6-25-1992

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Jianhua Wu
Wayne State University

Louis S. Tisa
University of New Hampshire, Durham, louis.tisa@unh.edu

Barry P. Rosen
Wayne State University

Recommended Citation
Wu, J.H., L.S. Tisa, and B.P. Rosen. 1992. Membrane topology of the ArsB protein, the membrane subunit of an anion-translocating ATPase. J. Biol. Chem. 267:12570-12576.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 267, No. 18, Issue of June 25, pp. 12570-12576, 1992
Printed in U.S.A.

Membrane Topology of the ArsB Protein, the Membrane Subunit of an Anion-translocating ATPase*

(Received for publication, December 20, 1991)

Jianhua Wu, Louis S. Tisa‡, and Barry P. Rosen§

From the Department of Biochemistry, School of Medicine, Wayne State University, Detroit, Michigan 48201

The *ars* operon of the conjugative R-factor R773 encodes an oxyanion pump that catalyzes extrusion of arsenicals from cells of *Escherichia coli*. The oxyanion translocation ATPase is composed of two polypeptides, the catalytic ArsA protein and the intrinsic membrane protein, ArsB. The topology of regions of the ArsB protein in the inner membrane was determined using a variety of gene fusions. Random gene fusions with *lacZ* and *phoA* were generated using transposon mutagenesis. A series of gene fusions with *blaM* were constructed *in vitro* using a β-lactamase fusion vector. To localize individual segments of the ArsB protein, a ternary fusion method was developed, where portions of the *arsB* gene were inserted in-frame between the coding regions for two heterologous proteins, in this case a portion of a newly identified *arsD* gene and the *blaM* sequence encoding the mature β-lactamase. The location of a periplasmic loop was determined from V8 protease digestion of an ArsA-ArsB chimera. From analysis of data from 26 fusions, a topological model of the ArsB protein with 12 membrane-spanning regions is proposed.

The clinically isolated R-factor R773 mediates bacterial resistance to arsenical and antimonial salts (Kaur and Rosen, 1992). The *ars* operon of the conjugative plasmid R773 encodes an oxyanion pump. The pump is a novel oxyanion-translocating ATPase with a minimal composition of two polypeptides, ArsA and ArsB (Chen *et al.*, 1986). The catalytic component, the ArsA protein, is an oxyanion-stimulated ATPase (Hsu and Rosen, 1989; Rosen *et al.*, 1988). The membrane component, the ArsB protein, has been identified as an integral membrane protein localized in the inner membrane of *Escherichia coli* (San Francisco *et al.*, 1989). The ArsB protein presumably forms the oxyanion-conductive pathway and is the membrane-binding site for the ArsA protein (Tisa and Rosen, 1990). The ArsB protein is composed of 429 amino acid residues and was predicted to have at least 10 hydrophobic regions that could be membrane-spanning α-helices (Chen *et al.*, 1986).

The membrane topology of bacterial membrane proteins can be examined using gene fusions (Jennings, 1989; Broome-Smith *et al.*, 1990; Manoil, 1990). Three types of gene fusions have proven useful. In-frame fusions with the *phoA* gene (for alkaline phosphatase) and the *lacZ* gene (for β-galactosidase) give complementary information in which high alkaline phosphatase activity reflects fusion in the coding region for the periplasmic portion of a membrane protein (Boyd *et al.*, 1987; Manoil and Beckwith, 1985, 1986; Akiyama and Ito, 1987), and high β-galactosidase activity indicates fusion in the coding region for the cytosolic region of a membrane protein (Froshauer *et al.*, 1988). β-Lactamase (*blaM*) fusions have similar features to *phoA* fusions in that chimeras with fusions in the sequence for the periplasmic regions of the membrane protein will facilitate the translocation of the β-lactamase moiety to the periplasmic space and provide resistance to high concentrations of ampicillin (Ap^r)[1] (Broome-Smith and Spratt, 1986). The degree of Ap^r in single colonies is proportional to the amount of β-lactamase in the periplasmic space, but production of cytoplasmic β-lactamase can give Ap^r in patches of cell growth on Petri dishes. Thus *blaM* fusions are useful for localization of membrane elements on both sides of the membrane.

In-frame *arsB-phoA* and *arsB-lacZ* fusions were produced by *in vivo* transposition of Tn*phoA* and *Mud*II 1681. A number of *arsB-phoA* and *arsB-lacZ* fusions exhibited high alkaline phosphatase or β-galactosidase activities, respectively. The *arsB* gene was cloned into a plasmid in front of a *blaM* gene lacking a ribosome-binding site. A series of exonuclease deletions were blunt end-ligated to the *blaM* gene, and strains producing chimeric proteins were identified by immunoblotting with antibody against β-lactamase and screening for ampicillin resistance. Some *arsB-blaM* fusion plasmids conferred high level Ap^r in single colonies. Others produced resistance only in patches of cell growth. The results of the three types of fusions were all consistent with each other.

A new strategy involving ternary fusions composed of parts of three genes was devised. First, a small hydrophilic protein serves as a cytosolic anchor. The second part is a portion of the membrane protein, and the third part is the localization reporter. For the cytoplasmic anchor protein, we chose the ArsD protein, a newly identified 13-kDa regulatory protein, the product of the second gene of the *ars* operon.[2] For the localization marker, the *blaM* gene was used. Gene fusions with membrane-spanning segments of proteins were found to be frequently lethal when attempts were made to overexpress them. An advantage of these ternary fusions is that their expression is tightly regulated under control of the *ars* promoter. When fully induced, no Ap^r ternary fusions with membrane-spanning α-helices were observed; in contrast, Ap^r could be selected at submaximal concentrations of inducer. Ternary fusions may also be useful for isolation of cytoplasmic

* This work was supported by United States Public Health Service Grant AI19793. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Biochemistry, University of Wisconsin, Madison, WI 53706.

§ To whom reprint requests should be addressed.

[1] The abbreviations used are: Ap^r, ampicillin resistant; Ap^s, ampicillin sensitive; Asi^r, sodium arsenite-resistant; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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

and periplasmic domains of membrane proteins for preparation of antibodies and for biochemical analysis.

EXPERIMENTAL PROCEDURES

Materials—All restriction enzymes and nucleic acid modifying enzymes were obtained from GIBCO/BRL. Antibodies to β-lactamase, *E. coli* alkaline phosphatase, and β-galactosidase were purchased from 5 Prime → 3 Prime, Inc. Oligonucleotides were synthesized in the Macromolecular Core Facility of the 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.

DNA Manipulations—The conditions for plasmid isolation, DNA restriction endonuclease analysis, *Bal*31 digestion, ligation, transformation, and sequencing have all been described (Sambrook *et al.*, 1989). Computer analysis of the sequence information was performed with the GENEPRO program from Riverside Scientific Enterprises (Seattle, WA).

Media and Growth Conditions—Cells were grown in LB medium (Miller, 1972) at 37 °C. Antibiotics were added to 40 μg/ml, unless otherwise noted. Sodium arsenite (5 mM) was added to test for resistance. When used as an inducer of the intact *ars* operon, 50 μM sodium arsenite was added. When used as an inducer of *arsA-arsB* and ternary fusions, 1 μM sodium arsenite was added, since levels of 10 μM or more arrested growth of these strains.

Isolation of arsB-phoA Gene Fusions—The procedure of Manoil and Beckwith (1985) was followed for Tn*phoA* transposition. *E. coli* strain CC118 was first transformed with pUM3, selecting for Ap^r and Asi^r. Tn*phoA* was introduced into CC118(pUM3) by infection with phage λ Tn*phoA*. The products of transposition were selected on plates containing 200 μg/ml kanamycin. Transductants were pooled, and a mixture of plasmid DNA was extracted. The mixture was used to transform strain CC118, and cells were spread on plates containing ampicillin, kanamycin, and 20 μg/ml of 5-bromo-4-chloro-3-indolyl phosphate, the chromogenic alkaline phosphatase substrate. Plasmid DNA from the blue colonies was analyzed by restriction nuclease digestion with the appropriate enzymes to confirm the constructions. The fusion junctions of the *arsB-phoA* fusions were determined by double-stranded DNA sequencing using the *phoA*-specific primer, 5′-CAGGGCAAAACGGGAAAGG-3′.

Isolation of arsB-lacZ Gene Fusions—The *arsB-lacZ* gene fusions were the result of *in vivo* transposition of Mu dII1681, as previously described (Castilho *et al.*, 1984). Strain PoII1681 containing a mini-Mu defective prophage and a complementing Mu*cts* prophage was transformed with pWSU1, which contains the entire *ars* operon. Transformants were induced for Mu phage lytic growth, and the lysate was used to infect strain M8820 (Muc^+), and lac^+ transfectants were selected on plates containing ampicillin, kanamycin, and 20 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Analysis of the restriction endonuclease sites of the fusion plasmids were used to confirm the constructions. The fusion junctions of the *arsB-lacZ* plasmids were determined by DNA sequencing of double-stranded plasmid DNA using the primer 5′-GTTTTCCCAGTCACGAC-3′, which is complementary to the *lacZ* gene.

One *arsB-lacZ* fusion (pLZB182) was constructed by direct cloning of the *Sma*I-*Sal*I fragment of pMC1871 into pWSU1 that had been digested with both *Eco*RV and *Sal*I. This plasmid encodes a chimeric protein with the first 182 residues of the ArsB protein.

Construction of arsB-blaM Gene Fusions—Plasmid pWSU1 was digested with *Eco*RI and *Hin*dIII, and a 5-kilobase fragment containing the entire *ars* operon was cloned into plasmid pJBS633 that had been digested with both *Eco*RI and *Hin*dIII, yielding plasmid pJHW100 (Fig. 1). To create a unique restriction site for exonuclease digestion, plasmid pJHW100 was digested with *Sal*I, and the ends were filled in with the Klenow fragment of DNA polymerase I to recreate the *Sal*I site. The linearized plasmid was then partially digestion with *Pvu*II to remove the *Sal*I-*Pvu*II fragment between the *ars* insert and *blaM*, and the DNA was circularized by intramolecular ligation. The resulting plasmid was transformed into strain TG1 with selection for Asi^r. Plasmids from Asi^r transformants were analyzed by restriction enzyme digestion. In the resulting construct, pJHW101, the *Sal*I-*Pvu*II fragment (Fig. 1) has been deleted, and the *Sal*I site regenerated in front of the *blaM* coding region. In-frame *arsB-blaM* fusions were isolated according to the method of Broome-Smith and Spratt (1986). Isolated DNA from pJHW101 was linearized with *Kpn*I or *Pvu*II and digested with exonuclease *Bal*31. Samples were

TABLE I
Strains, plasmids, and phage

Strain/plasmid/phage	Genotype/description	Reference
CC118	*araD139 Δ(ara-leu)7697 ΔlacX74 galE galK ΔphoA20 thi rpsE rpoB argE(Am) recA1*	Manoil and Beckwith (1985)
LE392	F^-, *supF supE hsdR galK trpR metB lacY*	Manoil and Beckwith (1985)
Muc^+M8820	M8820 with Mu*cts*	Castilho *et al.* (1984)
HB101	F^-, *hsdR hsdM supE44 ara14 galK2 lacY1 proA2 rspL20 xyl-5 mtl-1 recA13 mcrB*	Sambrook *et al.* (1989)
TG1	K12*Δ(lac-pro) supEF′ traD36 proAB lacI*^{q} *ΔlacZM15*	Amersham Corp
PoII1681	M8820 with Mu dII1681 *ara*::(Mu*cts*)3 *araD*$^-$ *leu*$^+$ *lac*$^+$ *pro*$^+$	Castilho *et al.* (1984)
pUM3	The *arsABC* genes of R773 cloned into the *Hin*dIII site of pBR322	Mobley *et al.* (1983)
pWSU1	pBR322 with the entire *ars* operon cloned into *Eco*RI-*Hin*dIII-digested pBR322	San Francisco *et al.* (1990)
pJBS633	*blaM* gene fusion vector (Km^r, Tc^r)	Broome-Smith and Spratt (1986)
pMC1871	*lacZ* fusion vector (Tc^r)	Shapiro *et al.* (1983)
pBLC114	*arsC*::*blaM* fusion of pJHW100	This study
pJHW100	5-kilobase *Eco*RI-*Hin*dIII fragment containing the *ars* operon cloned into *Eco*RI- and *Hin*dIII-digested pJBS633	This study
pJHW101	pJHW100 with the *Sal*I-*Pvu*II deletion, and a unique *Sal*I site recreated in front of *blaM*	This study
pJHW110	pBLC114 with the *Bal*I fragment deletion	This study
pDBB222	Ternary gene fusion of *arsD-arsB-blaM*	This study
pDBB245	Ternary gene fusion of *arsD-arsB-blaM*	This study
pDBB277	Ternary gene fusion of *arsD-arsB-blaM*	This study
pUM211	MudII1734(Km^r) in pUM3	San Francisco *et al.* (1989)
pAPB series	Tn*phoA*(Km^r) in pUM3	This study
pLZB series	MudII168(Km^r) in pWSU1	This study
pBLB series	pJBS633 derivatives with *arsB-blaM* fusions	This study
λTn*phoA*	Tn5 $IS50_L$::*phoA*(Km^r)	Gutierrez *et al.* (1987)

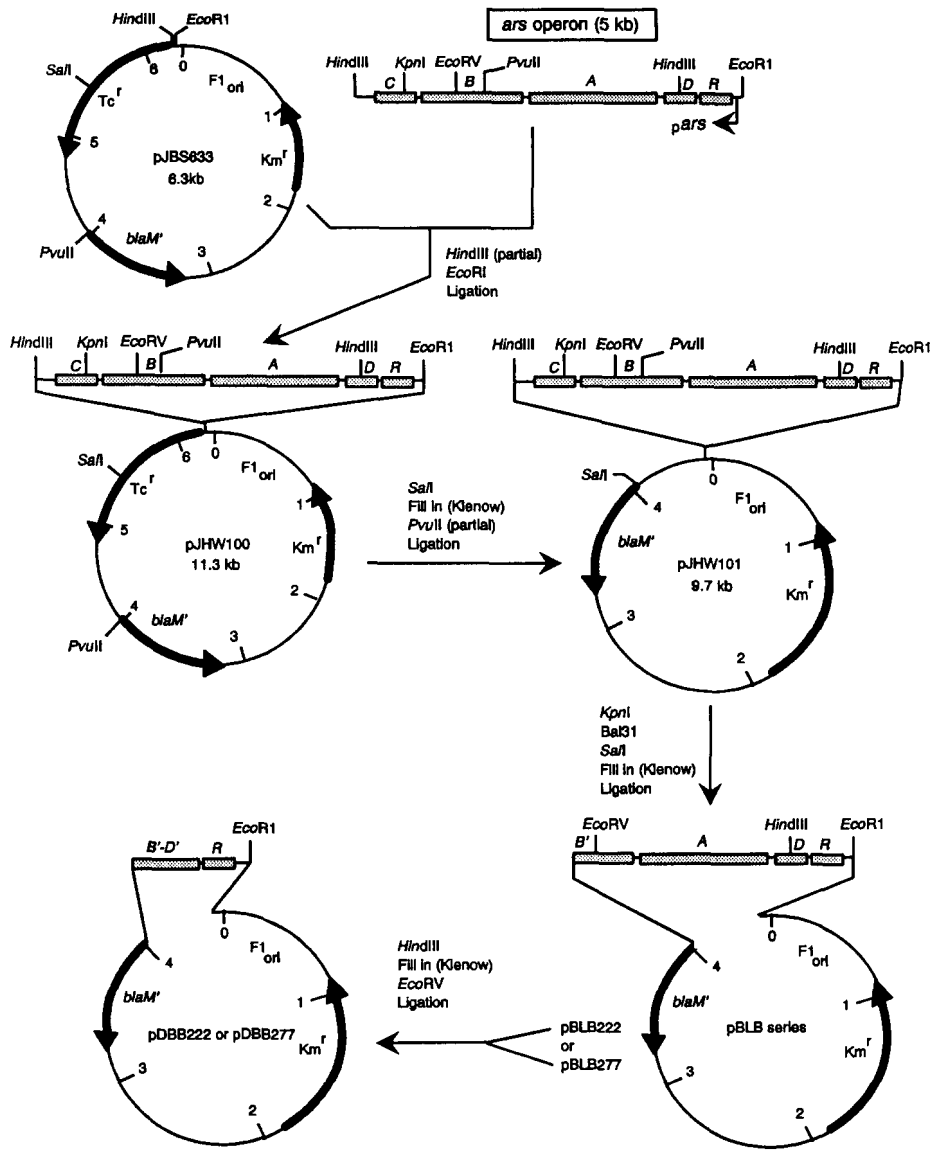

FIG. 1. **Construction of plasmid pJHW101, *bla*, and ternary fusion plasmids.** The *ars* operon of R-factor R773 was inserted into the *Eco*RI-*Hin*dIII site of plasmid pJBS633. Removal of a *Sal*I-*Pvu*II fragment resulted in plasmid pJHW101. A series of *Bal*31 exonuclease deletion (pBLB series) clones were constructed, in which 5′ portions of the *arsB* gene were fused in-frame with the coding sequence of the mature *blaM* gene. Removal of the *Hin*dIII-*Eco*RV fragment resulted in ternary fusion plasmids pDBB222 and pDBB277.

removed at intervals and pooled. Precipitated DNA was further digested with *Sal*I, and the ends were filled in with the Klenow fragment of DNA polymerase I. The DNA was circularized by intramolecular ligation. *E. coli* strain TG1 was transformed with the ligation mixture, and Km^r transformants were selected. In-frame fusions of the *arsB* gene to the coding region of the mature form of β-lactamase were identified by applying approximately 5×10^6 cells in patches to LB agar containing 40 μg/ml ampicillin. Plasmids containing in-frame fusions were analyzed by restriction endonuclease digestion. The fusion junctions between the *arsB* and *blaM* genes were determined by dideoxy sequencing on the single-stranded plasmid DNA obtained from pJBS633 derivatives prepared by infecting cells with helper phage RV1 (Stratagene) and using an oligonucleotide 5′-dCTCGTGCACCCAACTGA-3′ as a *blaM* primer. The minimal inhibitory concentration of ampicillin for single cells of *E. coli* strain TG1 harboring *arsB-blaM* fusion plasmids was determined by spotting 10 μl of a 10^{-6} dilution of an overnight culture (about 10 cells) on LB agar plates containing various concentrations of the antibiotic. The single cell minimal inhibitory concentration is defined as the lowest concentration of ampicillin that prevented growth of the bacteria.

Construction of Ternary Fusions—The *arsB-blaM* fusions plasmids pBLB222 and pBLB277 were digested with both *Hin*dIII and *Eco*RV. The *Hin*dIII ends were filled in with DNA polymerase I Klenow fragment, and the DNA was circularized by intramolecular ligation, producing ternary fusions pDBB222 and pDBB277, respectively (Fig. 1). These plasmids have in-frame gene fusions producing chimeric proteins consisting of the first 67 residues of ArsD, a portion of ArsB, and the mature β-lactamase. In pDBB222 the ArsB portion is residues 201–222. In pDBB277, the ArsB portion is residues 201–277.

To construct ternary fusion pDBB245, the *Eco*RV-*Bam*HI fragment of the *ars* operon, encoding residues 201–245 of the ArsB protein, was isolated from pWSU1, and the ends were filled in with DNA polymerase Klenow fragment. The fragment was cloned into pJHW100 that was digested with both *Hin*dIII and *Pvu*II and treated with DNA polymerase I Klenow fragment, producing an in-frame fusion of *arsD*, *arsB*, and *blaM*. The fusion junctions between *blaM* and *arsB*, as well as *arsB* and *arsD*, were confirmed by DNA sequencing analysis.

Construction of the arsA-arsB Gene Fusion—The *arsC-blaM* fusion plasmid pBLC114 was digested with *Bal*I, followed by intramolecular ligation, producing the *arsA-arsB* fusion plasmid pJHW110. The chimera encoded by the fusion gene contains the first 260 residues of the ArsA protein and the last 169 residues of the ArsB protein.

Assays of Enzymatic Activity—β-Galactosidase and alkaline phosphatase activity were assayed at 37 °C essentially as described (Miller, 1972). Portions (0.2 ml) of a logarithmic phase culture were mixed with 1.8 ml of a buffer consisting of 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol, pH 8.0 (β-galactosidase) or 1 M Tris-HCl, pH 8.0 (alkaline phosphatase), 50 μl of 0.1% SDS (sodium dodecyl sulfate), and 50 μl of chloroform. Reactions were started by the addition of 0.4 ml of 0.4% *o*-nitrophenyl galactoside (β-galactosidase) or *p*-nitrophenyl phosphate (alkaline phosphatase). After centrifugation in a microcentri-

fuge, the $A_{405\,nm}$ of the supernatant was measured. A unit of activity is defined as 1 μmol of substrate hydrolyzed/min/$A_{600\,nm}$ of cells. Molar extinction coefficients of 4860 (*o*-nitrophenol) and 16,000 (*p*-nitrophenol) were used.

Protease Treatment of Spheroplasts—Spheroplasts were prepared essentially as described by Witholt *et al.* (1976). When spheroplast formation was almost complete, as determined by phase contrast microscopy, $MgSO_4$ was added to 10 mM, and V8 protease was added to 100 μg/ml and incubated for 30 min at 37 °C. The reaction was terminated by boiling the spheroplasts in SDS sample buffer for 5 min.

Polyacrylamide Gel Electrophoresis and Immunological Blotting—Samples were prepared by boiling in SDS sample buffer for 5 min. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (1970). Immunological blotting (Gershoni and Palade, 1983) was carried out as described previously (Tisa and Rosen, 1990). Antibodies to β-lactamase (5 Prime → 3 Prime, Inc.) was used at a 1:2000 dilution to detect the β-lactamase-containing chimeric proteins.

RESULTS

Characterization of arsB-phoA Fusions—Five *arsB-phoA* fusions were obtained (Table II). Colonies of cells with each fusion plasmid were blue on 5-bromo-4-chloro-3-indolyl phosphate plates, indicating a periplasmic location of the alkaline phosphatase moiety of the five chimeric proteins. The fusion sites were all within the first 157 residues of the ArsB protein (Table II). Four of the chimeras, containing residues 1–23, 1–27, 1–33, and 1–67 of ArsB had high alkaline phosphatase activity. The fifth, containing residues 1–157 of ArsB, had low alkaline phosphatase activity. Immunoblotting of cells expressing the fusion plasmids demonstrated reactive material of only 45 kDa (data not shown), indicating that the chimeras were not stable. Upon osmotic shock treatment of the cells, all of the antigenic material and all of alkaline phosphatase activity were recovered in the periplasmic fractions, although the amount of antigenic material recovered from pAPB157 was very low. This indicates that each of the chimeras produced an alkaline phosphatase moiety that was translocated to the periplasmic space, where it was proteolytically cleaved. Thus, residues 23, 27, 33, and 67 are likely to be outwardly directed, as shown diagrammatically in Fig. 2. Residue 157 is more likely to be located within a membrane-spanning region.

Characterization of arsB-lacZ Fusions—Seven *arsB-lacZ* fusions were isolated (Table III). Four chimeras, containing ArsB residues 1–40, 1–50, 1–168, and 1–182, had low β-galactosidase activity. The other three, chimeras with ArsB residues 1–155, 1–281, and 1–338, had high β-galactosidase activity. These results suggest that residues 40, 50, 168, and 182 are outwardly directed, whereas residues 155, 281, and 338 are inwardly directed (Fig. 2). Note that in a *lacZ* fusion, residue 155 is clearly cytosolic, whereas residue 157 in a *phoA* fusion is indeterminate. This result could be obtained if residues 155 and 157 are located near the center of a membrane-spanning region (Fig. 2).

TABLE II

Alkaline phosphatase activity of arsB-phoA fusions

Plasmid	Fusion site (amino acid residue)	Junction sequence[a]	Alkaline phosphatase activity
			units/$A_{600\,nm}$
None			0.0
pAPB23	23	TTA GGG ACT GAC TCT	43.5
pAPB27	27	TGG AGC GCT GAC TCT	134.0
pAPB33	33	GCT GTA CCT GAC TCT	129.8
pAPB67	67	ATC AGC CCT GAC TCT	53.8
pAPB157	157	TCG AAC TCT GAC TCT	3.4

[a] The sequence from the *arsB* gene is underlined.

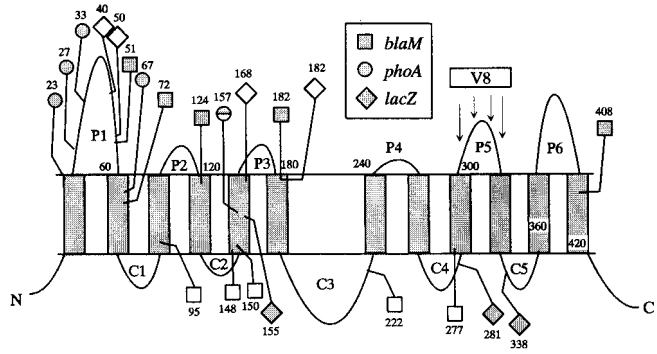

FIG. 2. **Location of fusion sites within the proposed ArsB transmembrane structure.** The topological model identifies the location of fusion junctions (given as the ArsB residue number above or below each fusion site) as determined by DNA sequencing of the fusion plasmids. ■, Ap^r fusions; □, Ap^s fusions; ●, chimeras with active alkaline phosphatase; ○, chimeras with inactive alkaline phosphatase; ◆, chimeras with active β-galactosidase; ◇, chimeras with inactive β-galactosidase. *Arrows* indicate potential V8 protease sites. *C1–C5* and *P1–P6* are proposed cytoplasmic and periplasmic loops, respectively, joining membrane spanning α-helices.

TABLE III

β-Galactosidase activity of arsB-lacZ fusions

Plasmid	Fusion site (amino acid residue)	Junction sequence[a]	β-Galactosidase activity
			units/$A_{600\,nm}$
None			0.0
pLZB40	40	GGT GTG CTG AAG	0.5
pLZB50	50	GTG TGG CTG AAG	1.7
pLZB155	155	ATC GTA CTG AAG	26.2
pLZB168	168	TTC AAA CTG AAG	0.5
pLZB182	182	GTG GAT GGG GAT	1.2
pUM211	281	CCC TGG CTG AAG	48.9
pLZB338	338	ATG CCG CTG AAG	42.6

[a] The sequence from the *arsB* gene is underlined.

TABLE IV

Analysis of arsB::bla fusions

Plasmid	Fusion site (amino acid residue)	Junction sequence[a]	Ap concentration to inhibit single cell growth
			μg/ml
None			4
pBLB51	51	TGG AAT CTG CGT	>600
pBLB72	72	GAT GAG CTG CGT	20
pBLB95	95	CTG TTT CTG CGT	4
pBLB124	124	GCC ATG CTG CGT	160
pBLB148	148	GAT ACT CTG CGT	4
pBLB150	150	GCT AGC CTG CGT	4
pBLB182	182	GTG GAT CTG CGT	120
pBLB222	222	GCG ACG CTG CGT	4
pBLB277	277	GGC GCG CTG CGT	4
pBLB408	408	GTC ATG CTG CGT	20

[a] The sequence from the *arsB* gene is underlined.

Characterization of arsB-blaM Fusions—Ten in-frame *arsB-blaM* fusions were obtained (Table IV). Five of these (chimeras with ArsB residues 1–95, 1–148, 1–150, 1–222, and 1–277) gave ampicillin resistance to patches of cells but no resistance to single colonies. Three (chimeras with ArsB residues 1–51, 1–124, and 1–182) gave high level Ap^r in single colonies. Two (chimeras with ArsB residues 1–72 and 1–408)

produced resistance in single colonies at intermediate levels. Immunoblots of the chimeric proteins were used to demonstrate that the difference in Ap^r was due to localization and not the amount of chimera produced (data not shown). These results suggest that residues 95, 148, 150, and 222 are inwardly directed, residues 51, 124, and 182 are outwardly directed, and 72 and 408 are either outwardly directed or near the surface of the membrane (Fig. 2).

Characterization of Ternary Fusions—Based on the hydropathic profile of the ArsB protein (Chen *et al.*, 1986), there are two regions of high hydrophobicity in the region from residues 220–270. Neither *arsB-blaM* fusion pBLB222 nor pBLB277 confer Ap^r on single cells, indicating that the regions around residues 220 and 270 are exposed to the cytosolic side. The regions between residues 220 and 270 are therefore reasonably predicted to contain two membrane-spanning stretches. From residues 201–220, there is a highly charged hydrophilic domain that would be predicted to be located on the cytosolic side.

To test this prediction, three ternary gene fusion plasmids, pDBB222, pDBB245, and pDBB277, were constructed (Fig. 3). Cells harboring pDBB222, which would be expected to produce a 37-kDa chimera with ArsB residues 201–222, expressed a protein of 37 kDa, which was only found in the cytosolic fraction (Fig. 4). Cells harboring this fusion plasmid exhibited Ap^r in patches of growth but not in single colonies.

In contrast, the hybrid proteins encoded by pDBB245, containing ArsB residues 201–245, or pDBB277, with ArsB residues 201–277, were found only in the membrane fraction (Fig. 4), suggesting that one or both of the hydrophobic regions could promote translocation of the hybrid proteins into the membrane. Although both chimeric proteins were membrane-bound, only pDBB245 conferred Ap^r on single cells. Cells with pDBB277 were Ap^r only in patches of growth. These results indicate that the β-lactamase moiety in the pDBB245-encoded chimera was translocated to the periplasmic side, whereas the β-lactamase moiety in the pDBB277 encoded chimera resided on the cytosolic side (Fig. 3).

Expression of all three ternary fusions was under the control of the *ars* promoter. It should be pointed out that Ap^r could be observed only with 1 μM arsenite as inducer. Levels of inducer of 10 μM or greater stopped growth, even though cells without *ars* genes can grow in media containing as much as 1 mM sodium arsenite. Hypersensitivity to arsenite is probably a result of overexpression of the membrane-bound chimeras. In contrast, when cells with pDBB222 were induced with 10 μM arsenite, a concentration that inhibited growth of cells with the other two ternary fusions, growth of the cells was not affected, and the chimeric ternary protein accumulated in inclusion bodies (data not shown). This illustrates the difference between overexpression of soluble chimeras and chimeras with even a single membrane-spanning unit.

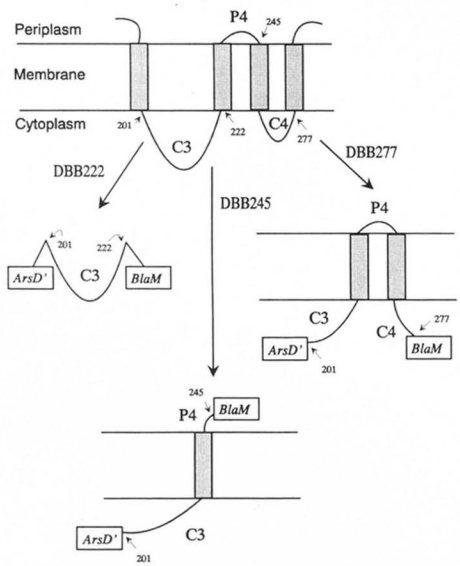

FIG. 3. **Construction of ternary fusions.** The DNA corresponding to the portions of the *arsB* gene encoding proposed cytoplasmic loops C3 and C4 and periplasmic loop P4 were isolated as the indicated restriction endonuclease fragments. These DNA fragments were ligated between a portion of the *arsD* gene and the *blaM* gene, producing ternary fusions DBB222, DBB245, and DBB277.

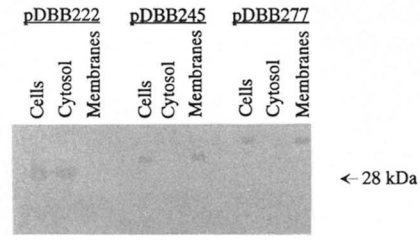

FIG. 4. **Cellular localization of the gene products of ternary fusions.** Cultures of *E. coli* strain TG1 bearing individual ternary fusion plasmids were induced by 1 μM arsenite for 60 min. Cells were fractionated into cytosol and membranes as previously described (Tisa and Rosen, 1990). Samples for SDS gel electrophoresis were prepared by boiling in SDS sample buffer cells derived from 0.2 ml of a culture of A_{600} = 1.2 and the cytosol and membrane fractions from a corresponding number of cells. The solubilized samples were analyzed on a 10% polyacrylamide gel followed by immunoblotting with an antibody to β-lactamase. *Lanes 1–3*, TG1 + pDBB222; *lanes 4–6*, TG1 + pDBB245; *lanes 7–9*, TG1 + pDBB277; *lanes 1, 4,* and *7*, cells; *lanes 2, 5, and 8*, cytosolic fraction; *lanes 3, 6,* and *9*, total membrane fraction.

Characterization of the arsA-arsB Fusion—To explore the topological arrangement of the C-terminal region of the ArsB protein, an *arsA-arsB* gene fusion was constructed. The fusion encodes a 45-kDa chimeric product with amino-terminal residues 1–260 of the ArsA protein and residues 261–429 of the ArsB protein. The fusion gene was under control of the *ars* promoter and inducible by addition of 1 μM arsenite. Higher concentrations of arsenite inhibited growth. Cell fractions from induced cells were run on an SDS gel and immunoblotted with antiserum against the ArsA protein. A 45-kDa protein was found in the membrane fraction (data not shown).

Spheroplasts from induced cells were treated with V8 protease, and the products were analyzed by SDS-PAGE and immunoblotting with anti-ArsA serum (Fig. 5). Following proteolysis, the 45-kDa ArsA-ArsB chimera was converted to a polypeptide of 32 kDa. This result indicates that a protease V8-accessible site is about 50 amino acid residues after the fusion site of the ArsA and ArsB proteins. Between ArsB residues 300 and 320 are 3 aspartyl residues and 1 glutamyl residue, each potential V8 protease sites (Fig. 2). Thus, it is likely that this region of the ArsB protein faces the periplasmic side.

DISCUSSION

To understand the mechanism of active transport requires knowledge of the structure of the transport protein. From the hydropathic profile of the ArsB protein, there are 10–12 regions of 18 or more amino acid residues in length with a hydropathy index greater than 1.5, which is indicative of possible membrane-spanning α-helices (Kyte and Doolittle, 1982). To determine experimentally the number and arrangement of those membrane-spanning regions, we used a genetic approach for construction of fusions to the genes for alkaline

phosphatase, β-galactosidase, and β-lactamase. The results of the three types of fusions were all consistent with each other and were used to propose the topological model shown in Fig. 6. This model suggests 12 membrane-spanning α-helices, five cytoplasmic loops, and six periplasmic loops. Three (C1, C3, and C5) of the five cytoplasmic loops have a net positive charge, whereas five of the six periplasmic loops are either uncharged (P1 and P3) or have a net negative charge (P2, P4, and P5). This is consistent with positively charged loops serving as cytoplasmic anchors for membrane-spanning regions (Boyd and Beckwith, 1990; Dalbey, 1990; Nilsson and von Heijne, 1990; von Heijne, 1986, 1989; Yamane *et al.*, 1990).

The location of loops P1-P3 and C1, C2, C4, and C5 were substantiated by the fusions. However, the paucity of fusions in certain regions necessitated other methods to determine the location of some regions. An *arsA-arsB* fusion was created consisting of the 3′ half of the *arsB* gene, encoding ArsB residues 261–429, in-frame with the 5′ half of the *arsA* gene, encoding the first 260 residues of the ArsA protein. The chimera appeared only in the membrane, suggesting that the C-terminal portion of the ArsB protein was incorporated into the membrane without need for topogenic information from the N terminus. This result is consistent with the idea of independent insertion of individual membrane-spanning regions (Bibi and Kaback, 1990; Ehrmann and Beckwith, 1991).

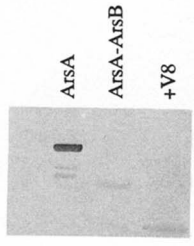

FIG. 5. ***In situ* proteolysis of an ArsA-ArsB chimera.** Expression of the *arsA-arsB* fusion gene was induced by adding 1 μM arsenite for 60 min at 37 °C to a culture of *E. coli* HB101 harboring plasmid pJHW110. Spheroplasts derived from 0.2 ml of a culture of $A_{600} = 1.0$ were treated with protease V8. Samples for SDS-PAGE were prepared by boiling the spheroplasts in sample gel buffer followed by electrophoresis on a 10% polyacrylamide gel and immunoblotting with antibody against the ArsA protein. *Lane 1*, purified 63-kDa ArsA protein standard; *lane 2*, spheroplasts expressing the *arsA-arsB* fusion plasmid; *lane 3*, spheroplasts expressing the *arsA-arsB fusion* treated with V8 protease.

The location of the P5 loop of the ArsB protein could be determined biochemically by V8 protease treatment of spheroplasts expressing the ArsA-ArsB chimera. The size of the chimera was decreased by 13 kDa when spheroplasts were treated with V8 protease. This would represent a loss of approximate 120 residues from the C terminus. This would place the V8 site at approximately ArsB residue 309. ArsB residues Asp^{303}, Asp^{307}, Glu^{311}, and Asp^{314} are potential V8 sites. The next potential V8 site within ArsB residues 261–429 is Asp^{349}, which is too distant to be the site of protease cleavage. This would place the P5 loop in the periplasm. It should be noted that trypsin treatment under the same conditions did not affect the size of the chimera, even though there are potential trypsin sites within residues 261–429. The reason for this inaccessibility of trypsin sites is not known.

Gene fusions of *arsB-blaM* at ArsB residues 222 and 277 did not confer Ap^r on single cells, suggesting that loops C3 and C4 are exposed on the cytosolic side of the membrane and that the two hydrophobic regions between ArsB residues 220 and 275 could be membrane-spanning helices. To confirm these predictions, three ternary *arsD-arsB-blaM* fusions were used to determine the orientation of individual helices. In these fusions, a portion of the *arsB* gene was cloned between two heterologous genes. In this case, a 5′ piece of the *arsD* gene was fused to regions of the *arsB* gene, which was fused to the *blaM* gene. These ternary gene fusions encoded chimeras of ArsD-ArsB-β-lactamase. Cells harboring a ternary fusion with only the C3 domain expressed a hybrid protein of 37 kDa that was found only in the cytosol. Thus, the hydrophilic C3 domain could not mediate translocation of the hybrid protein into the membrane. Consistent with the cellular location of the chimera, this fusion plasmid failed to confer Ap^r on single cells. In contrast, the hybrid proteins containing the region from ArsD-ArsB(C3-P4)-β-lactamase or ArsD-ArsB(C3-P4-C4)-β-lactamase appeared only in the membrane fraction, suggesting the hydrophobic regions have topogenic information for translocation of the hybrid proteins through the membrane. Moreover, the ternary chimera containing C3-P4 conferred Ap^r on single cells, indicating the β-lactamase moiety was translocated to the periplasmic side. In contrast, the ternary protein containing C3-P4-C4 resulted in single cell Ap^s, indicating that the β-lactamase moiety remained on the cytosolic side.

Ternary fusions have the potential to resolve many questions of membrane topology, allowing identification of topo-

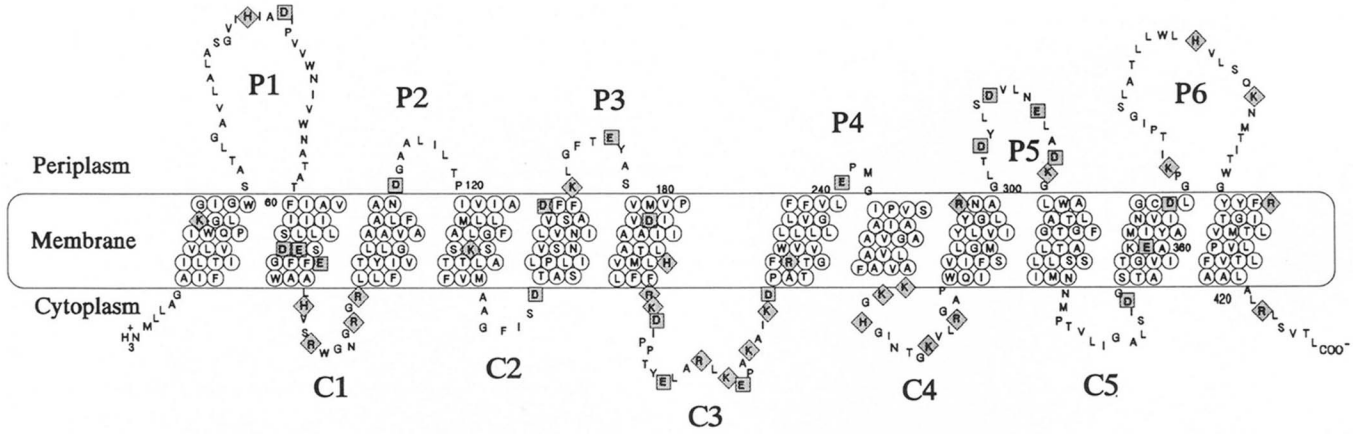

FIG. 6. **Topological model of the ArsB protein.** The model proposes 12 membrane-spanning α-helices joined by six periplasmic loops (*P1–P6*) and five cytoplasmic loops (*C1–C5*). The N and C termini are suggested to be cytosolic. The precise placement of each residue cannot be assigned from the data. The suggested placement includes equal numbers of intramembranal positive and negative charges, with glycyl or prolyl residues placed in turn regions where possible. Acid (□) and basic (◇) residues are indicated.

genic signals in small segments of membrane and extracellular proteins. In addition, they can be used to produce small segments in substantial quantities for biochemical or immunological purposes. For example, the ArsD-ArsB(C3)-BlaM chimera can be overproduced as the major protein of the cell and forms inclusion bodies when induced with 10 μM arsenite. Inclusion bodies from 100 ml of culture yielded 20 mg of nearly pure chimeric protein after washing several times with buffer.[2] In this respect, ternary fusions are similar to the tribrid fusion expression vectors described by Weinstock *et al.* (1983), which could also be used for antibody production. However, tribrid fusions were not designed for analysis of the topology of membrane proteins.

It has been shown that the ArsB protein is the membrane anchor of this oxyanion-translocating ATPase (Tisa and Rosen, 1990). Both *in vivo* and *in vitro* experiments demonstrated the *arsB* gene product is required for the ArsA protein, the ATPase catalytic component, to bind to the *E. coli* inner membrane. This necessitates direct contact of the ArsA and ArsB proteins on the cytosolic side of the membrane. Although all cytosolic loops are potential regions of interaction, larger loops with charged residues would seem more likely candidates. Based on the proposed topological model of the ArsB protein, the cytoplasmic C3 loop has 22 residues, 10 of which are charged, and so C3 would be a logical region in which to look for interactions with the ArsA protein. It should be pointed out that many secondary porters have a large central cytoplasmic loop separating two groups of six membrane-spanning α-helices. Although the significance of this 6+6 arrangement is unknown, it probably represents a fundamental structural motif. Thus, it is not clear whether the C3 loop in ArsB protein is part of the ArsA anchor site or fulfills some other function. There are two other cytosolic loops, C1 and C4, which contain 4 and 5 charged residues, respectively, and thus are also candidates for ArsA interaction sites.

Acknowledgments—We thank M. Casadaban, J. K. Broome-Smith, and C. Manoil for supplying the plasmids and strains required for this study. We are grateful to J. K. Broome-Smith and S. Silver for comments and suggestions.

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