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Topology Analysis of the Colicin V Export Protein CvaA in *Escherichia coli*

RACHEL C. SKVIRSKY,* SHOBA REGINALD, AND XIAOYU SHEN†

Department of Biology, University of Massachusetts-Boston, Boston, Massachusetts 02125

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The antibacterial protein toxin colicin V is secreted from *Escherichia coli* cells by a dedicated export system that is a member of the multicomponent ATP-binding cassette (ABC) transporter family. At least three proteins, CvaA, CvaB, and TolC, are required for secretion via this signal sequence-independent pathway. In this study, the subcellular location and transmembrane organization of membrane fusion protein CvaA were investigated. First, a series of CvaA-alkaline phosphatase (AP) protein fusions was constructed. Inner and outer membrane fractionations of cells bearing these fusions indicated that CvaA is inner membrane associated. To localize the fusion junctions, the relative activities of the fusion proteins, i.e., the amounts of phosphatase activity normalized to the rate of synthesis of each protein, as well as the stability of each fusion, were determined. These results indicated that all of the fusion junctions occur on the same side of the inner membrane. In addition, the relative activities were compared with that of native AP, and the protease accessibility of the AP moieties in spheroplasts and whole cells was analyzed. The results of these experiments suggested that the fusion junctions occur within periplasmic regions of CvaA. We conclude that CvaA is an inner membrane protein with a single transmembrane domain near its N terminus; the large C-terminal region extends into the periplasm. This study demonstrates the application of AP fusion analysis to elucidate the topology of a membrane-associated protein having only a single transmembrane domain.

In gram-negative bacteria, proteins secreted to the extracellular environment must traverse a complex envelope consisting of an inner membrane, periplasm, and an outer membrane. To accomplish this, the cell requires strategies in addition to the signal sequence-dependent process used to secrete cell envelope proteins (45). In many cases of extracellular secretion, dedicated mechanisms mediate protein transport directly across both membranes; a major class of such dedicated transporters is constituted by the bacterial ATP binding cassette (ABC) exporters (16, 55). These exporters actually constitute a subfamily within a diverse superfamily of ABC transporters occurring in eukaryotic as well as prokaryotic cells (30). Members of the ABC superfamily share a highly conserved, cytoplasmic, 200-amino-acid ATP-binding region, as well as characteristic membrane-spanning domains. Besides the bacterial exporters, other subfamilies within the superfamily are the bacterial ABC importers (periplasmic permeases) (1, 31) and the eukaryotic ABC transporters (30). The latter group includes several exporters of clinical importance such as the multidrug resistance protein (15) and the cystic fibrosis transmembrane regulator (46).

The present study focuses on a protein exporter within the rapidly expanding bacterial ABC export subfamily. Although the transported substrates within this subfamily are diverse, many are protein virulence factors (14, 19, 23, 24, 27, 38, 52). The systems that transport extracellular proteins in gram-negative bacteria generally include, in addition to the ABC protein, an accessory export protein encoded by a linked gene, as well as an outer membrane protein. The accessory protein belongs to a recently identified, novel class of export proteins designated the membrane fusion protein (MFP) family (13). In addition to functioning in ABC export systems, members of the MFP family act in conjunction with exporters of the major facilitator superfamily and exporters of the heavy metal resistance-nodulation-cell division family for the export of drugs and other molecules from gram-negative cells (13). Proteins in the MFP family have been hypothesized to cause localized fusion of the two bacterial membranes, allowing passage of substrates directly across the cell envelope in one step. However, the exact structure of the MFP, its interaction with the other export proteins, and its role in the export process have not been established. Furthermore, the mechanism of substrate translocation is not known for any of the ABC systems.

To address the structure and role of the MFP, this study examines the transport protein CvaA, the MFP component of the ABC system mediating export of colicin V in *Escherichia coli*. Colicin V, which is produced by diverse strains of enteric bacteria, is a small (88-residue) extracellular protein toxin with antibacterial activity (22). The determinants for colicin V activity, immunity, and export are encoded on low-copy-number virulence plasmids. *cvaC* encodes the 103-amino-acid toxin precursor; the precursor contains a 15-amino-acid leader peptide of the double-glycine type, which is removed concomitantly with export (18, 29). The *cvi* gene confers immunity to the producing cell, and *cvaA* and *cvaB* encode two dedicated export proteins (22). The signal sequence-independent transport process also requires the outer membrane protein TolC (23), which is encoded by a chromosomal gene.

The colicin V transport protein CvaB is the ABC component. Sequence analysis predicts CvaB to be an integral inner membrane protein with a C-terminal cytoplasmic region that contains a typical ATP-binding cassette (23). Acting in conjunction with this ABC export protein is CvaA, a largely hydrophilic, 413-amino-acid MFP with a hydrophobic N-terminal region (see Fig. 1). The role of CvaA in the export process is unknown. Moreover, its location within the cell, its membrane association and overall structure, and its interactions with the

* Corresponding author. Mailing address: Department of Biology, University of Massachusetts-Boston, 100 Morrissey Blvd., Boston, MA 02125. Phone: (617) 287-6617. Fax: (617) 287-6650. Electronic mail address: skvirsky@umbsky.cc.umb.edu.

† Present address: Cubist Pharmaceuticals, Inc., Cambridge, MA 02139.

other export components have not been demonstrated. In this paper, we describe the experimental characterization of the CvaA protein, in particular its subcellular location and membrane topology, using protein fusions of CvaA to alkaline phosphatase (AP). This paper also demonstrates the application of AP fusion analysis to determine the topology of a membrane-associated protein having only a single transmembrane domain.

MATERIALS AND METHODS

Media and culture conditions. In experiments to isolate Tn*phoA* insertions, LB (44) was used for both solid and liquid growth media. In other experiments, cells were grown in minimal M63 (44), supplemented with 0.2% glucose–1 mM $MgSO_4$–1 μg of thiamine per ml. In experiments with M63, the cultures were first grown overnight in M63 and then diluted 10-fold into fresh medium and grown to late exponential phase. Except when noted, antibiotics were used at the following final concentrations: ampicillin, 150 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 20 μg/ml. The chromogenic substrate for AP, XP (5-bromo-5-chloro-3-indolyl phosphate [obtained from Bachem Fine Chemicals, Torrance, Calif.]), was used at a final concentration of 40 μg/ml in solid medium. Unless otherwise specified, the cultures were grown at 37°C.

Bacterial strains and plasmids. All bacterial strains were *E. coli* K-12 derivatives. Except when noted, the strain used in these experiments was KS300, which was supplied by K. Strauch and J. Beckwith. This strain is MC1000 *recA1* Δ*phoA-pvuII*. To isolate Tn*phoA* insertions, strain ZK126 was infected with λTn*phoA* (25). ZK126, which was provided by R. Kolter, is W3110 *tna2* Δ*lacU169* (9). SM529, which was supplied by J. Beckwith, is MC1000 *phoR*. The *phoA* gene is present in single copy on the chromosome in this strain, which was used as a source of native AP in some experiments. In addition, colicin V bioassays were carried out on lawns of strain 71-18, which is Δ(*lac-proAB*) *thi supE*/F′ *lacI*^{q} *lacZM15*. Plasmids pLY11 and pLY21 were described previously (23). Plasmid pLY11, a derivative of pBR322 and pHK11, contains functional *cvaA* and *cvaB* genes, while pLY21, a derivative of pACYC184 and pHK22, contains functional *cvaC* and *cvi* genes (22).

Isolation of *cvaA-phoA* fusions. Insertions of Tn*phoA* in pLY11 were obtained by a procedure modified from the one described by Manoil (41). Specifically, strain ZK126 bearing plasmid pLY11 (*cvaA cvaB* Ap^r) was grown to 5×10^8 cells per ml and was infected with λTn*phoA* at a multiplicity of infection of 4. The culture was allowed to adsorb for 30 min at 30°C, was diluted fivefold with LB, and then was subdivided into 10 tubes; each subculture was grown separately for 1.5 h at 37°C. The cells were concentrated, and aliquots were plated onto ampicillin-kanamycin (30 μg/ml). The resulting colonies were then replica plated onto a mixture of ampicillin, XP, and an increased concentration of kanamycin (300 μg/ml) to select for transposition into plasmids. Individual blue colonies were purified, and plasmids were isolated from these clones. In addition, cells from each plate were pooled, and plasmid DNA was isolated from each pool. The plasmids were used to transform strain KS300 containing pLY21 (*cvaC cvi* Cm^r), with plating on a mixture of kanamycin (30 μg/ml), chloramphenicol, and XP. Individual clones were then screened for a colicin V export-negative phenotype by colicin V bioassays (50). Transpositions were mapped with restriction digests, and fusion junctions within *cvaA* were localized precisely by double-stranded DNA sequencing. The sequencing primer corresponded to the sequence between bases 71 and 86 at the 5′ end of Tn*phoA*.

Enzyme assays. (i) Assay of AP. Cells bearing CvaA-AP fusions were grown to stationary phase in M63 medium with ampicillin and kanamycin. The cultures were then diluted 10-fold and grown to late exponential phase. After growth, 0.5-ml culture samples were treated with 1 mM iodoacetamide (final concentration) to prevent spontaneous activation of cytoplasmic phosphatase (12). The cells were then washed twice with 6.6 mM potassium-morpholinepropanesulfonic acid–83 mM NaCl–16 mM NH_4Cl–10 mM $MgCl_2$–1 mM iodoacetamide (pH 7.3). Washed cells were diluted fivefold in the same buffer for determination of the optical density at 600 nm (OD_{600}). The rate of *p*-nitrophenyl phosphate hydrolysis in washed samples was measured as described elsewhere (6), except that the assay buffer was 1 M Tris-HCl (pH 8.0)–1 mM $ZnCl_2$, and cells in 0.1-ml samples were permeabilized with 1 drop of chloroform and 25 μl of 0.1% sodium dodecyl sulfate (SDS) before being assayed. Assays were performed at 28°C and were stopped by the addition of 120 μl of 80 mM EDTA (pH 8.0)–2 M K_2HPO_4. AP activity units were calculated as optical density at 420 nm [$OD_{420} - (1.75 \times OD_{550}) \times 1{,}000$]/[time (min) × OD_{600} × volume of culture assayed (ml)]. All activity values shown are the results of at least two independent sets of assays.

(ii) Assay of β-lactamase. The *bla* gene encoding β-lactamase was carried by the plasmid pLY11, a derivative of pBR322. The ability of β-lactamase to hydrolyze the C-N bond in the β-lactam ring of penicillin G was measured in a kinetic assay (47) performed at room temperature. Aliquots (0.2 ml each) of the cell fractions were added to 1 ml of reaction mix (0.1 M phosphate buffer [pH 7.3] plus 0.5 mg of penicillin G per ml) in a quartz cuvette. After 30 s of incubation, the change in OD_{250} was monitored for 2 to 3 min. Units of enzyme activity were calculated by (change in OD_{250}/min × 1,000)/(OD_{600} × volume [ml] of culture assayed). Activity values shown are the results of at least two independent sets of assays.

(iii) Assay of CAT. The *cat* gene encoding chloramphenicol acetyltransferase (CAT) was introduced into cells by transformation with plasmid pACYC184. CAT activity was determined by measuring chloramphenicol-dependent acetyl coenzyme A deacetylation with 5-5′-dithiobis(2-nitrobenzoic acid) as described elsewhere (49), except that the assay was performed at room temperature. The reaction was started with the addition of 10 μl of 5 mM chloramphenicol to a cuvette containing 20 μl of a cell fraction together with 800 μl of reaction buffer at room temperature. Enzyme activity units were calculated by (change in OD_{412}/min × 1,000)/(OD_{600} × volume [ml] of culture assayed). Activity values shown are the results of at least two independent sets of assays.

Pulse-labeling and immunoprecipitation of AP fusion proteins. Cells were pulse-labeled by incubating 0.5-ml aliquots of late-exponential-phase cultures in prewarmed tubes with [^{35}S]Met (30 μCi/ml of culture), as described previously (50). The cells were aerated at 37°C during the 30-s pulse. For experiments in which a chase was required, 4 ml of cells were pulsed-labeled for 30 s and then chased at 37°C with unlabeled Met to a final concentration of 0.05%. The chase was terminated at various time points up to 1 h. Extraction of cell pellets and immunoprecipitation were performed as described elsewhere (50), with 40 μl of AP-specific antisera (purchased from 5′ to 3′, Inc., Boulder, Colo.) and IgGsorb, a *Staphylococcus aureus* cell preparation (The Enzyme Center, Inc. Malden, Mass.). Equivalent amounts of each sample were analyzed by electrophoresis through an SDS-10% polyacrylamide gel (37) and then by autoradiography. The proteins were quantified by scanning bands on nonsaturated autoradiograms with a Bio-Rad 620 Video Densitometer (see Table 1 footnotes).

Cell fractionations. Separation of inner and outer membranes was based on the method described by Guzman-Verduzco and Y. M. Kupersztoch (26). Cultures of 100 ml were grown overnight in M63 with antibiotics. The cells were pelleted and resuspended in 2 ml of 100 mM Tris-HCl (pH 8.0)–0.5 M sucrose–0.5 mM EDTA–60 μg of lysozyme per ml, and the suspension was then incubated for 30 min on ice. The samples were then centrifuged, and the spheroplast pellets were resuspended in 20 ml of 10 mM Tris-HCl (pH 8.5)–5 mM EDTA–0.2 M KCl–5 mM β-mercaptoethanol. Spheroplast suspensions were then sonicated on ice (approximately four cycles, each with 40 s of sonication and 30 s of rest) until the suspension was clarified. The samples were centrifuged at 3,000 × *g* for 20 min to remove unbroken cells, and the resulting supernatants were centrifuged at 100,000 × *g* for 1 h at 4°C. Cell envelope pellets were then fractionated, on the basis of the differential solubility of inner and outer membranes in Sarkosyl (3, 20). Specifically, membrane pellets were resuspended in 500 μl of Sarkosyl buffer (0.5% Sarkosyl, 10 mM Tris-HCl [pH 8.0], 5 mM EDTA), and the suspensions were then incubated for 30 min at room temperature. The membranes were spun at 135,000 × *g* for 2 h at 15°C. The supernatant, containing inner membrane proteins, was saved, and the pellet was resuspended in Sarkosyl buffer and respun at 135,000 × *g* for 1 h at 15°C. The supernatant was discarded, and the pellet, containing outer membrane proteins, was resuspended in SDS sample buffer (50 mM Tris-HCl [pH 7.6], 1 mM EDTA [pH 8.0], 1% SDS). Samples of inner and outer membrane preparations were electrophoresed through 10% polyacrylamide gels, and Western blots (immunoblots) were probed with AP-specific antisera. To gauge the accuracy of fractionation, Western blots were also probed with antibodies directed against marker proteins. These were MalF for inner membranes and OmpF for outer membranes. (Antisera were provided by B. Traxler and J. Beckwith.)

Spheroplast preparation and proteolysis treatment. Spheroplasts were prepared as described elsewhere (43) from cells bearing a CvaA-AP fusion plasmid and pACYC184. Briefly, late-exponential-phase cells were pulse-labeled and then pelleted, washed in spheroplast buffer (40% sucrose, 33 mM Tris [pH 8.0]), and resuspended in 1.2 ml of the same buffer. Lysozyme and EDTA were then added to 5 μg/ml and 1 mM, respectively, and the samples were incubated on ice for 15 min. The samples were subjected to proteinase K treatment as described, except that proteolysis was extended to 30 min. In addition, after the treated spheroplasts were pelleted, they were washed once in spheroplast buffer to reduce contamination from the periplasmic fraction. Pelleted spheroplasts were resuspended (43) and lysed by three cycles of freeze-thawing with dry ice-ethanol. Final proteinase-treated spheroplast samples, as well as untreated spheroplast controls and treated nonfractionated controls, were incubated with AP-specific antibodies for immunoprecipitation. The proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% resolving gels and were visualized by autoradiography. Fractionation efficiency was monitored by determining CAT and β-lactamase activity levels in periplasmic and spheroplast fractions of nonradiolabeled control samples.

Western blots. Proteins were transferred from SDS-10% PAGE gels to Pro-Blott membranes (Applied Biosystems) in CAPS buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid [pH 11.0] in 10% methanol). Western blots were performed according to the protocol of the Vectastain ABC kit from Vector Laboratories, Inc., with 40 μl of AP-specific antisera.

RESULTS

Generation of AP fusion proteins. AP fusions to CvaA were made by transposition of Tn*phoA* into pLY11, resulting in a

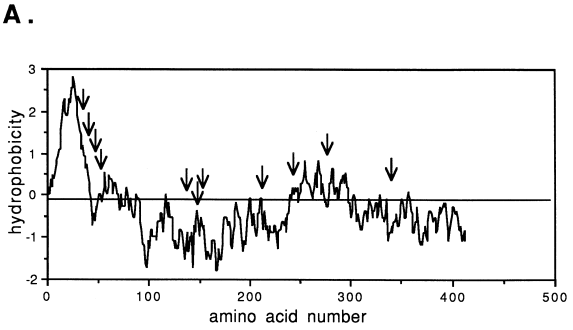

FIG. 1. Hydrophobicity and amino acid sequence of CvaA. (A) Hydrophobicity plot of CvaA. Arrows indicate CvaA-AP fusion junctions. Plot was based on Kyte and Doolittle (36). (B) Amino acid sequence of CvaA from Gilson et al. (23). Superscripts indicate fusion junctions. The numbers refer to the last amino acid in CvaA before the junction. The putative membrane-spanning domain is underlined.

series of eleven plasmids bearing in-frame *cvaA-phoA* fusions. The resulting protein fusions are designated A41, A43, A47, A53, A138, A147, A151, A213, A242, A275, and A341. In these designations, the numbers correspond to the last amino acid in CvaA before the fusion junction to AP. (The corresponding plasmids are referred to as pRS-A41, etc.) Analysis of a hydropathy plot of CvaA (Fig. 1) indicated that all 11 fusion junctions occur downstream of a putative transmembrane domain.

Since the AP moiety appeared to occur on the same side of the membrane in all fusions, it was not possible to determine the orientation of the protein in the membrane simply by analyzing differences in AP activities. Furthermore, the distribution of positively charged residues in CvaA (Fig. 1) did not provide clues about the orientation (5, 54). Thus, we considered constructing an additional fusion with a precise junction upstream of the putative transmembrane domain to compare the fusions described above with one containing a junction on the opposite membrane face. However, we realized that such a fusion would not discriminate between an N-terminal domain which is periplasmic from one which is cytoplasmic. Specifically, if this domain were situated outside the inner membrane, the topological signals required for its export would be absent in the hybrid protein (4, 58). Thus, we would be unable to distinguish whether this domain is cytoplasmic, periplasmic, or extracellular, because in all cases it would behave as a cytoplasmic domain.

Specific AP activities of fusion proteins. Assays of AP activity were performed on cells bearing CvaA-AP fusions. Results from these experiments revealed that the fusions that are more upstream (shorter) have higher enzyme activities than the fusions that are more downstream (longer) (Table 1). The larger drops in AP activity are seen between fusions with the greatest differences in size. The ratio between the highest and lowest

TABLE 1. AP activities of fusion proteins

AP fusion plasmid[a]	AP activity U	Synthesis U[b]	Relative activities[c]
pRS-A41	308	0.245	1,257
pRS-A43	328	0.271	1,210
pRS-A47	358	0.242	1,479
pRS-A53	333	0.211	1,578
pRS-A138	145	0.165	879
pRS-A147	149	0.120	1,241
pRS-A151	173	0.081	2,136
pRS-A213	102	ND[d]	ND[d]
pRS-A242	98	0.098	1,000
pRS-A275	81	0.135	600
pRS-A341	58	0.109	532

[a] All plasmids encode CvaA-AP fusion proteins. The number within the plasmid designation indicates the last amino acid in CvaA before the AP fusion junction.

[b] To quantify the synthesis of fusion proteins, autoradiograph bands were analyzed by densitometry. The area under the peak of the fusion protein band, which was integrated by weighing, was normalized to the number of Met residues per fusion protein. Synthesis unit is defined as the density of protein band × (10/number of Met residues). To verify the linearity of the exposures, autoradiographs at bracketing exposures were also measured and were found to give consistent results.

[c] Relative activity is defined as AP activity normalized to the rate of synthesis (AP activity units/synthesis units).

[d] ND, value not determined.

AP enzyme activity units among the fusion proteins is approximately 6. For most proteins examined previously, the ratio of AP activity units between periplasmic and cytoplasmic fusion junctions is 10 or higher (4, 41). This lends support to the possibility that in all of the CvaA-AP fusions generated, the junctions occur on the same side of the membrane, as suggested by hydropathy analysis.

To distinguish whether the observed differences in AP activity of the fusions reflect different intrinsic activities or different synthesis rates, quantitative measurements of the amounts of labeled fusion protein synthesized during a 30-s pulse with [^{35}S]Met were undertaken. (Even unstable proteins are not expected to degrade during a 30-s pulse.) Cells bearing fusions were pulse-labeled for 30 s, and proteins were immunoprecipitated with AP-specific antisera and separated by SDS-PAGE. The synthesis levels of the first four upstream fusions were considerably higher than those of the remaining downstream fusions (Fig. 2). For each fusion protein, the relative activity, i.e., the amount of AP activity normalized to the rate of synthesis of the fusion protein (4), is shown in Table 1. The ratio between the highest and lowest relative activities is approximately 4. This result reinforces the hypothesis that all of the CvaA-AP fusion junctions occur on the same side of the inner membrane.

The relative activities of a representative upstream fusion (A47) and a representative downstream fusion with reduced synthesis levels (A275) were then compared with that of native, periplasmic AP (Fig. 3). For each strain, a single culture was used for both pulse-labeling and determination of AP activities. The results show that the relative activity of the more upstream fusion is 2.8-fold lower than that of native AP and that the relative activity of the more downstream fusion is 3.7-fold lower than that of native AP. AP fusions with junctions situated outside the inner membrane are expected to have relative activities similar to that of native AP, while most properly localized cytoplasmic fusion junctions have relative activities that are 10-fold lower or less (4). Therefore, these results suggest that all of the CvaA-AP fusion junctions occur within periplasmic regions of the CvaA protein.

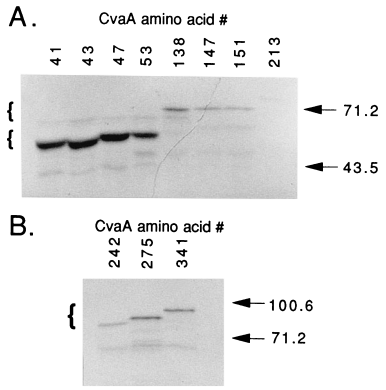

FIG. 2. Synthesis levels of CvaA-AP fusion proteins. Cells bearing fusions were pulse-labeled for 30 s as described, and proteins were immunoprecipitated with AP-specific antisera and separated by SDS-PAGE. Numbers over the lanes indicate the last amino acid in CvaA before the fusion junction. Brackets at left indicate expected positions of fusion proteins. Molecular masses (in kilodaltons) are shown on the right. (A) Fusion proteins with upstream junctions; (B) Fusion proteins with downstream junctions.

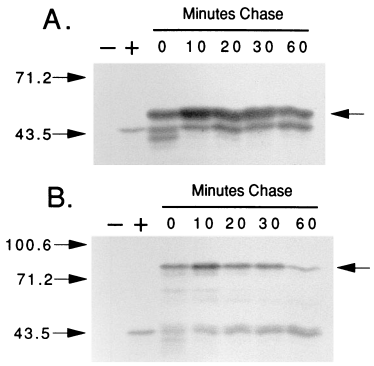

FIG. 4. Determination of CvaA-AP fusion stabilities. Cells were pulse-labeled for 30 s as described and then chased for 1 h with unlabeled Met. After different chase times, the proteins were immunoprecipitated with AP-specific antisera and were separated by SDS-PAGE. Strains bearing upstream fusion A47 (A) and downstream fusion A275 (B) were used. Arrows to the right of the lanes indicate fusion proteins. (Major lower band corresponds to the AP moiety from degraded fusion or native AP.) −, a negative control strain with no AP; +, a strain with native AP. Molecular masses (in kilodaltons) of marker proteins are shown at the left. Chase times are indicated.

Determination of fusion stabilities. Cells expressing representative upstream (A47) and downstream (A275) fusions were pulsed for 30 s with [^{35}S]Met and then chased for 1 h with unlabeled Met. After different chase times, samples were immunoprecipitated with AP-specific antisera and were analyzed by SDS-PAGE (Fig. 4). Both the upstream and the downstream fusion proteins were relatively stable for as long as 30 min. This experiment supports the conclusion that the smaller amounts of the downstream fusion proteins, as visualized by autoradiography, are due to reduced synthesis levels of these fusions, rather than to rapid degradation during the 30-s pulse. The latter possibility would be more likely were the downstream fusion junctions localized to the cytoplasm, since in this reducing environment, the intrachain disulfide bonds of AP, which are required for folding into an active and protease-resistant state, are unable to form (12). Also supporting the model of periplasmic fusion junctions is evidence of a breakdown product that is the size of AP for both hybrid proteins (Fig. 4). Occurrence of such a breakdown product is characteristic of fusions with periplasmic rather than cytoplasmic junctions.

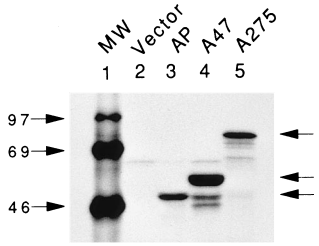

FIG. 3. Comparison of synthesis levels of CvaA-AP fusion proteins with those of native AP. Cells were pulse-labeled for 30 s as described, and proteins were immunoprecipitated with AP-specific antisera and separated by SDS-PAGE. Lanes: 1, molecular weight (MW) markers; 2, strain with no fusion protein (vector only); 3, strain SM529 carrying native AP; 4, strain with fusion protein A47; 5, strain with fusion protein A275. Molecular masses (in kilodaltons) of marker proteins are shown at left. Arrows on the right, from top to bottom, indicate expected positions of the A275 fusion protein, the A47 fusion protein, and the native AP. In this experiment, AP activity units of cells carrying native AP, A47, and A275 were 409, 391, and 116, respectively, and the synthesis units (defined in Table 1, footnote *b*) were 0.053, 0.140, and 0.055. Relative activities (defined in Table 1, footnote *c*) were 7,717, 2,793, and 2,109, respectively.

Localization of CvaA by fractionation of inner and outer membranes. Inner and outer membrane fractions were prepared from strains bearing representative upstream (A43) and downstream (A341) fusions, in order to localize the fusions within these fractions. In enzyme assays, inner membrane fractions showed eightfold higher levels of AP than outer membrane fractions (data not shown). The fractions were further analyzed by SDS-PAGE, and Western blots were prepared. The accuracy of fractionation was verified by probing the blots with antisera directed against MalF and OmpA, which are marker proteins for the inner and outer membranes, respectively. When the blots were probed with AP-specific antisera, the AP cross-reacting material was detected in inner membrane samples (Fig. 5). These results suggest that the CvaA protein is anchored in the inner membrane.

Proteinase K accessibility of the fusion junctions. Cells carrying representative upstream and downstream fusions were radiolabeled, and spheroplasts were prepared and then treated with proteinase K. Proteins in the spheroplast and periplasmic fractions were immunoprecipitated with AP-specific antisera and separated by SDS-PAGE. While AP itself is particularly resistant to proteinase K, the fusion junction is labile, provided it is accessible to the protease (53). In spheroplasts, fusion junctions occurring in the periplasm are potentially accessible.

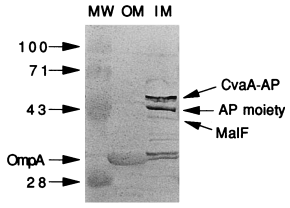

FIG. 5. Western analysis of inner (IM) and outer (OM) membrane fractions. Inner and outer membrane fractions were prepared from cells bearing fusion A43. Western blots were probed with antibodies directed against AP, MalF (inner membrane marker), and OmpA (outer membrane marker). MW, molecular weight markers. Molecular masses (in kilodaltons) of marker proteins are shown to the left. The unlabeled bands in the IM lane are the result of nonspecific interaction with the polyclonal antibodies.

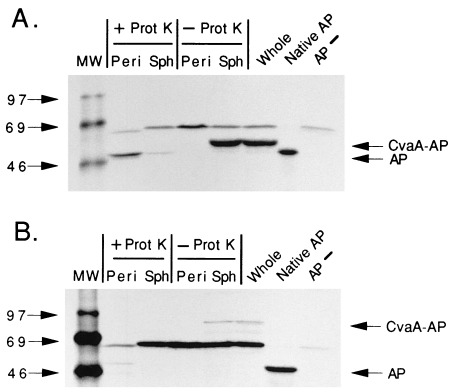

FIG. 6. Protease accessibility of the fusion junctions. Spheroplasts of fusion-carrying strains were prepared and then treated with proteinase K as described. (A) Representative upstream fusion A41; (B) representative downstream fusion A341. Proteins in the spheroplast and periplasmic fractions were immunoprecipitated with AP-specific antisera. MW, molecular weight markers; +Prot K and −Prot K, with and without proteinase K treatment, respectively; Peri, periplasmic fraction; Sph, spheroplast fraction; whole, untreated whole cells of fusion-bearing strain; native AP, untreated whole cells with native AP; AP$^-$, a negative control strain lacking AP. Molecular mass (in kilodaltons) of marker proteins are shown to the left. The band at approximately 65 kDa is a strain-specific contaminant that is recognized by our polyclonal antibodies.

In such cases, upon treatment of spheroplasts with proteinase K, the AP moiety can be released into the supernatant (i.e., the periplasmic fraction). On the other hand, if the AP moiety of the fusion protein is cytoplasmic, the AP will remain uncleaved and pellet with the spheroplasts. In experiments with both upstream (A41) and downstream (A341) fusions, a moiety with the size of AP was released into the periplasmic fraction (Fig. 6). This indicates that both upstream and downstream fusion junctions occur within regions of CvaA that are situated outside the inner membrane, presumably in the periplasm. Activities of CAT and β-lactamase, which are marker proteins for the cytoplasm and periplasm, respectively, were determined for spheroplast and periplasmic fractions, which confirmed the accuracy of fractionation (Table 2).

To verify that the fusion junctions are periplasmic rather than exposed to the extracellular medium, a parallel control experiment was performed with unfractionated cells. This experiment was conducted with cells of the two strains described in Table 2 (KS300 with pACYC184 and either pRS-A41 or pRS-A341, upstream and downstream fusion plasmids, respectively). When whole cells of each type were treated with proteinase K, an average of 99.5% of the AP activity remained in the cells, and only 0.5% of the AP activity could be detected in the supernatant. Thus, in unfractionated cells, the fusion junctions were not accessible to proteinase K in the extracellular medium. We conclude that both upstream and downstream fusion junctions occur within periplasmic regions of CvaA.

DISCUSSION

This study analyzed experimentally the subcellular location and topology of CvaA as a step toward deciphering its specific role in colicin V export, as well as the overall structure of its ABC apparatus. Previously, Gilson et al. (23) noted that the CvaA N terminus exhibits some properties of a signal sequence but does not have a typical leader peptidase cleavage site. Thus, at the outset, it was unclear whether CvaA is translocated by the signal sequence-dependent pathway to the periplasm or outer membrane, whether the protein is cytoplasmic, or whether its hydrophobic N-terminal region associates with the inner membrane. To resolve this question experimentally, we fractionated inner and outer membranes of cells bearing CvaA-AP fusions and demonstrated that CvaA is anchored in the inner membrane.

The enzymatic activities of the CvaA-AP fusion proteins were analyzed to determine the transmembrane organization of CvaA. This approach is based on the observation that AP activity depends on its localization outside the inner membrane; thus, AP fusions to periplasmic domains of a target protein are enzymatically active, while fusions to cytoplasmic domains are inactive (42). This approach has been used widely to analyze the topology of membrane proteins with multiple membrane-spanning regions. However, particular complications arise when proteins such as CvaA with a single membrane-spanning region are analyzed. For example, fusions to regions upstream of the sole membrane-spanning region would lack export signals. Such fusions would have low activity and appear cytoplasmic, regardless of whether the junction occurs in a normally cytoplasmic or periplasmic region of the native protein; these fusions would thus provide little useful information. In this situation, caution must be used in evaluating whether fusions downstream of the membrane-spanning region show high (periplasmic) or low (cytoplasmic) activities, since this interpretation must be made without the benefit of comparing fusions on opposite faces of the membrane (4).

In the present study, multiple lines of evidence were used to demonstrate the orientation of CvaA. Fusion expression levels were measured to determine relative activities (4), that is, the amount of enzymatic activity normalized to the rate of synthesis of the hybrid protein. In these experiments, the long and short fusions showed different levels of synthesis. A step down in expression between early and late fusions has been documented previously for *malF-lacZ* hybrids (21). However, it is possible in the present case that the longer CvaA-AP fusions were synthesized in lower quantities because of the shortness of the pulse. Relative activity determinations were supported

TABLE 2. CAT and β-lactamase activities in proteinase K-treated samples

Plasmid[a]	Enzyme	Activity[b] with proteinase K		Activity[b] without proteinase K	
		Spheroplast fraction	Periplasmic fraction	Spheroplast fraction	Periplasmic fraction
pRS-A41	CAT	2,044	133	2,190	177
	β-Lactamase	28	161	ND[c]	148
pRS-A341	CAT	386	90	454	45
	β-Lactamase	ND	74	7	67

[a] In both cases, the strain used was KS300, carrying pACYC184 as well as the plasmids listed.
[b] CAT activity units were calculated as (change in OD_{412}/min × 1,000)/(OD_{600} × volume [ml] of culture assayed). Units of β-lactamase were calculated as (change in OD_{250}/min × 1,000)/(OD_{600} × volume [ml] of culture assayed).
[c] ND, value not determined.

by experiments that demonstrated the stabilities of the fusions. Comparison of relative activities of the hybrid proteins suggested that all of the fusion junctions occur on the same face of the membrane, and comparison with the relative activity of native AP suggested that all fusion junctions occur within periplasmic domains. These conclusions were corroborated further by experiments that showed release of AP moieties from spheroplasts after proteinase K treatment. In addition, since proteinase K treatment of whole cells bearing downstream fusions failed to release the AP moiety into the supernatant, it is unlikely that CvaA extends into the extracellular space. Therefore, CvaA appears to be anchored to the inner membrane by a single transmembrane domain, possibly between amino acids 15 to 36, with the major portion of the protein extending into the periplasm. The possibility that the extreme carboxy terminus of CvaA is anchored in the outer membrane has not been ruled out.

Although the precise function of CvaA and other MFP proteins has not been determined, it is noteworthy that these proteins are required in gram-negative systems in which substrates are secreted across both membranes to the extracellular medium. In addition to CvaA, other MFP proteins that act in such ABC systems include HlyD (required for export of α-hemolysin from *E. coli*) (40), PrtE (protease export [*Erwinia chrysanthemi*]) (10), AppD (hemolysin export [*Actinobacillus pleuropneumoniae*]) (7), LktD (leukotoxin export [*Pasteurella haemolytica*]) (52), CyaD (cyclolysin export [*Bordetella pertussis*]) (24), AprE (alkaline protease export [*Pseudomonas aeruginosa*]) (14), and HasE (heme acquisition protein export [*Serratia marcescens*]) (39). The colicin V transporter shares many features with these export systems, especially the Hly and Prt systems, which have been examined in most detail. For example, export occurs without any detectable periplasmic intermediate (16, 35) and in many cases requires an outer membrane factor such as TolC in the Cva and Hly systems (23, 56) and the TolC homolog PrtF in the Prt system (38). Furthermore, complementation studies have demonstrated a functional relationship between the colicin V transporter and the Hly and Prt systems (17).

In addition, the results presented here indicate that CvaA is structurally similar to HlyD and PrtE. Like CvaA, HlyD and PrtE both fractionate completely or primarily with the inner membrane; for each, an N-terminal membrane-spanning region and a large C-terminal periplasmic domain have been reported (10, 48, 57). The proposed structures are consistent with the hypothesis that the MFP may help form a bridge that connects the two membranes and enables transport directly to the cell exterior (13, 16, 32). Also consistent with this model is recent evidence from hybrid ABC systems of interaction between the MFP and the outer membrane component (2).

However, while the parallels among these systems point to a conserved mechanism, there are also significant distinctions between the colicin V transporter and other gram-negative ABC systems, which may be relevant to CvaA function. Indeed, sequence comparisons of the MFPs indicate that CvaA forms a distinct subbranch of the MFP protein export cluster (13). On a functional level, Schulein et al. (48) presented evidence that the 10 C-terminal residues of HlyD mediate association with the outer membrane; while HlyD, LktD, CyaA, and PrtE have significant sequence similarities within this region (48), CvaA does not share this homology. In addition, in contrast to the C-terminal secretion signals of α-hemolysin (34) and the *E. chrysanthemi* proteases (11), the export signal in CvaC has been localized to the N-terminal region (23). It is noteworthy that CvaC has actually been identified as a member of a class of peptide bacteriocins with double-glycine-type leader peptides, which were found previously only in gram-positive bacteria (18, 29). Furthermore, like other ABC proteins that mediate export of related bacteriocins, the colicin V transporter CvaB has an N-terminal extension proposed to have leader peptidase activity (28). Finally, the hypothesized function of CvaA must be reconciled with the observation that accessory proteins are also required for extracellular secretion in certain gram-positive ABC systems (8, 28, 33, 51), and that CvaA is homologous to the LagE protein, which is required for export of the bacteriocin lactococcin G across a single gram-positive membrane (28). Thus, the mechanism of CvaA must be considered in light of the features it shares with both gram-positive and -negative systems.

The composite structure of the colicin V export apparatus remains unelucidated, as do the interactions among the protein components of this and other ABC export systems. In the case of the Cva proteins, for example, it is not known whether CvaA interacts physically with CvaB in an export complex or whether it is an independent component. However, evidence that a mutation in *cvaA*, *cvaB*, or *tolC* results in cytoplasmic accumulation of colicin V suggests that the three export proteins may act coordinately in a single functional complex (16). The topology model of CvaA presented in this study is consistent with this protein being accessible for physical interaction with CvaB and TolC at the inner and outer membranes, respectively. As evidence accumulates for possible interactions between the exporters and for the specific role of each component, the molecular mechanism whereby bacterial ABC export systems mediate translocation can be deciphered.

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