblot analysis of the overproduced chimeric ArsAB2 protein. A polyclonal antiserum prepared against purified ArsA protein was used. The contents of each lane are the same as described for *A*.

Since antibodies to the ArsB protein have never been obtained, the level of expression of the *arsB* constructs was determined with antibodies to β-lactamase as a reporter group using a series of *blaM* gene fusions. Three binary *arsB-blaM* fusions were created with either the sequence of the *arsB* gene encoding the first 51 residues of the wild-type ArsB protein (pBL10) or the corresponding regions of the *arsB1* (pBL20) or *arsB2* (pBL30) gene. A ternary fusion was constructed by fusing the *arsAB2* gene of pJUN4 with the *blaM* gene, creating

plasmid pBL40. The amount of anti-β-lactamase-reacting material produced by cells expressing each of the chimeras was compared using immunoblotting. The results demonstrate that the β-lactamase moiety of the ternary fusion is produced in at least 100-fold greater amounts than any of the other constructs. Fig. 4 illustrates the level of expression with a comparison of immunodot blots from cells expressing the *arsB-blaM* and *arsA-arsB2-blaM* fusion genes.

Effect of Expression of arsAB2 Gene on Cell Growth—In some cases, attempts to express the genes for membrane proteins at high levels cause loss of viability (28, 29). In other cases, membrane proteins can be produced in high amounts with little deleterious effect on cell growth (27, 30, 31). Growth of cells expressing the *arsAB2* fusion gene was inhibited by overexpression of the chimeric ArsAB2 protein (Fig. 5*A*). In spite of the decrease in growth rate, the culture density after overnight growth was not decreased, suggesting that production of the chimeric protein is not toxic.

Cellular Localization of Chimeric ArsAB2 Protein—Integral membrane proteins are not extracted into 0.1 M sodium bicarbonate, whereas peripherally bound proteins are (19). *E. coli* cells bearing pJUN4 were induced with arsenite in the logarithmic phase of growth as described under "Experimental Procedures." From the time course of induction, the chimera appeared in <10 min and reached maximal induction within 50 min. At various times following induction, intact cells were extracted with 0.1 M sodium bicarbonate, and the alkali-soluble and -insoluble fractions were analyzed by SDS-PAGE (Fig. 5, *B* and *C*). The chimeric ArsAB2 protein was found primarily in the alkali-insoluble fraction, indicating that it is an integral membrane protein. Washing the membranes with urea to remove extrinsically bound proteins did not remove the chimera, again indicating that it is an integral membrane protein (Fig. 6*A*). Total membranes containing the ArsAB2 protein were fractionated into inner and outer membrane by isopycnic centrifugation as described under "Experimental Procedures," and the membrane fractions were analyzed by SDS-PAGE and immunoblotting (Fig. 6, *B* and *C*). The ArsAB2 protein was found only in the inner membrane fraction (Fig. 6, *B* and *C*). When the chimeric protein was maximally induced, inclusion bodies containing the chimera formed. This form of the protein could be solubilized with 4 M urea (Fig. 6*A*). A similar phenomenon has been observed with the lactose permease (32).

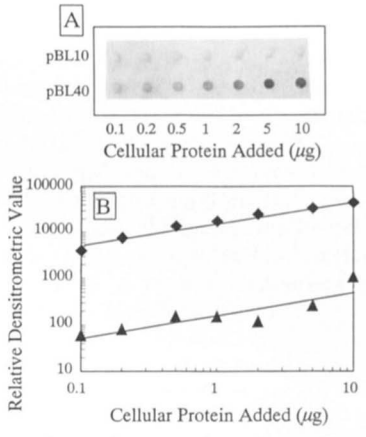

FIG. 4. **Comparison of expression of *arsAB2* gene with *arsB* gene.** Cells of *E. coli* TG1 bearing plasmid pBL40 (ternary *arsA-arsB2-blaM* fusion) or pBL10 (binary *arsB-blaM* fusion) were grown as described under "Experimental Procedures." The cells were solubilized with 0.5% SDS, and the extracts were vacuum-blotted onto nitrocellulose membranes and immunodetected with antiserum to β-lactamase (*A*). In *B*, the amount of antigenic material was quantified by densitometry (Stratagene).

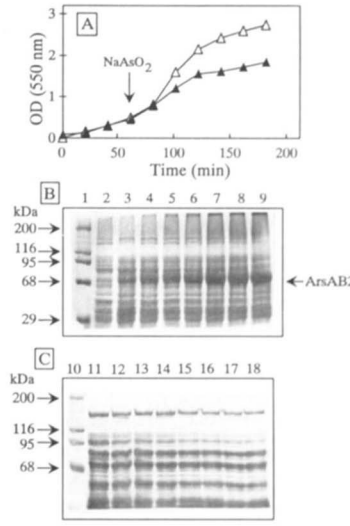

FIG. 5. **Alkaline fractionation of *E. coli* protein from cells expressing the *arsAB2* gene.** *A*, cells of *E. coli* strain HB101 carrying plasmid pJUN4 were grown at 37 °C and either not induced (△) or induced with 0.1 mM sodium arsenite (▲) at the time indicated by the *arrow*. After alkaline treatment of the cells, the insoluble (*B*) and soluble (*C*) fractions were analyzed by SDS-PAGE and stained with Coomassie Blue. In *B* and *C*, the cells were induced for the following times: *lanes 2* and *11*, uninduced; *lanes 3* and *12*, 10 min; *lanes 4* and *13*, 20 min; *lanes 5* and *14*, 30 min; *lanes 6* and *15*, 40 min; *lanes 7* and *16*, 50 min; *lanes 8* and *17*, 60 min; *lanes 9* and *18*, 120 min. Each lane contained material representing ~30% of the cells from a 20-ml culture (see text). *Lanes 1* and *10* contain marker enzymes, with masses indicated in kilodaltons. The position of the ArsAB2 protein is indicated by the *arrow*.

ArsAB2 Protein Is Functional—The functional state of the chimeric ArsAB2 protein was investigated in three ways. First, its ability to confer arsenical resistance was determined. As described above, expression of the *arsAB2* gene is not lethal, even though it decreases the growth rate. Arsenical resistance requires expression of only the *arsA* and *arsB* genes (33). The chimera, which contains the entire ArsB protein and a portion of the ArsA protein, is unable to confer resistance by itself (Fig. 7*A*). When a wild-type *arsA* gene was coexpressed from a compatible plasmid, the cells were resistant to arsenite.

The second measure of function of the chimeric protein is its ability to catalyze arsenite extrusion. Exclusion of arsenite from cells in an uptake assay reflects extrusion activity (23). The uptake of arsenite by intact cells expressing the *arsAB2* gene was examined using $^{73}AsO_2^-$ (Fig. 7*B*). When expressed either alone or in *trans* with a wild-type *arsA* gene, the fusion gene conferred the ability to exclude arsenite. It is not clear whether the transport activity of cells with the *arsAB2* gene alone requires the remaining portion of the ArsA protein. Some arsenite extrusion has been observed in *E. coli* cells that have only a wild-type *arsB* gene from either plasmid R773 or pI258 without an *arsA* gene,[3] so it is not surprising that cells with only the *arsAB2* gene exhibit a similar property. It may be that the ArsB protein can function as a secondary porter in the absence of the catalytic ArsA protein. Alternatively, a chromosomally encoded ATPase may substitute for the ArsA protein. These possibilities are under investigation. In any case, these results demonstrate that the ArsAB2 protein retains its resistance and transport functions.

Third, the ability of the chimeric ArsAB2 protein to bind

[3] S. Dey, D. Dou, G. Ji, S. Silver, and B. P. Rosen, manuscript in preparation.

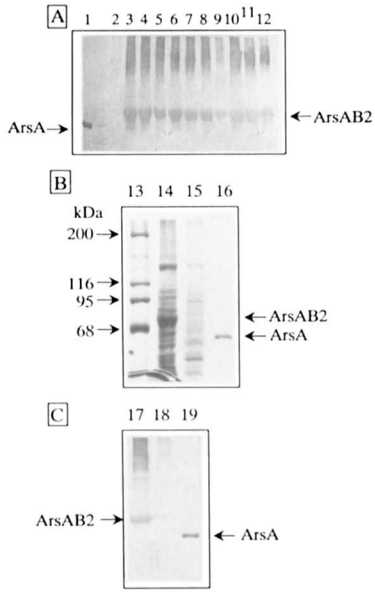

FIG. 6. **Cellular localization of overexpressed chimeric ArsAB2 protein.** *A*, immunoblot of an SDS gel (7% acrylamide) of cells fractionated into the cytosol (*lane 2*), total membranes (*lane 3*), and inclusion bodies (*lane 8*). The membranes and inclusion bodies were extracted with urea at concentrations of 2 M (*lanes 4* and *9*), 4 M (*lanes 5* and *10*), 6 M (*lanes 6* and *11*), and 8 M (*lane 7* and *12*). Urea extraction was performed for 60 min for membranes and for 30 min for inclusion bodies at 4 °C, and the suspensions were centrifuged. The pelleted material from the membranes and the urea-solubilized material from the inclusion bodies were analyzed by immunoblotting. *B*, total membranes were fractionated into inner (*lane 14*) and outer (*lane 15*) membranes by sucrose gradient centrifugation. The membrane fractions were analyzed by SDS-PAGE (7% acrylamide) and stained with Coomassie Blue. *C*, the proteins from *B* were immunoblotted. *Lanes 17–19* are the same as *lanes 14–16* in *B*. *Lanes 1, 16*, and *19* contain purified ArsA protein (2 μg). *Lane 13* contains protein standards. Immunoblotting was performed with anti-ArsA serum. Polyacrylamide gels were 7% acrylamide.

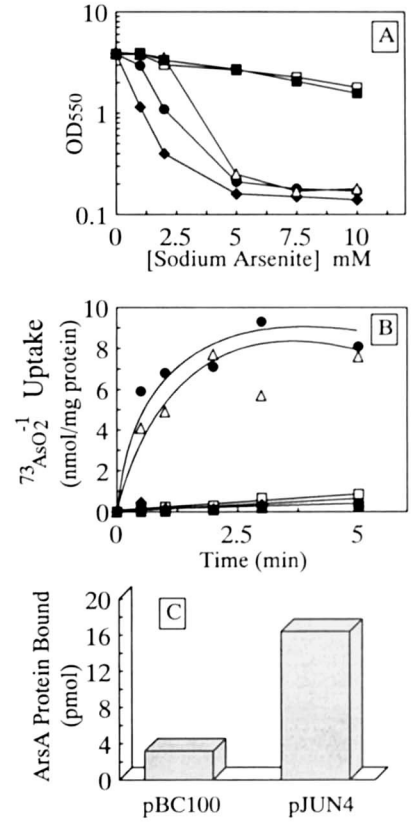

FIG. 7. **Biological activity of ArsAB2 protein.** The function of the *arsAB2* fusion gene in arsenite resistance and $^{73}AsO_2^-$ exclusion from *E. coli* cells was analyzed. *A*, arsenite resistance. Overnight cultures of *E. coli* strain HB101 carrying plasmids were diluted 50-fold into LB medium containing the indicated concentrations of sodium arsenite and incubated at 37 °C with shaking for 20 h. In *B*, cells were grown in enriched medium and assayed for accumulation of $^{73}AsO_2^-$ as described under "Experimental Procedures." In *A* and *B*, cells had the following plasmids: ●, pBR322 (vector); □, pUM3 (*arsA arsB*); ♦, pJUN4 (*arsAB2*); △, pArsA (*arsA*); ■, pJUN4 + pArsA (*arsA* + *arsAB2*). In *C*, the amount of purified ArsA protein bound to everted membrane vesicles from cells expressing plasmid pBC100 (*arsB*) (*left*) or pJUN4 (*arsAB2*) (*right*) was determined as described under "Experimental Procedures."

purified ArsA protein *in vitro* was examined (Fig. 7*C*). The amount of ArsA protein bound to everted membrane vesicles (1 mg of membrane protein) prepared from cells expressing the chimeric ArsAB2 protein from plasmid pJUN4 was considerably higher than the amount bound to membranes prepared from cells expressing the wild-type *arsB* gene from plasmid pBC100 (Fig. 7*C*), although not the 100-fold increase that might have been expected from the data in Fig. 4, suggesting that only a portion of the ArsAB2 protein may be functional.

DISCUSSION

There are numerous gene products of biological interest that cannot be obtained from the natural sources in quantities sufficient for detailed biochemical and biophysical analysis. The levels of many soluble proteins have been increased to the point at which they become the predominant protein in the cell simply by expressing the respective genes in high-copy number plasmids or behind strong promoters. Since the inner membrane of *E. coli* has a more limited volume than the cytosol, membrane proteins are frequently more difficult to produce in large quantities than are soluble proteins (34). Some membrane proteins have been produced in amounts 10–50-fold higher than normal, including the *E. coli* D-lactate and NADH dehydrogenases (35) and the *E. coli* lactose permease (32). Some membrane-bound complexes could be produced in amounts rivaling the most highly produced soluble proteins, for example, fumarate reductase (30). In that case, there was a concomitant increase in phospholipid production, so that the extra protein formed cytosolic phospholipid-protein structures, circumventing the problem of the two-dimensional nature of the inner membrane (36). In each case, the increase in membrane protein production resulted from increasing the rate of transcription by the use of multicopy vectors and strong promoters.

However, other membrane proteins are refractory to overproduction. Increasing the copy number of the tetracycline transporter encoded by transposon Tn10 (Class B *tet*) resulted in lower levels of resistance to tetracycline than did strains bearing the same *tet* gene in a low-copy state (37). Thus, raising transcriptional rates through gene dosage or promoter control is not a universal solution to the problem of production of cloned gene products.

The ArsB protein is an example of a membrane protein that cannot be increased in amount through transcriptional control. Even though the *ars* operon is a single transcriptional unit, the ArsA and ArsC proteins were produced in proportion to the number of plasmid copies of the operon, whereas the ArsB protein was not (2). The *arsB* gene was cloned into vectors using a variety of promoters, including tandem λp_L and λp_R promoters (15), the T7 RNA polymerase promoter (38), p_{lac} (39), and p_{tac} (40). All were ineffective in increasing

the level of arsenite resistance. The ArsB protein could only be detected as a labeled band on SDS-polyacrylamide gels when expressed as an [^{35}S]methionine-labeled protein under the T7 promoter (27).

Other possible reasons for the poor expression of the wild-type *arsB* gene could be due to 1) inefficient codon usage, 2) poor translational initiation, 3) instability of mRNA, and/or 4) instability of the ArsB protein. Previous studies had shown that the mRNA transcript was not stable, with degradation occurring within the *arsB* reading frame. The other reading frames were more stable, so that the ArsA and ArsC proteins continued to be made even after the full-length message was broken down. Analysis of the *arsB* TIR revealed several details. First, the second codon, UUA, is not the most frequently utilized leucine codon (9). Second, a GC-rich palindromic sequence of 8 base pairs between the third and twelfth codons could potentially form a stable (−17 kcal/mol) stem-loop structure (8). Third, the mRNA immediately before the initiating AUG codon of the *arsB* reading frame is UUU, which has been shown to have a drastically negative effect on translational initiation of the *lacZ* message (41). Expression of eukaryotic membrane protein in *E. coli* has also been linked to efficient translation (42). Thus, the rate of ArsB protein synthesis could be limited by the rate at which ribosomes moved past the start of the reading frame. The mRNA past the bottleneck would be relatively unpopulated by ribosomes and thus susceptible to endonuclease digestion. Two exoribonucleases, polynucleotide phosphorylase (*pnp*) and RNase II (*ams*), have been shown to be involved in mRNA degradation (43). There was no increase in arsenite resistance when the *ars* operon was expressed in *E. coli* strain SK5671 carrying mutations in both the *ams* and *pnp* genes,[4] so that mRNA degradation alone may not be responsible for the observed differential translation. It could, however, result from the combination of these factors. These speculations were the basis for the modifications of the *arsB* gene, where the second codon was changed to the more frequently utilized leucine codon CUG and the stem-loop structure was destabilized. A restriction site was created at the beginning of the *arsB* gene to allow cloning into a vector with the highly expressed *atpE* TIR (15). The results of these manipulations were unstable plasmids and no increase in production of the ArsB protein. For whatever reasons, the desired outcome was not achieved by this approach.

A more fruitful result was obtained by fusing the re-engineered *arsB* gene with a large portion of the *arsA* gene. This was not simply accidental. The *arsA* gene is highly expressed, with a TIR very similar to that of *lacZ* (41). The chimeric ArsAB2 protein could be expressed at levels comparable to that of the ArsA protein, which is 100-fold greater than that of the wild-type ArsB protein. The chimeric protein is functional in resistance and transport, but the amount of functional protein required to produce a resistance phenotype is not known. Isolated membranes with the chimeric protein bound only 5–8-fold more purified ArsA protein than did membranes with the wild-type ArsB protein, suggesting that not all of the chimeric protein may be active. In conclusion, these results establish that the difficulty in expressing genes for membrane proteins in *E. coli* can be related in some cases to translational initiation. However, even when the cause is recognized, the solution is not always obvious. Modifications of *arsB* that were anticipated to increase expression produced lethality and plasmid instability. In fact, it is not clear why expression of the *arsAB2* construct is not toxic to the cell. The ability to make large amounts of the chimeric ArsAB2 protein demonstrates that conditions can be found for production of even the most refractory membrane proteins, although in this case the approach was more brute-force than systematic.

[4] D. Dou and B. P. Rosen, unpublished results.

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