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Characterization of the *arcD* Arginine:Ornithine Exchanger of *Pseudomonas aeruginosa*

LOCALIZATION IN THE CYTOPLASMIC MEMBRANE AND A TOPOLOGICAL MODEL*

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The *arcDABC* operon of *Pseudomonas aeruginosa* encodes the enzymes of the arginine deiminase pathway and is induced by oxygen limitation. The *arcD* gene specifies a 53-kDa protein with arginine: ornithine exchange activity. The ArcD protein of *P. aeruginosa*, like the LysI lysine transporter of *Corynebacterium glutamicum*, has 13 hydrophobic regions which could span the cytoplasmic membrane. Fusion of a Caa (colicin A) epitope to the N-terminal part of ArcD permitted the localization, by immunoblotting, of the hybrid protein in the inner membrane of *P. aeruginosa*. Fusion of PhoA (alkaline phosphatase) to the very C terminus of ArcD produced another hybrid protein, which exhibited PhoA activity. Both ArcD hybrid proteins retained arginine transport activity and served to support a topological model which proposes that the N terminus is oriented toward the cytoplasm and the C terminus faces the periplasm. Further ArcD-PhoA fusions were consistent with this model. When the Caa epitope was fused to a C-terminal ArcD fragment consisting of only 5 hydrophobic domains, the resulting hybrid protein could be recovered intact from the inner membrane, suggesting that the C-terminal part of ArcD contains sufficient information for insertion into the membrane. This study illustrates the utility of the Caa epitope to tag membrane proteins.

Under anaerobic conditions, *Pseudomonas aeruginosa* obtains metabolic energy from nitrate respiration or, in the absence of nitrate, from arginine degradation via the arginine deiminase pathway. This pathway intracellularly converts arginine to ornithine, with concomitant formation of one ATP from ADP; ornithine is released into the medium (1). The enzymes of the arginine deiminase pathway are encoded by the *arcDABC* operon (1–3) and coordinately induced by oxygen limitation (2, 4, 5). Induction requires the transcriptional activator protein ANR, which recognizes a sequence motif in the −40 region of the *arc* promoter (5–7). The *arcABC* genes encode the soluble enzymes arginine deiminase, catabolic ornithine carbamoyltransferase, and carbamate kinase, respectively (1, 2, 8, 9). The *arcD* gene specifies a highly hydrophobic protein, which can be detected in the total membrane fraction of *Escherichia coli* maxicells after overexpression of the cloned *arcD* gene (3). A hydrophobicity plot of the deduced ArcD amino acid sequence shows 13 potential transmembrane segments (3). Point mutations in *arcD* render *P. aeruginosa* unable to utilize extracellular arginine for anaerobic growth although in these *arcD* mutants the enzymes of the arginine deiminase pathway remain active and inducible (1, 3). Under aerobic conditions, *P. aeruginosa* utilizes arginine as a carbon and nitrogen source, primarily via the N^2-succinylarginine pathway (10). The *arcD* mutants are not impaired in aerobic arginine catabolism (1). Taken together, these findings suggest that the ArcD protein is involved in a specific uptake system that delivers arginine to the arginine deiminase pathway during conditions of oxygen limitation.

The arginine deiminase pathway allows the utilization of arginine as an energy source in a variety of bacteria other than *P. aeruginosa, e.g.* in *Lactococcus* species. In lactococci, a specific membrane transport system catalyzes the stoichiometric, electroneutral exchange between arginine and ornithine, which is produced in the cytoplasm by the arginine deiminase pathway (11–13). The driving force for arginine uptake and ornithine excretion is provided by the concentration gradients of these amino acids. The arginine-ornithine exchanger of *Lactococcus lactis* has been extracted and functionally reconstituted in proteoliposomes (13). This has permitted an analysis of the kinetic mechanism; the exchanger appears to have a single substrate binding site which may be exposed alternately to the inner and outer surface of the cytoplasmic membrane (14). The exchange activity in proteoliposomes is not affected by the electrical potential across the membrane or the protonmotive force (13, 14). Thus, the energetic costs for arginine uptake and ornithine release are minimized in the arginine deiminase pathway. The gene encoding the exchanger, however, has not yet been identified in lactococci.

Recent experiments have shown that the ArcD protein of *P. aeruginosa* has arginine:ornithine antiporter activity which is similar to that found in lactococci. An *E. coli* strain lacking arginine permease has been transformed with a recombinant

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plasmid expressing the *arcD* gene of *P. aeruginosa*. Membrane vesicles from this recombinant strain exchange intracellularly accumulated ornithine for external arginine. The ArcD protein can be solubilized from membrane vesicles and reconstituted into liposomes in a functionally active state (15). In addition to the exchange reaction, ArcD also catalyzes arginine and ornithine uptake driven by the protonmotive force (15). In this study, we report the construction of gene fusions of *arcD* to *phoA* (alkaline phosphatase) or *caa* (colicin A). The properties of the resulting hybrid proteins lead us to propose a model for the topology of the ArcD protein in the cytoplasmic membrane.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—*P. aeruginosa* strains were: PAO1 (wild type), PAO25*phoA* (*argF10 leu-10 phoA*; obtained from A. Lazdunski, Marseille), PTO6138 (*arcD6138 recA102 strA* (3)), and PTO6235 (*arcA*::Ω*Hg recA102* (5)). We used *E. coli* strains W3110(Nal^r) (prototrophic (16)), JC182–5 (*thi-1 purF1 argP* (17)), CC118 (*araD139* Δ(*ara-leu*)*7697* Δ*lacX174 phoA*Δ*20 galE galK thi rpsE rpoB argE$_{am}$ recA1* (18)), CC202 (=CC118/F′*42ts*114 *lacI3 zzf-2*::Tn*phoA* (18)), S17–1 (*pro thi hsdR recA* chromosomal RP4–2 (19)), ED8654 (*metB supE supF hsdR* (20)), and TG1 (Δ(*lac pro*) *supE thi hsdS*/F′*traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15 (Stratagene)). The following plasmids have been published: pBLS (=Bluescript II KS+ (Stratagene)), pCH39, pCH40 (21), pJRD7pol, pJRD253 (22), pKT240 (23), pME183 (2), pVBC18 (24), and pVL1 (16). Newly constructed plasmids are shown in Fig. 1 and Table I. Plasmids were introduced into *E. coli* and *P. aeruginosa* by transformation (25); pJRD7pol and some IncQ plasmids were transferred from *E. coli* S17–1 to *P. aeruginosa* by mobilization (19, 26).

Growth Conditions—Media, anaerobic growth in the Gas-Pak jar, conditions of good or limiting aeration, and selective antibiotic concentrations have been described (1, 2, 7, 25, 26). The pJRD253 derivative pME3608 was selected in *P. aeruginosa* on minimal medium containing streptomycin (75 μg/ml).

DNA Manipulations—The conditions for plasmid isolation, restriction, BAL31 digestion, ligation, and DNA sequencing by the chain termination method have all been described (2, 7, 25, 27).

Construction of 'phoA Fusions—The target plasmid for Tn*phoA* insertions was constructed by inserting the 1.95-kb[1] *Hin*dIII-*Bam*HI fragment encompassing the *arc* promoter, *arcD*, and part of *arcA* (Fig. 1*A*) into pBLS. In the resulting plasmid pME3750, the *arc* genes are expressed from the *lac* promoter on the vector. *E. coli* CC202 was transformed with pME3750. Transposition of Tn*phoA* was obtained by selection for high level kanamycin resistance on nutrient agar containing kanamycin (1000 μg/ml), ampicillin (100 μg/ml), XP (40 μg/ml), and isopropyl thiogalactoside (100 μM; added to induce the *lac* promoter). 20 blue or white colonies were purified; 3 had a Tn*phoA* insertion in *arcD* (Table I). From the latter plasmids, the segments carrying the *arc* promoter and the *arcD*′-′*phoA* open reading frame were excised by *Hin*dIII + *Xho*I digestion and inserted into pKT240 (23) cut by the same enzymes. The kanamycin promoter of pKT240, which might have interfered with *phoA* expression, was eliminated by a 1.6-kb *Xho*I-*Hpa*I deletion, giving pME3761, pME3762, and pME3765 (Table I). *In vitro phoA* fusions were constructed in *E. coli* CC118. The *Aat*II site in *arcD* (position 910) carried by pME3750 and the *Pst*I site upstream of *phoA* on pCH39 were both rendered blunt by T4 DNA polymerase (27) to allow in-frame joining; subcloning into pKT240 gave pME3763 (Table I). The *Pst*I site in *arcD* (position 1019) and the *Pst*I site of pCH40 served to construct pME3764 (Table I). Finally, pME3750 was cleaved at the *Bam*HI site in *arcA* (Fig. 1), digested with exonuclease III + mung bean nuclease (Stratagene) + calf intestinal alkaline phosphatase (27), and ligated with a *Bgl*II linker (Boehringer). A deletion derivative of pME3750 was chosen in which the *Bgl*II linker was inserted precisely into the TGA stop codon of *arcD*. The resulting 1.7-kb *Hin*dIII-*Bgl*II fragment carrying *arcD* (Fig. 1) and the 3.2-kb *Bam*HI-*Hin*dIII fragment carrying *'phoA* from pVBC18 (24) were inserted together into pKT240 (cleaved with *Hin*dIII), giving pME3806 (Table I).

Construction of caa′ Fusions—The expression vector pEPlink (Fig. 1*A*) was derived from pVL1 (16), from which a 0.5-kb *Nco*I-*Ava*I fragment was removed and replaced by a multiple cloning site. The *Nco*I site spans codons 29–31 of the *caa* gene (Fig. 1*B*). For the construction of pME3801, the proximal part of the *arcD* gene on pME183 (Ref. 2) was deleted by BAL31 digestion to position 1220 in *arcD* and ligated to a *Hin*dIII linker, as described previously (3). A 19-base pair fragment from pBLS was joined to the *Hin*dIII site. This created a new *Sal*I site upstream of *arcD*. The 0.75-kb *Sal*I-*Bam*HI fragment carrying *'arcD-arcA'* was then inserted into the corresponding sites in pEPlink, giving pME3801 (Fig. 1, *A* and *B*). The construction of pME3802 involved cloning of the 1.7-kb *Mae*I-*Bam*HI fragment containing *'arcD-arcA'* into the *Eco*RV and *Bam*HI sites of pEPlink (Fig. 1, *A* and *B*). Plasmid pME3808 was derived from pME3802 as follows: the *caa* promoter was removed by a 0.25-kb BAL31 deletion initiated from the unique *Pvu*II site; after insertion of a phosphorylated *Bam*HI linker (Pharmacia LKB Biotechnology Inc.), the promoterless 1.8-kb *caa*′-′*arcD arcA* fragment was inserted into pJRD253 such that the T7Φ10 promoter reads into *caa*′-′*arcD* (Fig. 1*A*).

Induction of the caa Promoter—*E. coli* cells carrying pEPlink derivatives were grown to $OD_{600\,nm} \approx 0.5$ in LB medium (27) and then induced with mitomycin C (0.3 μg/ml; Sigma (16)) or with an analog of nalidixic acid, norfloxacin (4 μg/ml; Merck Sharp & Dohme), for 2 to 4 h (28). Alternatively, induction was achieved by UV irradiation (20 J/m^2 (28)).

Membrane Preparations of P. aeruginosa—Total membranes of strain PAO were prepared by the method of Hancock and Nikaido (29) and fractionated on two discontinuous sucrose gradients (29, 30) in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF).

SDS-PAGE and Immunoblotting—Protein samples were solubilized in 160 mM Tris (base), 4 mM EDTA, 3.6% (w/v) SDS, 60 mM dithiothreitol, 1% (v/v) β-mercaptoethanol, 0.8 M sucrose, and 0.01% bromphenol blue by heating at 95 °C for 5 min. Samples containing Caa′-′ArcD were not heated (31) as heat treatment was found to enhance protein degradation. 12.5% polyacrylamide gels were 0.75 mm thick; they were run according to Laemmli and Favre (32). Marker proteins were prestained (Bio-Rad). Note that the addition of the dye causes these proteins to migrate more slowly than expected from their true molecular masses (Bio-Rad). Apparent molecular masses of the markers are 16, 24, 33, 47, and 84 kDa rather than the true values of 14, 20, 29, 43, and 66 kDa, which are indicated in Figs. 2–4. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) in a Hoefer semi-dry Blotter (27). Immunochemical detection of Caa′-′ArcD proteins was performed using monoclonal antibody 1C11 (33), goat anti-mouse IgG conjugated with peroxidase (Bio-Science Products, Emmenbrücke, Switzerland), and peroxidase reaction mixture (27).

Enzyme and Protein Assays—Alkaline phosphatase was determined in *P. aeruginosa* cells permeabilized with 0.005% SDS and 5% (v/v) chloroform in 1 M Tris-HCl, pH 8; the substrate was *p*-nitrophenyl phosphate (34). NADH oxidase was assayed by the method of Osborn *et al.* (35). One enzyme unit is defined as 1 nmol of product formed per min. Anaerobic arginine consumption by whole cells of *P. aeruginosa* was measured by a modified Sakaguchi reaction (36). Protein concentrations were estimated by the method of Bradford (37) using a commercial reagent (Bio-Rad) and bovine serum albumin as a standard.

RESULTS

Construction of Caa′-′ArcD Fusion Proteins and Their Expression in E. coli—We wished to detect the ArcD protein of *P. aeruginosa* specifically in membrane fractions. As no antibodies against ArcD are available, we tagged ArcD with an epitope which is derived from the colicin A (Caa) protein and which is recognized by a specific monoclonal antibody (16, 33). The epitope consists of amino acids 13 to 26 of the Caa protein.[2] The construction of Caa′-′ArcD hybrid proteins was facilitated by the expression vector pEPlink (Fig. 1*A*). This plasmid carries the strong σ^{70}-dependent, LexA-regulated *caa* promoter, the *caa* ribosome binding site, and the 31 N-terminal amino acids of the *caa* structural gene (38, 39),

[1] The abbreviations used are: kb, kilobase pair(s); PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SLS, sodium lauryl sarcosinate; XP, 5-bromo-4-chloro-3-indolyl phosphate.

[2] V. Géli, R. Lloubès, S. A. J. Zaat, R. M. L. van Spaendonk, J. Najib, H. Benedetti, and C. Lazdunski, manuscript in preparation.

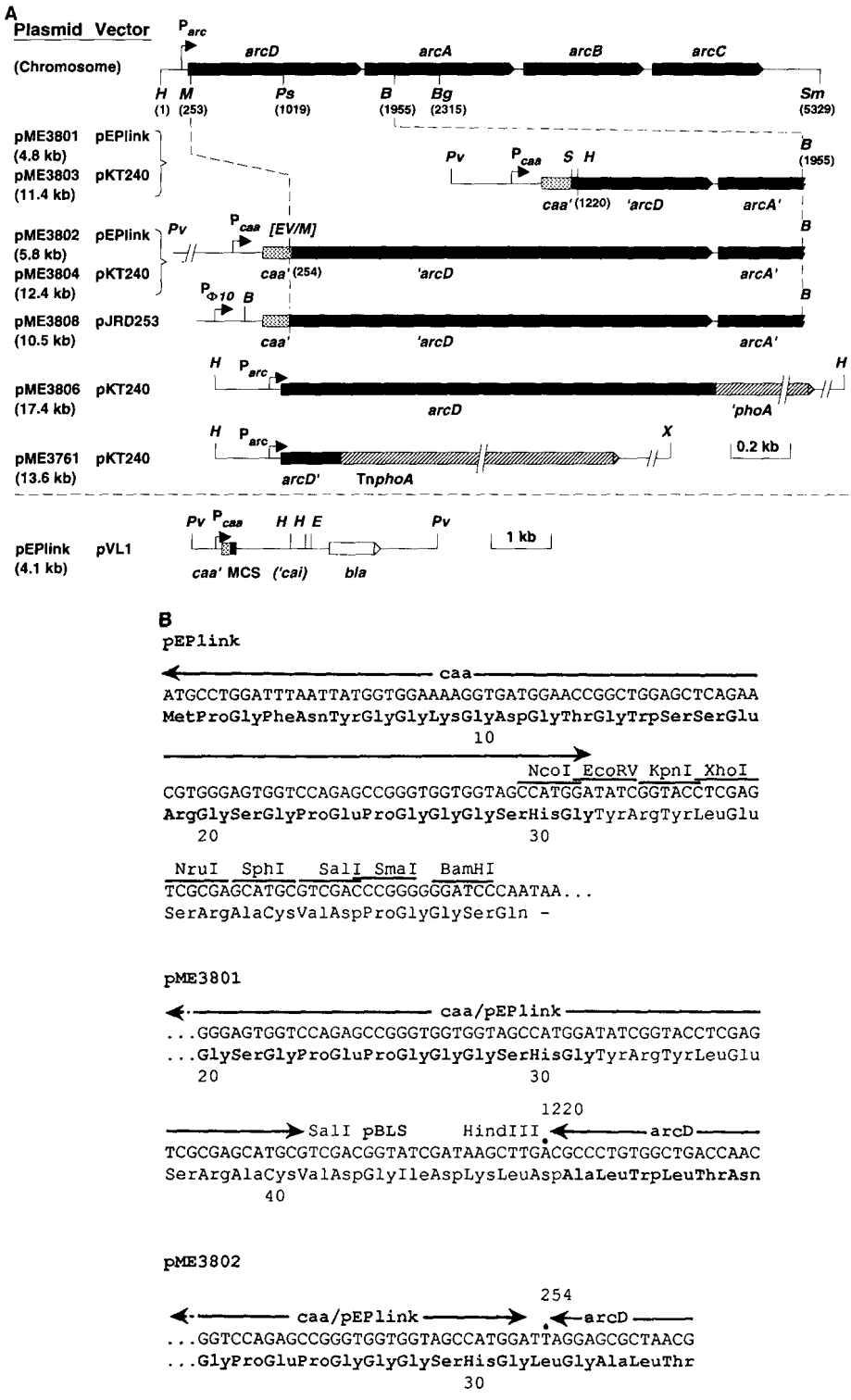

FIG. 1. **Recombinant plasmids coding for Caa'-'ArcD and ArcD'-'PhoA hybrid proteins.** *A*, the construction of these plasmids is described under "Materials and Methods." Two representative *phoA* constructs are shown (pME3806 and pME3761); the others are listed in Table I. *Horizontal arrows* designate promoters of the *arc* operon (P_{arc}), the *caa* gene (P_{caa}), or the T7Φ10 sequence ($P_{\Phi 10}$). *Numbers* in *brackets* below restriction sites refer to the complete nucleotide sequence of the *arcDABC* operon (3, 8, 9). Restriction sites are abbreviated: *B*, *Bam*HI; *Bg*, *Bgl*II; *E*, *Eco*RI, *EV*, *Eco*RV; *H*, *Hin*dIII, *M*, *Mae*I; *Ps*, *Pst*I; *Pv*, *Pvu*II; *S*, *Sal*I; *Sm*, *Sma*I; *X*, *Xho*I. *MCS*, multiple cloning site (see *B*). *B*, the 31 N-terminal amino acid residues (*boldface*) of the *caa* gene (38, 39) are connected to the MCS in pEPlink. All restriction sites (*Nco*I to *Bam*HI) in the MCS are unique. The monoclonal antibody 1C11 (33) recognizes amino acid residues 13 to 26.[2] In pME3801, the first *arcD* amino acid residues Ala-Leu-Trp-Leu-Thr-Asn are in the putative transmembrane helix IX (Fig. 6). In pME3802, the first *arcD* residues Leu-Gly-Ala-Leu-Thr are in the putative transmembrane helix I (Fig. 6).

followed by a multiple cloning site (Fig. 1*B*). To construct the first Caa'-'ArcD fusion protein, we removed the 10 N-terminal codons from the *arcD* coding sequence by digestion with *Mae*I, which cleaves the *arcD* gene once at position 253 (Ref. 3). The *Mae*I site was filled in with Klenow polymerase and the truncated *'arcD* gene was fused, in frame, to the 31 N-terminal codons of the *caa* gene on pEPlink (cleaved at the unique *Eco*RV site; Fig. 1*B*). Thus, the recombinant plasmid pME3802 (Fig. 1, *A* and *B*) was obtained which has a *caa'-'arcD* open reading frame consisting of 503 codons (31 *caa'* codons + 472 *'arcD* codons). The molecular mass of the deduced Caa'-'$ArcD_{472}$ hybrid polypeptide was calculated to be 54 kDa. Note that the first putative transmembrane segment in the wild type ArcD protein is believed to start at Leu-11 (3; *cf.* Fig. 6). As the Caa epitope replaces the first 10 amino acid residues of ArcD, the Caa'-'$ArcD_{472}$ hybrid should have an intact transmembrane segment 1.

A second hybrid *caa'-'arcD* gene was constructed on pME3801 (Fig. 1*A*). This derivative of pEPlink also expresses the *caa* N terminus under the control of the *caa* promoter. The 31 N-terminal codons of *caa* were fused, via a 17-codon linker fragment, to the 149 C-terminal codons of *arcD*. Details

of construction are explained under "Materials and Methods." The resulting fusion protein, designated Caa′-′ArcD$_{149}$, has a calculated molecular mass of 21 kDa. Its probable topology will be discussed later (Fig. 6).

In *E. coli* W3110(Nal^r) carrying pME3801 or pME3802, these Caa′-′ArcD fusion proteins were expressed after SOS induction with mitomycin C, UV irradiation, or norfloxacin, and detected by Western blotting using the specific monoclonal antibody directed against the Caa epitope. Without SOS induction, no proteins were revealed in Western blots (data not shown), demonstrating the tight LexA control of the vector promoter on pEPlink.

The Caa′-′ArcD proteins could be localized in the cytoplasmic membrane of *E. coli*. Cells of strain W3110(Nal^r) harboring pME3801 or pME3802 were induced with mitomycin C and disrupted by sonication. The total membrane fraction purified by sucrose block gradient centrifugation (40) was extracted with sodium lauryl sarcosinate (SLS), a detergent known to solubilize selectively inner membrane proteins of *E. coli* (41). The total membrane fractions and the SLS extracts were subjected to SDS-PAGE. A Western blot of an SLS extract revealed the Caa′-′ArcD$_{472}$ protein as a single band corresponding to an apparent molecular mass of 40 kDa (Fig. 2, *lane 1*). In the total membrane preparation, this protein was also visible, along with some supposed degradation products (Fig. 2, *lane 3*). It appears that SLS extraction resulted in a purification of the Caa′-′ArcD$_{472}$ protein, removing the contaminating degradation polypeptides (Fig. 2, *lane 1*). The Caa′-′ArcD$_{149}$ protein lacks more than two-thirds of ArcD; this hybrid was nevertheless found in the total membrane fraction (Fig. 2, *lane 4*). Extraction with SLS gave a single 18-kDa band (Fig. 2, *lane 2*), indicating that this protein was located in the cytoplasmic membrane as well. The membrane fraction contained the 18-kDa Caa′-′ArcD$_{149}$ protein together with an aggregated form, perhaps representing a dimer (Fig. 2*A*, *lane 4*). The experimental M_r values for Caa′-′ArcD$_{472}$ and Caa′-′ArcD$_{149}$ are both smaller than the values calculated from sequence data. This discrepancy was observed previously for the ArcD protein (3) and is typical of integral membrane proteins. These hydrophobic proteins bind excessive amounts of SDS, resulting in an anomalously high electrophoretic mobility (42).

The orientation of the Caa epitope in the inner membrane was probed by two methods. First, right-side-out membrane vesicles of strain W3110(Nal^r) carrying pME3801 or pME3802 were prepared from osmotically lysed spheroplasts (35). Inner membrane vesicles were treated with proteinase K (25 μg/ml) on ice for 15 min. This treatment did not affect the integrity of the Caa′-′ArcD$_{149}$ and Caa′-′ArcD$_{472}$ proteins (Fig. 3, *lanes 1* and *3*). Apparently, the periplasmic loops of the ArcD recombinant proteins were not accessible to proteinase K. After lysis of the membrane vesicles with Triton X-100, however, the Caa epitope was proteolytically degraded (Fig. 3, *lanes 2* and *4*). We infer from this result that in both fusion proteins the Caa epitope faces the cytoplasm.

This finding was confirmed with *E. coli* cells whose outer membrane had been permeabilized with EDTA in Tris-HCl buffer (44). This procedure allows access of trypsin to the periplasm (43, 44). When the Caa epitope is exposed to the periplasm, as is the case in a Caa′-′TolQ hybrid protein,

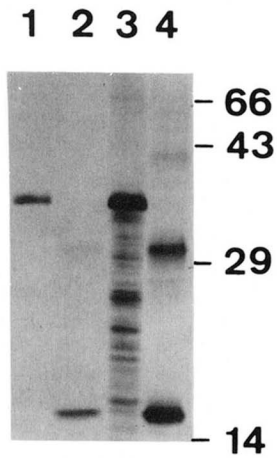

FIG. 2. **Solubilization of Caa′-′ArcD hybrid proteins with SLS.** *E. coli* W3110(Nal^r) carrying pME3801 or pME3802 was induced with mitomycin C. Cells of a 50-ml culture were resuspended in 2 ml of 35 mM Tris-HCl, pH 7.8, 20% (w/v) sucrose, 0.2 mM PMSF, 10 μg of DNase/ml and broken by sonification (3 pulses of 30 s). EDTA was added to 5 mM final concentration. Total membranes were purified by sucrose block gradient centrifugation (40, 43). Part of this preparation was extracted with 0.5% (w/v) SLS (final concentration) in 500 μl of 100 mM Tris-HCl, pH 7.9, at room temperature for 20 min (41). The reaction was stopped by 20-fold dilution in 10 mM Tris-HCl, pH 7.9, 5 mM EDTA, 0.2 mM PMSF. After ultracentrifugation at 100,000 × *g* and 4 °C for 100 min, the supernatant with added carrier protein (bovine serum albumin, 50 μg/ml) was precipitated with 0.5 volume of 50% (w/v) trichloroacetic acid. The precipitate was dissolved in loading buffer (see "Materials and Methods"). The Caa epitope was visualized with 1C11 antibody by Western blotting of an SDS gel. *Numbers* indicate size standards (kDa). *1*, Caa′-′ArcD$_{472}$ encoded by pME3802 after SLS extraction; *2*, Caa′-′ArcD$_{149}$ encoded by pME3801 after SLS extraction; *3*, total membrane fraction containing Caa′-′ArcD$_{472}$; *4*, total membrane fraction containing Caa′-′ArcD$_{149}$.

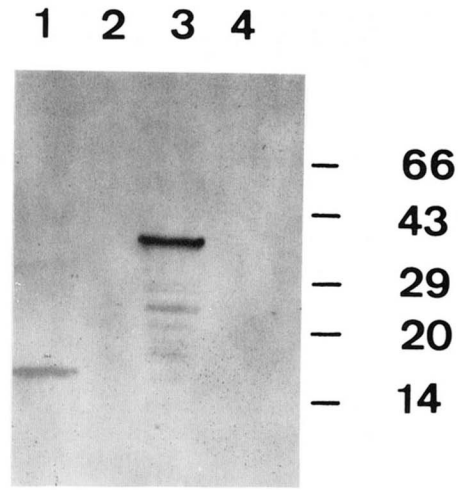

FIG. 3. **Accessibility of Caa′-′ArcD hybrid proteins to proteinase K in right-side-out membrane vesicles.** *E. coli* W3110(Nal^r) carrying pME3801 or pME3802 was induced with mitomycin C. Cells from a 500-ml culture were converted to spheroplasts and osmotically lysed (35). After centrifugation at 1,200 × *g* for 20 min (to remove whole cells) and ultracentrifugation at 360,000 × *g* for 1 h, the membrane pellet was resuspended in 2 ml of 10 mM Tris, 5 mM EDTA, pH 7.8, 25% (w/v) sucrose, and purified by sucrose block (60 to 30% (w/v)) gradient centrifugation (40) in a Beckman SW41 rotor at 250,000 × *g* for 18 h. Fractions containing inner membrane vesicles were pooled. Proteinase K (25 μg/ml, final concentration) was added to this preparation (100 μg of protein/ml) at 0 °C; Triton X-100 (0.5% (v/v), final concentration) was further included in two samples. After incubation at 0 °C for 15 min, the reaction was stopped by addition of PMSF (10 mM, final concentration). The entire trichloroacetic acid-precipitated samples were immediately submitted to SDS-PAGE and analyzed by Western blotting using 1C11 antibody. *Numbers* indicate the molecular masses of size standards (kDa). *1*, Caa′-′ArcD$_{149}$ specified by pME3801, without Triton X-100; *2*, same as *1* but with Triton X-100; *3*, Caa′-′ArcD$_{472}$ specified by pME3802, without Triton X-100; *4*, same as *3* but with Triton X-100.

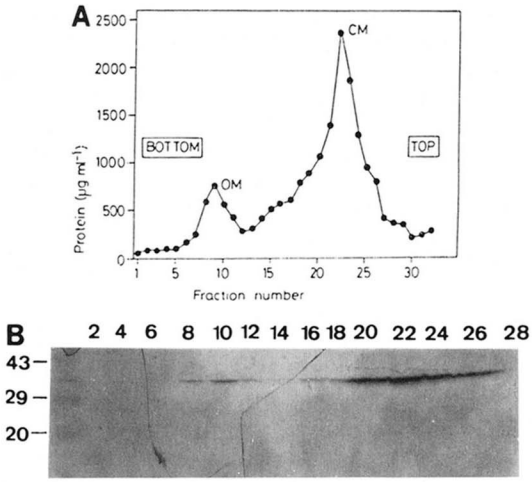

FIG. 4. **Distribution of the Caa′-′ArcD$_{472}$ fusion protein in membrane fractions of *P. aeruginosa*.** *A*, *P. aeruginosa* PAO harboring pME3808 and pJRD7pol was grown in 1 liter of LB medium supplemented with tetracycline (50 μg/ml) and streptomycin (75 μg/ml) at 30 °C. When the cell density had reached $OD_{600\,nm} \approx 0.5$, the culture was shifted to 42 °C for 30 min and then the incubation was continued in the presence of rifampicin (200 μg/ml) at 30 °C for 100 min. Cells were disrupted in a French Press, and total membranes were fractionated by two sucrose gradients (29, 30). Fractions (400 μl) of the second sucrose gradient were collected from the bottom of the tube and tested for protein concentration. Peak fractions were assayed for NADH oxidase activity; fractions 9 (*OM*, outer membrane) and 22 (*CM*, cytoplasmic membrane) contained 11 units/ml and 166 units/ml, respectively. *B*, from each even-numbered fraction, 100 μg of protein was precipitated with trichloroacetic acid and then subjected to SDS-PAGE. Immunoblot analysis with antibody 1C11 revealed the Caa′-′ArcD$_{472}$ protein.

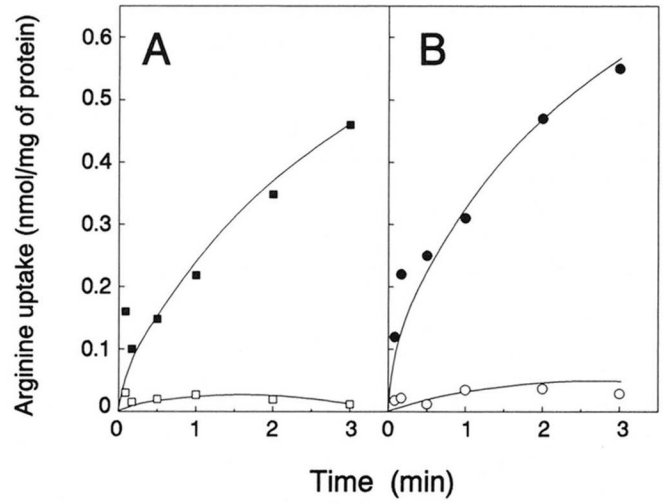

FIG. 5. **Arginine:ornithine exchange activity of the Caa′-′ArcD$_{472}$ and ArcD-′PhoA hybrid proteins.** *A*, *E. coli* JC182–5 (*argP*) carrying pME3802 was induced with norfloxacin in LB medium for 4 h. Membrane vesicles were prepared as described (15) and loaded with 500 μM L-ornithine in 50 mM potassium phosphate, pH 6.0, at 0 °C overnight, the protein concentration being 0.5 mg/ml. The membrane vesicles were exposed to 37 °C for 5 min, concentrated by centrifugation at 120,000 × *g* and 4 °C, and stored on ice. The exchange reaction was started by diluting the membrane vesicles (approximately 6 to 7 mg of protein per ml) 50-fold in 50 mM potassium phosphate, pH 6.0, containing 0.375 μM L-[^{14}C]arginine (11 GBq/mmol). The reaction was stopped at intervals with 0.1 M LiCl. Radioactivity was determined in vesicles collected on a nitrocellulose filter (13). *E. coli* JC182–5/pME3802 vesicles loaded (■) or nonloaded (□) with ornithine. *B*, *E. coli* JC182–5/pME3806 was grown in LB medium, and membrane vesicles were prepared as described (15). Reaction conditions are as in *panel A*. *E. coli* JC182–5/pME3806 vesicles loaded (●) or nonloaded (○) with ornithine.

trypsin/EDTA treatment leads to rapid digestion of the epitope (43). In contrast, we could not detect any damage of the Caa epitope by trypsin/EDTA in Caa′-′ArcD$_{149}$ or Caa′-′ArcD$_{472}$, suggesting that indeed the epitope in these fusion proteins was not directed toward the periplasmic side of the inner membrane (data not shown).

Expression of Caa′-′ArcD Fusion Proteins in P. aeruginosa—The *caa* promoter-operator region and the *caa′-′arcD* coding sequences of the *E. coli* plasmids pME3801 and pME3802 were subcloned into the broad-host-range vector pKT240 (cleaved with *Hpa*I and *Bam*HI). The resulting recombinant plasmids pME3803 and pME3804 allowed expression of Caa′-′ArcD$_{149}$ and Caa′-′ArcD$_{472}$, respectively, in *E. coli* after induction with mitomycin C, norfloxacin, or UV irradiation (data not shown). After conjugative transfer of these plasmids to *P. aeruginosa* PAO1, we attempted to obtain expression of the fusion proteins by exposure to mitomycin C (1.5 μg/ml), UV irradiation (20 J/m^2), or norfloxacin (4 μg/ml). It is known that such DNA damaging agents induce the RecA protein and pyocins in *P. aeruginosa* (28, 45, 46). However, the PAO1/pME3803 or pME3804 transconjugants did not express the Caa′-′ArcD proteins under these conditions, suggesting that the *caa* promoter of *E. coli* is not inducible in *P. aeruginosa*. The reasons for the lack of induction are not clear; *P. aeruginosa* contains a LexA-like repressor protein (47).

To determine the cellular location of the Caa′-′ArcD$_{472}$ protein in *P. aeruginosa*, we therefore decided to use the T7Φ10 promoter on the broad-host-range vector pJRD253 (22) as a substitute for the *caa* promoter. We constructed the recombinant plasmid pME3808 (Fig. 1*A*) which contains the *caa′-′arcD* open reading frame of pME3802 fused to the T7Φ10 promoter. The LexA-repressible *caa* promoter, but not the *caa* ribosome binding site, was removed by BAL31 deletion. Strain PAO1 was transformed with pME3808 and pJRD7pol (helper plasmid coding for the T7 RNA polymerase under the control of the thermoinducible λP_L promoter (22)). After a temperature shift from 30 °C to 42 °C, the Caa′-′ArcD$_{472}$ protein could be detected by Western blotting. Moreover, this protein was found to be associated mostly with the cytoplasmic membrane, as judged by Western blot analysis of a sucrose gradient that was used to separate the inner and outer membrane fractions (Fig. 4). Thus, it appears that the Caa′-′ArcD$_{472}$ protein is localized in the cytoplasmic membrane in *P. aeruginosa* as well as in *E. coli*.

Arginine:Ornithine Exchanger Activity of the Caa′-′ArcD$_{472}$ Protein in E. coli—To demonstrate transport activity of the Caa′-′ArcD$_{472}$ fusion protein, we introduced pME3802 into the arginine permease-negative *E. coli* strain JC182–5. After induction with norfloxacin (4 μg/ml) for 4 h, membrane vesicles were prepared from osmotically lysed spheroplasts. (Induction with mitomycin C or UV interfered with the isolation of membrane vesicles.) The vesicles were loaded with unlabeled ornithine at 0 °C overnight. Incubation in the presence of [^{14}C]arginine at 25 °C resulted in arginine uptake (Fig. 5*A*; ■), whereas arginine uptake was low in vesicles not loaded with ornithine (Fig. 5*A*, □). In a control experiment, membrane vesicles from strain JC182–5 without plasmid did not take up arginine (data not shown). We conclude that the 10 N-terminal amino acids of ArcD are not essential for transport activity in that they can be replaced by the Caa epitope.

Construction of ArcD′-′PhoA Fusion Proteins and Their Expression in P. aeruginosa—Alkaline phosphatase of *E. coli* has been widely used to study the topology of membrane proteins (18, 21, 48, 49). Six *arcD′-′phoA* gene fusions were

TABLE I

Characteristics of ArcD'-'PhoA hybrid proteins

Plasmid[a]	Source of *phoA*	Sequence of *arcD*'-'*phoA* junctions and deduced amino acid sequence[b]	Color of colonies on XP plates[c]	Alkaline phosphatase sp.act.[d] $+O_2$	Alkaline phosphatase sp.act.[d] $-O_2$
pME3761	Tn*phoA*	367 • ——— * CTG ATC GGA TGG GCG ACT □ CCT GTT **Leu Ile Gly Trp Ala** Thr X *Pro*$_6$ *Val*	blue	1.1	47.5
pME3762	Tn*phoA*	655 • ——— * CTG CAC TTC CTG GTG CCT □ CCT GTT **Leu His Phe Leu Val** Pro X *Pro*$_6$ *Val*	pale blue	<0.1	1.1
pME3763	pCH39	910 • —— * GCG GAA AAA CGT TCC GGC CCT GTT **Ala Glu Lys Arg Ser** Gly *Pro*$_6$ *Val*	white	<0.1	0.1
pME3764	pCH40	1019 • ——— * CTG GCC AAG CTG CAG CCT CAG GGC **Leu Ala Lys Leu Gln** Pro *Gln*$_{14}$ *Gly*	blue	0.9	24.4
pME3765	Tn*phoA*	1090 • ——— * CTG ATC AGC GTC GGC CCT □ CCT GTT **Leu Ile Ser Val Gly** Pro X *Pro*$_6$ *Val*	white	<0.1	0.5
pME3806	pCH40[e]	1070 • ——————————— ACC CTC TGC AGA TCC GTC GAC CTG **Thr Leu** Cys Arg Ser Val Asp Leu ——— * CAG CCT CAG GGC Gln Pro *Gln*$_{14}$ *Gly*	blue	8	65

[a] The vector was pKT240. Care was taken to avoid read-through from vector promoters into the *arcD'-'phoA* region. The exception is pME3806 where the promoter of the kanamycin resistance gene of pKT240 lies 0.8 kb upstream of the *arc* promoter, resulting in moderate phosphatase activity even when the *arc* promoter is not induced.

[b] Numbers and dots shown above the sequence indicate the last *arcD* nucleotides (3). Horizontal lines above the sequence represent linkers; □ stands for a 16-codon sequence at the end of Tn*phoA* (18): GAC TCT TAT ACA CAA GTA GCG TCC TGG ACG GAA CCT TTC CCG TTT TGC Asp Ser Tyr Thr Glu Val Ala Ser Trp Thr Glu Pro Phe Pro Phe Cys. * refers to the first nucleotide in mature alkaline phosphatase (50). Amino acid sequences represent ArcD (**boldface**), linkers, and PhoA (*italics*), in this order. Numbers designate the amino acid residue in wild type PhoA (50).

[c] The host strain was *P. aeruginosa* PTO6235 (*arcA*::ΩHg *recA102*) except for pME3806 which had been transferred to strain PTO6138 (*arcD6138 recA102*). Nutrient agar plates containing the chromogenic substrate XP (40 μg/ml) were incubated aerobically at 30 °C for 3 days.

[d] Alkaline phosphatase levels are expressed in nmol per min and ml of culture at $OD_{600\ nm}$ = 1.0. Cells were grown in YEA medium with good aeration ($+O_2$) or oxygen limitation ($-O_2$).

[e] The linker sequence consists of a *Bgl*II linker spliced to a *Bam*HI-*Pst*I fragment of pVBC18 (Ref. 24) and the *Pst*I site of pCH40.

constructed by two approaches. First, Tn*phoA* insertions (18) were obtained in the multicopy *E. coli* plasmid pME3750 carrying *arcD* (see "Materials and Methods"). Most Tn*phoA* insertions occurred in the ampicillin resistance gene of the vector and only three different *arcD*::Tn*phoA* fusions were isolated, which gave blue colonies on XP plates. To test the *arcD'-'phoA* fusions in *P. aeruginosa*, we subcloned the fragments containing the *arc* promoter and the *arcD'-'phoA* open reading frame into the broad-host-range vector pKT240. The resulting plasmids pME3761 (Table I and Fig. 1*A*), pME3762, and pME3765 (Table I) could be transferred to *P. aeruginosa*. It turned out that the *arcD*::Tn*phoA* insertions of pME3762 and pME3765 gave very low alkaline phosphatase activities in *P. aeruginosa* (Table I). Hence, the corresponding precursor plasmids in *E. coli* had indicated a false positive $PhoA^+$ phenotype.

In the second approach, a *'phoA* fragment (21) was fused, *in vitro*, to the *arcD* gene at three different sites, the vector being again pKT240 (Table I). Two *arcD*-internal sites (*Aat*II at position 910 and *Pst*I at position 1019) were used in pME3763 and pME3764, respectively. For the construction of a full-length ArcD-'PhoA hybrid protein, a *Bgl*II linker was inserted precisely at the natural TGA stop codon behind the *arcD* gene (3), by an *in vitro* deletion of the distal *arcA* gene. This permitted the fusion of *'phoA* to the C terminus of *arcD* (in pME3806, Fig. 1*A* and Table I). All *arcD'-'phoA* fusions were constructed in-frame and checked by DNA sequencing.

The *phoA* constructs were introduced into *P. aeruginosa* by transformation. Since all *arcD'-'phoA* hybrid genes are under the control of the *arc* promoter, they should be expressed during oxygen limitation. This was found to be the case. *P. aeruginosa* carrying pME3761, pME3764, or pME3806 had high alkaline phosphatase activities, which were inducible by lack of aeration (Table I). On XP plates, these strains formed blue colonies (Table I). This indicates that the PhoA moiety of the corresponding hybrid proteins is directed toward the periplasm. In contrast, *P. aeruginosa* transformed with the

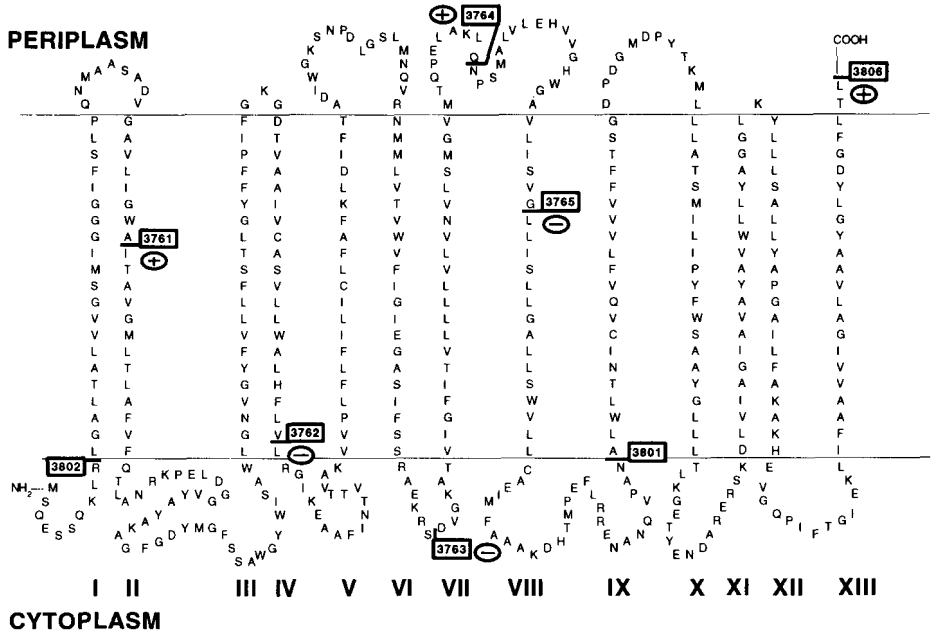

FIG. 6. **Topological model of the ArcD protein in the cytoplasmic membrane.** The model is based on a hydrophobicity plot (3, 53) and the inside-positive rule (55, 56). *Arrows* mark the fusion sites of Caa′ or ′PhoA hybrid proteins; *numbers* indicate the plasmid constructs shown in Fig. 1*A* and Table I. +, high PhoA activity; −, low PhoA activity.

plasmids pME3762, pME3763, or pME3765 yielded very low alkaline phosphatase activities and produced pale blue or white colonies on XP plates (Table I), suggesting that the PhoA part of the hybrid proteins faces the cytoplasm or has no access to the periplasm. The topological model presented below (Fig. 6) incorporates these findings.

Plasmid pME3806 codes for a hybrid protein that has all ArcD amino acid residues and, therefore, could retain arginine:ornithine antiporter activity. This was tested by complementation. A recombination-deficient *arcD* mutant of *P. aeruginosa*, PTO6138, was transformed with pME3806. On yeast extract-arginine (YEA) medium, the transformant grew anaerobically in a Gas-Pak jar, whereas the *arcD* mutant could not grow. Moreover, in liquid medium containing 25 mM arginine, the transformant PTO6138/pME3806 consumed 98% of the arginine initially present within 24 h of incubation under oxygen limitation. Arginine consumption by the wild type PAO1 was similar (3), whereas the *arcD* mutant PTO6138 used only 26% of the arginine in the medium. To establish the transport activity of the ArcD-′PhoA fusion protein directly, we performed arginine uptake experiments with membrane vesicles from *E. coli* JC182–5 transformed with pME3806. Membrane vesicles prepared from these cells rapidly accumulated [^{14}C]arginine after loading with unlabeled ornithine (Fig. 5*B*, ●). Uptake of arginine was low when vesicles were not loaded with ornithine (Fig. 5*B*, ○). These results indicate that the ArcD moiety specified by pME3806 remains active for arginine uptake.

DISCUSSION

The ArcD protein of *P. aeruginosa* transports arginine, ornithine, and lysine and catalyzes their energy-independent exchange. The exchange reaction is much faster than the uptake driven by the protonmotive force (15). The physiological role of ArcD in the arginine deiminase pathway is to take up arginine from the medium in exchange for ornithine, which is formed from arginine in the cytoplasm (1, 3). The ArcD protein has 43% sequence identity with the LysI protein of *Corynebacterium glutamicum* (51). LysI mediates lysine uptake and exchanges lysine for alanine, valine, or isoleucine (52). The hydrophobicity profiles (53) of ArcD and LysI are very similar; both proteins have 13 hydrophobic regions (3, 51). Eleven hydrophobic segments of ArcD each have about 21 amino acids and are presumed to traverse the cytoplasmic membrane. The slightly shorter hydrophobic segments XI and XII might be embedded in the membrane or just span it (Fig. 6). It is of particular interest that ArcD appears to have 13 membrane-spanning segments; usually 12 membrane-spanning segments are found in bacterial secondary transport proteins (54). When we apply the "positive inside-rule" (56), the N terminus of ArcD should lie in the cytoplasm whereas the C terminus would be exposed to the periplasm (Fig. 6). According to this topology, there are 15.6% positively charged (arginine, lysine) residues in the cytoplasmic loops of ArcD *versus* 6.7% positive charges in the periplasmic loops (Fig. 6), which is in agreement with von Heijne's postulates (55, 56). In the present study, we have located the ArcD protein in the inner membrane and we have examined the plausibility of the topological model by Caa′ and ′PhoA fusions.

The ′PhoA fusion to full-length ArcD (encoded by pME3806) gave high alkaline phosphatase activity (Table I), and a substantial amount of the hybrid protein was associated with the cytoplasmic membrane (data not shown). This result is consistent with our model (Fig. 6). The Caa epitope fused to the 11th ArcD residue in Caa′-′ArcD$_{472}$ was protected from protease attack and hence is likely to face the cytoplasm (Fig. 3). The Caa N terminus contains 1 lysine and 1 arginine residue, as does the N-terminal fragment of ArcD which has been replaced by the Caa epitope. The Caa N terminus consists essentially of hydrophilic amino acids and should not influence the targeting of following hydrophobic segments. For these reasons we expect that the Caa′-′ArcD$_{472}$ hybrid should behave like native ArcD protein with respect to insertion into the membrane. If the assumption is correct, the topology proposed by our model (Fig. 6) is again supported. The ′PhoA and the Caa′ fusion proteins of pME3806 and pME3802 both retained ArcD transport activity (Fig. 5, *A* and *B*). The ′PhoA and Caa′ extensions may therefore provide useful handles for affinity purification of the ArcD protein.

The construction and testing of *arcD′-′phoA* fusions required certain precautions. Since some false positive results were observed in an *E. coli* background, all fusions were tested in *P. aeruginosa*. Direct Tn*phoA* insertions into an *arc*$^+$ broad-host-range plasmid of the IncQ group were avoided because

Tn*5* insertions in IncQ plasmids are notoriously unstable in *Pseudomonas* species (57, 58). Therefore, the fusions of *arcD* to *'phoA* or Tn*phoA* fragments were subcloned, *in vitro*, into pKT240 derivatives (Table I), and this procedure limited the number of constructs that could be analyzed. The PhoA activities of four internal ArcD'-'PhoA constructs (pME3761 to pME3764; Table I) were compatible with the model presented in Fig. 6. The PhoA$^-$ phenotype of a further fusion protein (encoded by pME3765) suggests that perhaps the fusion site lies closer to the cytoplasm than predicted by the hydrophobicity plot (Fig. 6). This seems possible because the periplasmic loop between segments VII and VIII contains several hydrophobic amino acids (VVGHWGA) that might be embedded in the membrane (Fig. 6). Clearly, further fusions would be necessary to confirm the proposed topology of the inner part of ArcD. However, an important implication of our present model, the uneven number of membrane-spanning segments in ArcD, ought not to be affected by the properties of additional 'PhoA fusions.

The Caa'-'$ArcD_{149}$ hybrid was recovered from the inner membrane (Figs. 2 and 3) and appeared to be stable, indicating that the five C-terminal hydrophobic segments are sufficient to direct the truncated protein into the membrane. C-terminal fragments of the LacY lactose carrier can also be incorporated into the cytoplasmic membrane but they need the presence of a complementary N-terminal fragment for stabilization (59, 60). Protease accessibility experiments suggest that the Caa epitope in the Caa'-'$ArcD_{149}$ hybrid protein is oriented toward the cytoplasm, as expected from the model (Fig. 6).

The usefulness of PhoA fusions in topological studies of membrane proteins has been amply documented (48, 49). Similarly, fusions of the Caa epitope (33) to membrane proteins can be exploited to determine the orientation of a protein in bacterial membranes. Previous applications of the Caa tag system have shown that the Caa epitope can either be located in the cytoplasm (as in the case of the immunity protein to colicin A (16)) or be translocated to the periplasm (as in the case of the TolQ protein (43)). Furthermore, it is also possible to insert the Caa epitope at an internal site of a polypeptide chain. For example, a hybrid PhoE-Caa outer membrane protein has been constructed in *P. putida*. In this hybrid, the epitope is exposed to the cell surface, allowing immunochemical detection of the recombinant bacteria.[2] The present study on the ArcD inner membrane protein confirms the utility of the Caa tagging method.

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REFERENCES

1. Vander Wauven, C., Piérard, A., Kley-Raymann, M., and Haas, D. (1984) *J. Bacteriol.* **160,** 928–934
2. Lüthi, E., Mercenier, A., and Haas, D. (1986) *J. Gen. Microbiol.* **132,** 2667–2675
3. Lüthi, E., Baur, H., Gamper, M., Brunner, F., Villeval, D., Mercenier, A., and Haas, D. (1990) *Gene (Amst.)* **87,** 37–43
4. Mercenier, A., Simon, J.-P., Vander Wauven, C., Haas, D., and Stalon, V. (1980) *J. Bacteriol.* **144,** 159–163
5. Gamper, M., Zimmermann, A., and Haas, D. (1991) *J. Bacteriol.* **173,** 4742–4750
6. Zimmermann, A., Reimmann, C., Galimand, M., and Haas, D. (1991) *Mol. Microbiol.* **5,** 1483–1490
7. Galimand, M., Gamper, M., Zimmermann, A., and Haas, D. (1991) *J. Bacteriol.* **173,** 1598–1606
8. Baur, H., Stalon, V., Falmagne, P., Lüthi, E., and Haas, D. (1987) *Eur. J. Biochem.* **166,** 111–117
9. Baur, H., Lüthi, E., Stalon, V., Mercenier, A., and Haas, D. (1989) *Eur. J. Biochem.* **179,** 53–60
10. Jann, A., Stalon, V., Vander Wauven, C., Leisinger, T., and Haas, D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 4937–4941
11. Poolman, B., Driessen, A. J. M., and Konings, W. N. (1987) *J. Bacteriol.* **169,** 5597–5604
12. Poolman, B. (1990) *Mol. Microbiol.* **4,** 1629–1636
13. Driessen, A. J. M., Poolman, B., Kiewiet, R., and Konings, W. N. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 6093–6097
14. Driessen, A. J. M., Molenaar, D., and Konings, W. N. (1989) *J. Biol. Chem.* **264,** 10361–10370
15. Verhoogt, H. J. C., Smit, H., Abee, T., Gamper, M., Driessen, A. J. M., Haas, D., and Konings, W. N. (1992) *J. Bacteriol.* **174,** 1568–1573
16. Géli, V., Baty, D., and Lazdunski, C. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 689–693
17. Celis, T. F. R., Rosenfeld, H. J., and Maas, W. K. (1973) *J. Bacteriol.* **116,** 619–626
18. Manoil, C., and Beckwith, J. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 8120–8133
19. Simon, R., O'Connell, M., Labes, M., and Pühler, A. (1986) *Methods Enzymol.* **118,** 640–659
20. Borck, K., Beggs, J. D., Brammar, W. J., Hopkins, A. S., and Murray, N. E. (1976) *Mol. & Gen. Genet.* **146,** 199–207
21. Hoffman, C. S., and Wright, A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 5107–5111
22. Davison, J., Chevalier, N., and Brunel, F. (1989) *Gene (Amst.)* **83,** 371–375
23. Bagdasarian, M. M., Amann, E., Lurz, R., Rückert, B., and Bagdasarian, M. (1983) *Gene (Amst.)* **26,** 273–282
24. Géli, V., Baty, D., Pattus, F., and Lazdunski, C. (1989) *Mol. Microbiol.* **3,** 679–687
25. Itoh, Y., Watson, J. M., Haas, D., and Leisinger, T. (1984) *Plasmid* **11,** 206–220
26. Jeenes, D. J., Soldati, L., Baur, H., Watson, J. M., Mercenier, A., Reimmann, C., Leisinger, T., and Haas, D. (1986) *Mol. & Gen. Genet.* **203,** 421–429
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
28. Miller, R. V., and Kokjohn, T. A. (1988) *J. Bacteriol.* **170,** 2385–2387
29. Hancock, R. E. W., and Nikaido, H. (1978) *J. Bacteriol.* **136,** 381–390
30. Bally, M., Ball, G., Badere, A., and Lazdunski, A. (1991) *J. Bacteriol.* **173,** 479–486
31. Ito, K. (1984) *Mol. & Gen. Genet.* **197,** 204–208
32. Laemmli, U. K., and Favre, M. (1973) *J. Mol. Biol.* **80,** 575–599
33. Cavard, D., Crozel, V., Gorvel, J.-P., Pattus, F., Baty, D., and Lazdunski, C. (1986) *J. Mol. Biol.* **187,** 449–459
34. Michaelis, S., Inouye, H., Oliver, D., and Beckwith, J. (1983) *J. Bacteriol.* **154,** 366–374
35. Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. (1972) *J. Biol. Chem.* **247,** 3962–3972
36. Ramos, F., Thuriaux, P., Wiame, J. M., and Bechet, J. (1970) *Eur. J. Biochem.* **12,** 40–47
37. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254
38. Morlon, J., Chartier, M., Bidaud, M., and Lazdunski, C. (1988) *Mol. & Gen. Genet.* **211,** 231–243
39. Lazdunski, C., Baty, D., Géli, V., Cavard, D., Morlon, J., Lloubès, R., Howard, S. P., Kniebiehler, M., Chartier, M., Varenne, S., Frenette, M., Dasseux, J.-L., and Pattus, F. (1988) *Biochim. Biophys. Acta* **947,** 445–464
40. Ishidate, K., Creeger, E. S., Zrike, J., Deb, S., Glauner, B., MacAlister, T. J., and Rothfield, L. I. (1986) *J. Biol. Chem.* **261,** 428–443
41. Filip, C., Fletcher, G., Wulff, J. L., and Earhart, C. F. (1973) *J. Bacteriol.* **115,** 717–722
42. Gennis, R. B. (1989) *Biomembranes. Molecular Structure and Function*, pp. 91–93, Springer-Verlag, New York
43. Bourdineaud, J.-P., Howard, S. P., and Lazdunski, C. (1989) *J. Bacteriol.* **171,** 2458–2465
44. Tommassen, J., and Lugtenberg, B. (1984) *J. Bacteriol.* **157,** 327–329
45. Shinomiya, T. (1984) *J. Virol.* **49,** 310–314
46. Sano, Y., and Kageyama, M. (1984) *J. Bacteriol.* **158,** 562–570
47. Garriga, X., Calero, S., and Barbé, J. (1992) *Mol & Gen. Genet.* **236,** 125–134
48. Manoil, C., Mekalanos, J. J., and Beckwith, J. (1990) *J. Bacteriol.* **172,** 515–518
49. Calamia, J., and Manoil, C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4937–4941
50. Chang, C. N., Kuang, W.-J., and Chen, E. Y. (1986) *Gene (Amst.)* **44,** 121–125
51. Seep-Feldhaus, A. H., Kalinowski, J., and Pühler, A. (1991) *Mol. Microbiol.* **5,** 2995–3005
52. Bröer, S., and Krämer, S. (1990) *J. Bacteriol.* **172,** 7241–7248
53. Eisenberg, D., Schwarz, E., Komaromy, M., and Wall, R. (1984) *J. Mol. Biol.* **179,** 125–142
54. Henderson, P. J. F. (1990) *Res. Microbiol.* **141,** 316–328
55. von Heijne, G. (1986) *EMBO J.* **5,** 3021–3027
56. von Heijne, G., and Manoil, C. (1990) *Protein Eng.* **4,** 109–112
57. Meyer, R., Laux, R., Boch, G., Hinds, M., Bayly, R., and Shapiro, J. (1982) *J. Bacteriol.* **152,** 140–150
58. Goldberg, J. B., Won, J., and Ohman, D. E. (1990) *J. Gen. Microbiol.* **136,** 789–796
59. Bibi, E., and Kaback, R. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4325–4329
60. Wrubel, W., Stochaj, U., Sonnewald, U., Theres, C., and Ehring, R. (1990) *J. Bacteriol.* **172,** 5374–5381