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Interactions of Dedicated Export Membrane Proteins of the Colicin V Secretion System: CvaA, a Member of the Membrane Fusion Protein Family, Interacts with CvaB and TolC

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The antibacterial peptide toxin colicin V uses a dedicated signal sequence-independent system for its secretion in *Escherichia coli* and requires the products of three genes, *cvaA*, *cvaB*, and *tolC*. As a member of the membrane fusion protein family, CvaA is supposed to form a bridge that connects the inner and outer membranes via interaction with CvaB and TolC, respectively. In this study, we investigated the possible interaction of these proteins. When CvaA or CvaB was absent, the corresponding amount of CvaB or CvaA, respectively, was decreased, and the amounts of both proteins were reduced when TolC was depleted. Translational *lacZ* fusions showed that TolC did not affect the synthesis of either CvaA–β-galactosidase or CvaB–β-galactosidase, and CvaA or CvaB did not affect the synthesis of CvaB–β-galactosidase or CvaA–β-galactosidase, respectively. However, the stabilities of CvaA and CvaB proteins were affected by the absence of one another and by that of TolC. The instability of CvaA was more severe in TolC-depleted cells than in CvaB-depleted cells. On the other hand, CvaB was less stable in the absence of CvaA than in the absence of TolC. In addition, using a cross-linking reagent, we showed that CvaA directly interacts with both CvaB and TolC proteins. Taken together, these data support the hypothesized structural role of CvaA in connecting CvaB and TolC.

In gram-negative bacteria, many proteins which lack a typical N-terminal signal sequence are secreted directly from the cytoplasm to the extracellular medium by dedicated export systems (38). One such cytoplasmic membrane transporter is the ATP-binding cassette (ABC) superfamily, which comprises both eukaryotic and prokaryotic proteins for the export or import of a wide range of substrates such as peptides, sugars, proteins, and antibiotics (11, 22). The majority of known ABC transporters in prokaryotes all possess highly conserved, cytoplasmic ATP-binding domains which provide the energy of ATP hydrolysis to pump substrates (5, 9). Generally, bacterial ABC export systems for protein secretion require two accessory proteins in addition to the transporter itself. One is in the group of proteins designated the membrane fusion protein (MFP) family which resides in the inner membrane, and the other is an outer membrane protein; the two proteins interact (8, 37).

Proteins in the MFP family are similar in size and hydropathy pattern, with two characteristic hydrophobic regions: one in the N terminus which spans the inner membrane, and the other near the C terminus which is highly conserved and is proposed to interact with the outer membrane protein (8). Besides acting in ABC export systems, proteins in the MFP family function in conjunction with a major facilitator superfamily permease or with transporters of the heavy metal resistance/nodulation/cell division family to export various drugs in gram-negative bacteria (8). It has been hypothesized that MFPs may connect the inner and the outer membranes to facilitate the passage of substrates. However, the role of MFPs is not yet characterized.

We have studied a bacterial ABC export system that secretes an antibacterial peptide toxin, colicin V (ColV). ColV, a small peptide toxin of about 9 kDa, is active against many *Escherichia coli* strains and other members of the family *Enterobacteriaceae*; it is encoded together with its immunity protein by a variety of low-copy-number plasmids (16, 19). The complete nucleotide sequences of four genes that are involved in the system have been determined, and the genes were found to be encoded in two converging operons, one with *cvi* and *cvaC* and the other with *cvaA* and *cvaB* (17). The *cvi* gene product confers immunity to cells that produce toxin ColV, which is encoded from the *cvaC* gene, directly downstream of *cvi*. The *cvaC* gene product, a 103-amino-acid protein, does not possess a typical signal peptide; however, the secreted ColV is processed to remove the N-terminal 15 amino acids, yielding the mature toxin (10, 13).

The transporter proteins are encoded in the *cvaAB* operon. The ABC transporter, CvaB, has six potential transmembrane domains with a typical ATP-binding cassette in a C-terminal cytoplasmic region (12). CvaA, a member of the MFP family, is anchored in the inner membrane with its N-terminal hydrophobic domain. It has been proposed to connect CvaB with outer membrane protein TolC, which also participates in ColV secretion in *E. coli*, similar to its role in the secretion of α-hemolysin (17, 33, 37). In this study, we examined the interaction of these proteins that are involved in the secretion of ColV. Using a cross-linking reagent, we showed that CvaA directly interacts with CvaB and TolC, thus supporting the structural role of MFP proposed by Dinh et al. (8).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 1. *E. coli* MC4100, ZK4, and ZK796 (16, 37) were used in most experiments. Strains 71-18 and BL21(λDE3) were used as lawns for the ColV assay and protein overproduction, respectively (34, 39). TB (10 g of tryptone and 8 g of NaCl per liter) was used as both a liquid and a solid (with 1.5% agar) growth medium for protein expression and activity tests. All cultures were grown at 37°C.

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TABLE 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Genotype or description	Reference or source
Strains		
MC4100	F⁻ *araD139* Δ*lacU169 rpsL deoC7 relA thiA*	16
ZK4	MC4100 *recA56*	16
ZK796	MC4100 *tolC*::Tn*10*	37
71-18	Δ(*lac-proAB*) *thi supE*/F′ *lacI*^{q} *lacZM15*	39
BL21(λDE3)	F⁻ *ompT hsdS*$_B$ λ*lacUV5*-T7 gene 1	34
Plasmids		
pHK11	pBR322 with *cvaABC* and *cvi* genes	16
pHK11-1	pHK11 with *cvaC*::Tn*5*	16
pHK11-4	pHK11 with *cvaB*::Tn*5*	16
pHK11-8	pHK11 with *cvaA*::Tn*5*	16
pTJ113	pHK11 with *Kpn*I site deletion; no CvaA expression	This study
pTJ114	pHK11-4 with Δ(*Bgl*II-*Bgl*II), only *cvaA*	This study
pTJ116	pHK11-1 with Δ(*Bgl*II-*Bgl*II) and *Bam*HI site mutation, only *cvaB*	This study
pHK22	pACYC184 with *cvaABC* and *cvi* genes	16
pHK22-2	pHK22 with *cvaA*::Tn*5*	16
pHK22-5	pHK22 with *cvaB*::Tn*5*	16
pHK22-6	pHK22 with *cvaC*::Tn*5*	16
pTJ224	pHK22-5 with Δ(*Sal*I-*Sal*I), only *cvaA*	This study
pTJ226	Same as pTJ116 except using pHK22-6, only *cvaB*	This study
pT7-6	Vector with T7 RNA polymerase promoter	35
pBR*tolC*	pBR322 with *tolC* gene	J. Fralick
pT7*tolC*	pT7-6 with *tolC* gene	This study
pAC*tolC*	pACYC184 with *tolC* gene	This study
pAC*tolC*-1	pAC*tolC* with mutated *tolC* (L193V)	This study
pQF52	Translational *lacZ* fusion vector	24
pQF52*cvaA*15	Translational *cvaA-lacZ* fusion at residue 15	23
pQF52*cvaB*16	Translational *cvaB-lacZ* fusion at residue 16	This study
pXZ6	pT7-6 with C-terminal cytoplasmic domain of *cvaB*	This study

Plasmid DNA isolation, digestion, transformation, and other routine DNA manipulations were as described by Sambrook et al. (31). Plasmids containing *cvaABC* and *cvi* genes in pHK11 (derived from pBR322) and its derivatives pHK11-1, pHK11-4, and pHK11-8 and in pHK22 (derived from pACYC184) and its derivatives pHK22-2, pHK22-5, and pHK22-6 were obtained from R. Kolter (Harvard Medical School, Boston, Mass.) (16). pBR*tolC*, which contains the *tolC* gene in pBR322, was a gift from J. Fralick (Texas Tech University Health Science Center, Lubbock).

For complementation, plasmids containing either *cvaA* or *cvaB* were constructed. Plasmid pTJ114, which contains only *cvaA*, was obtained by removing a *Bgl*II fragment from pHK11-4. Plasmid pTJ224, which also has only *cvaA*, was constructed by removing a *Sal*I fragment from pHK22-5. For the *cvaB*-containing plasmid, pHK11-1 was digested with *Bgl*II and self-ligated; the separated *Bgl*II pieces were reinserted into the *Bam*HI site of the self-ligated DNA to mutate *cvaA*, yielding pTJ116. Plasmid pTJ226, a derivative of pACYC184 which has only *cvaB*, was also obtained by the same construction procedure, except that pHK22-6 was used instead of pHK11-1.

For *tolC* plasmids, the *Hin*dIII-*Sal*I fragment containing *tolC* was obtained from pBR*tolC* and then subcloned into pT7-6 (35) and pACYC184, yielding pT7*tolC* and pAC*tolC*, respectively. To obtain a *tolC* mutant, pAC*tolC* was mutagenized by hydroxylamine (4) and screened by the ColV assay after transformation into ZK796/pHK11. Plasmid pAC*tolC*-1, which has a mutated *tolC*, was then isolated from transformants deficient in ColV activity.

To generate the antigen for anti-CvaB antibodies, a DNA fragment encoding amino acids 594 to 698 of CvaB (17) and containing an artificial Shine-Dalgarno sequence (AGGAG) and an internal methionine codon was amplified by PCR. The fragment was then subcloned into pT7-6 to yield pXZ6 and used to overproduce the cytoplasmic C-terminal domain of CvaB.

Preparation of anti-TolC and anti-CvaB antibodies. For preparations of anti-TolC and anti-CvaB antibodies, BL21(λDE3) cells containing pT7*tolC* and pXZ6 were used to overproduce TolC and the C-terminal domain of CvaB, respectively, as described previously (23). Two proteins of around 52 and 50 kDa were overproduced from cells containing pT7*tolC*, and two proteins of 12 and 11 kDa were overproduced from cells with pXZ6. Overproduced proteins were separated by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 10% gel and verified by N-terminal peptide sequencing. Gel pieces containing either TolC or the C-terminal domain of CvaB were excised, lyophilized, and injected into rabbits to raise antibodies. Serum preparations were performed by general standard procedures (20), and anti-CvaB antibodies were further purified by using a Reacti-Gel 6X affinity column (Pierce Biochemical Co.) that was conjugated with the overproduced 12-kDa C-terminal domain of CvaB.

Translational *lacZ* fusions and β-galactosidase activity assay. The translational *lacZ* fusion plasmids pQF52*cvaA*15 (23) and pQF52*cvaB*16 were used to examine the synthesis of CvaA and CvaB, respectively. To construct pQF52*cvaB*16, pHK11 was digested with *Kpn*I followed by S1 nuclease treatment to make an out-of-frame mutation in *cvaA*, yielding pTJ113; this *cvaA*-mutated plasmid was used as a template for PCR. The promoter region of *cvaAB* and the codons for the N-terminal 16 amino acids of CvaB were synthesized by PCR with *Sma*I sites at each end and inserted into the *Sma*I site of pQF52 (24). The fusion site of pQF52*cvaB*16 was verified by DNA sequence analysis. Transformants were selected on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates. For measuring β-galactosidase activities, exponentially growing cells at an optical density at 600 nm (OD_{600}) of ~0.5 were induced with 0.5 mM 2,2′-dipyridyl, an iron chelator, and then 1 ml of cells was cultured at the time indicated. β-Galactosidase activity was determined by the method of Miller (27).

Membrane preparation. Total membranes were used for determining protein expression and analysis of cross-linking products unless otherwise indicated. Cells were suspended in 50 mM Tris-HCl (pH 8.0)–50 mM NaCl–20% glycerol–5 mM EDTA and then lysed with a French press. Lysates were centrifuged for 5 min at 4,000 × *g* to remove cell debris, and then the total membrane fraction was pelleted by centrifugation at 100,000 rpm for 20 min in a Beckman TLA100.3 rotor.

Protein expression and Western blotting. Cells containing appropriate plasmids grown overnight were diluted to an OD_{600} of ~0.1 and incubated for growth. At an OD_{600} of ~0.5, the cells were induced with 0.5 mM 2,2′-dipyridyl and were harvested 2 h later. Protein concentrations of the lysates were measured by the micro-bicinchoninic acid method (Pierce). For the stability test, 200 μg of kanamycin or chloramphenicol per ml, depending on the strain used, was added to stop new protein synthesis after 30 min of induction, and samples were removed to determine CvaA or CvaB stability. Proteins, after electrophoresis, were transferred to a polyvinylidene difluoride membrane sheet (Applied Biosystems) for color development or an Immun-Lite membrane sheet (Bio-Rad) for chemiluminescence detection, and the membrane sheets were treated by established procedures for Western blotting (20). Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G was used as the secondary antibody. Immunoblot bands were then quantified by using the PDI image analysis system (Protein Databases Inc., Huntington Station, N.Y.).

In vivo formaldehyde cross-linking. Cells were induced for 30 min as described above and pelleted by centrifugation. The cell pellet was resuspended in the

same volume of 0.1 M sodium phosphate buffer (pH 6.8), and formaldehyde was added to a final concentration of 1% (wt/wt) as previously described (32). The samples were incubated at room temperature without shaking for various times and centrifuged immediately to pellet the whole cells. The membrane fraction was obtained as described above, solubilized in sample buffer in the presence of 4% SDS, and incubated at 37°C for 30 min. The sample was then electrophoresed in high-Tris SDS–10% polyacrylamide gels (3) and subjected to Western blotting.

ColV activity test. ColV activity was detected by a halo assay using TB plates overlaid with ColV-sensitive 71-18 cells as described previously (23).

Reagents. All chemicals and reagents were reagent grade and were purchased from Sigma Chemical Co. unless otherwise noted. Restriction enzymes, T4 DNA ligase, and S1 nuclease were from Boehringer Mannheim.

RESULTS

Expression of CvaA and CvaB in cells containing various plasmids. It was previously reported that in the absence of *cvaA*, *cvaB*, and *tolC* gene products, no extracellular ColV activity was detected (12). Furthermore, when an in vitro processing assay was performed with membranes of these mutants, no processing of the ColV precursor was observed (41). These results imply that these proteins, CvaA, CvaB, and TolC, are required and thus possibly interact with each other to form a complex to secrete ColV. To test this possibility, we have studied the interaction of these proteins. First, we analyzed the mutant membranes which were used for the in vitro processing assays (41) by Western blotting against anti-CvaA or anti-CvaB antibodies. The amount of CvaA or CvaB was significantly reduced when CvaB or CvaA, respectively, was depleted, and both proteins were drastically reduced in TolC-minus cells (Fig. 1A).

Since it is possible that some proteins can be degraded during the preparation of membranes for in vitro assays, we also analyzed the total cells and membranes. ZK4 cells containing pHK22 (*cvaABC* and *cvi* in pACYC184) and its derivatives pHK22-2 (pHK22 with *cvaA*::Tn*5*) and pHK22-5 (pHK22 with *cvaB*::Tn*5*) were used, and compatible pHK11 derivatives, pTJ114 (*cvaA* in pBR322) and pTJ116 (*cvaB* in pBR322), were used for complementation. Both CvaA and CvaB were much reduced in either CvaB- or CvaA-depleted cells and in TolC-depleted cells (Fig. 1B, lanes 3, 5, and 7), consistent with the results in Fig. 1A. The addition of a compatible plasmid which expressed only CvaA or CvaB partially restored the reduced CvaB or CvaA, respectively, compared to cells with the wild-type plasmid pHK22 (Fig. 1B, lanes 4 and 6). However, the recovery of CvaB was to a level much less than that of the wild type; this is probably due to translational coupling (15) since the peptide chain termination region of *cvaA* overlaps the initiation site of *cvaB* (17). Interestingly, the amount of CvaA was more reduced in TolC-minus cells than that in CvaB-minus cells (Fig. 1B, lanes 5 and 7). However, the recovery of CvaA was higher in the presence of TolC than CvaB, with the corresponding increased ColV activity in the supernatants (Fig. 1B, lanes 6 and 8). These results imply that TolC may affect the synthesis or stability of CvaA to a larger extent than that of CvaB. When TolC was supplied by a high-copy-number plasmid (pBR*tolC*) to ZK796 cells, the amounts of both CvaA and CvaB were still less than those in wild-type cells. This may be due to the decreased copy number or expression of the wild-type plasmid (pHK22) by the addition of the pBR322-derived plasmid, since when pBR322 was introduced to ZK4/pHK22 cells, the amounts of both CvaA and CvaB were reduced to levels similar to those of ZK796/pHK22/pBR*tolC* (Fig. 1C). The CvaA was also not fully recovered by the addition of a high-copy-number compatible plasmid (Fig. 1B, lane 4), probably due to the decreased amount of CvaB. These data suggest that CvaA and CvaB may affect the synthesis or stability of each other.

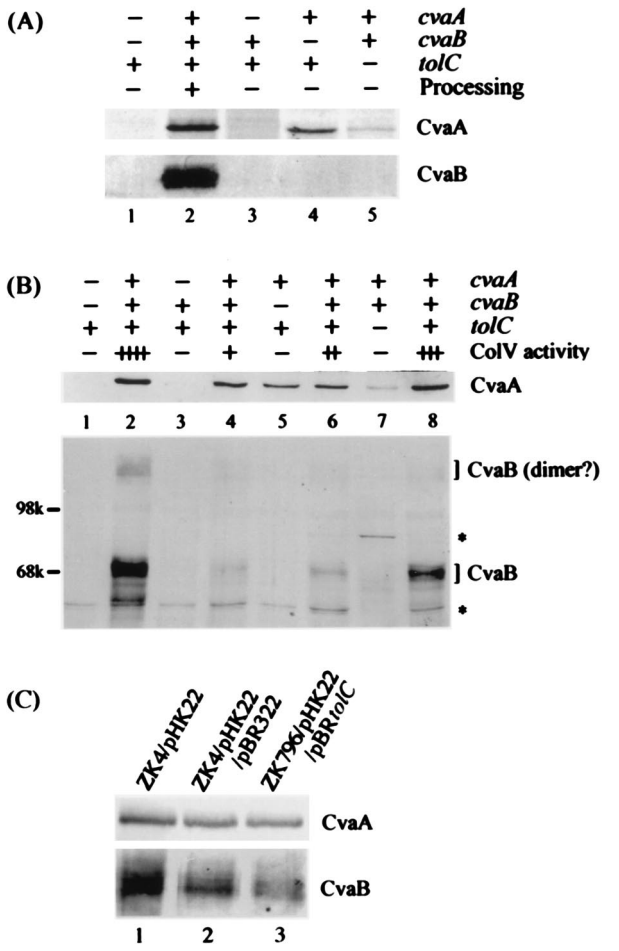

FIG. 1. Western blots of CvaA and CvaB. Cells containing various plasmids were used to determine the expression of CvaA and CvaB. (A) Both CvaA and CvaB were analyzed with membranes that were used for in vitro precursor processing in which active processing requires intact CvaA, CvaB, and TolC (41). Membrane fractions of MC4100 cells containing no plasmid (lane 1), pHK11 (lane 2), pHK11-8 (lane 3), pHK11-4 (lane 4), and ZK796 cells with pHK11 (lane 5) were used; 15 μg of protein of each sample was analyzed by PAGE on high-Tris SDS–10% polyacrylamide gels after being dissolved in 4% SDS-containing sample buffer for 30 min at 37°C. Immunoblot bands were detected by chemiluminescence methods. (B) For CvaA, total cell extracts were boiled in sample buffer and 15 μg was subjected to SDS-PAGE (10% gel). For CvaB, total membrane fractions were treated as in panel A, and then 30 μg was used for high-Tris SDS-PAGE (10% gel). CvaA and CvaB bands were detected immunologically by the color development methods. Lanes: 1, ZK4; 2, ZK4/pHK22; 3, ZK4/pHK22-2; 4, ZK4/pHK22-2/pTJ114; 5, ZK4/pHK22-5; 6, ZK4/pHK22-5/pTJ116; 7, ZK796/pHK22; 8, ZK796/pHK22/pBR*tolC*. Nonspecific bands are marked by asterisks. (C) The amounts of CvaA and CvaB in ZK4/pHK22 cells without (lane 1) and with (lane 2) plasmid pBR322. Lane 3 is same as lane 8 of panel B.

A higher-molecular-mass band (approximately 150 kDa) was recognized by the anti-CvaB antibodies (Fig. 1B, lane 2), and it disappeared upon heating to 100°C (data not shown). This band, which was also observed in other ABC transporters (7, 36), corresponds to the size of a CvaB dimer. The CvaB formed aggregates and could not be detected in the gels upon prolonged heating at 100°C (data not shown), much like another integral membrane protein, SecY (1).

Effects of TolC, CvaB, or CvaA on the synthesis of CvaA– or CvaB–β-galactosidase fusions. The results presented above showed that the synthesis or stability, or both, of CvaA and CvaB may be affected by one another and also by TolC. To

further investigate these possibilities, we measured the effects of TolC and CvaB or TolC and CvaA on the synthesis of CvaA and CvaB, respectively, using translational *lacZ* fusion plasmids. There was no difference in CvaA–β-galactosidase synthesis in the presence or absence of CvaB or TolC, and the synthesis of CvaB–β-galactosidase was also not affected by the presence or absence of CvaA or TolC (data not shown). Interestingly, the translational level of CvaB as measured by β-galactosidase activity was three to four times lower than that of CvaA, indicating the differential translation efficiencies of CvaA and CvaB. Alternatively, since pQF52*cvaB*16 was constructed by an out-of-frame deletion of *cvaA* in *Kpn*I sites, this is probably due to the translational coupling synthesis of CvaA and CvaB; the absence of CvaA translation may serve to reduce the levels of CvaB by preventing ribosome loading (15).

Effect of CvaB on CvaA stability. Translational *lacZ* fusion data showed that the presence or absence of CvaB and CvaA did not affect the synthesis of each other. Furthermore, the synthesis of each protein was not dependent on TolC. These results suggest that the reduced amounts of CvaA and CvaB (Fig. 1) are caused by reduced stability rather than reduced synthesis. To substantiate the possibility that both CvaA and CvaB proteins stabilize each other and are also stabilized by TolC, we examined the stabilities of CvaA and CvaB proteins. Since genes in the ColV operon, *cvaABC* and *cvi*, are negatively regulated by iron (2, 23), cells were induced with 2,2′-dipyridyl, an iron chelator, for 30 min, and then chloramphenicol (for pBR derivatives [Fig. 2A and 3]) or kanamycin (for pACYC derivatives [Fig. 2C]) was added to stop new protein synthesis. This method (23) was used to examine the stability of preexisting proteins in a rich medium simply by Western blotting, rather than by radiolabeling/pulse-chase and immunoprecipitation. Although equal protein amounts were used for electrophoresis, anti-EF-G antibodies were used to detect EF-G as an internal control to measure the ratio of CvaA amount to EF-G amount (Fig. 2). We examined the stability of CvaA in cells containing wild-type (pHK11) and *cvaB*-null (pHK11-4) plasmids to determine the effect of CvaB on CvaA stability. The CvaA in cells containing the *cvaB*-null plasmid was less stable than in cells with a wild-type plasmid (Fig. 2A). This result indicates that the presence of CvaB, which did not affect the synthesis of CvaA–β-galactosidase, stabilized the CvaA.

Effect of TolC on CvaA stability. The observation that the amounts of CvaA and CvaB were reduced in the *tolC* mutant (Fig. 1A, lane 5) but synthesis was not affected also raised the question of whether the absence of TolC affects the stabilities of CvaA and CvaB and whether the function of TolC in ColV secretion is merely to stabilize CvaA and CvaB. We therefore examined the effect of TolC on CvaA stability. To obtain a missense, nonfunctional TolC protein, we mutagenized the *tolC*-containing plasmid, pAC*tolC*, with hydroxylamine (4). One mutant plasmid, pAC*tolC*-1, was identified by the inability of the cells containing it to secrete ColV; this plasmid was verified by DNA sequence analysis to encode an L193V change in TolC. Figure 2B shows the expression of TolC and CvaA and ColV activity in ZK796/pHK11 cells with pAC*tolC* and pAC*tolC*-1. Cells containing pAC*tolC*-1 (Fig. 2B, lane 3) produced almost the same amounts of TolC and CvaA as cells containing pAC*tolC* (Fig. 2B, lane 2); however, ColV activity was reduced drastically (by clear zone area). Using the mutated plasmid, we measured the effect of TolC on CvaA stability (Fig. 2C). The CvaA protein was quite stable even in cells containing the less functional L193V TolC; however, it was very unstable in cells without TolC. These results demonstrate the importance of TolC on ColV export; functionally, it is required to secrete ColV, and structurally, it is needed to stabilize both CvaA and CvaB (see below).

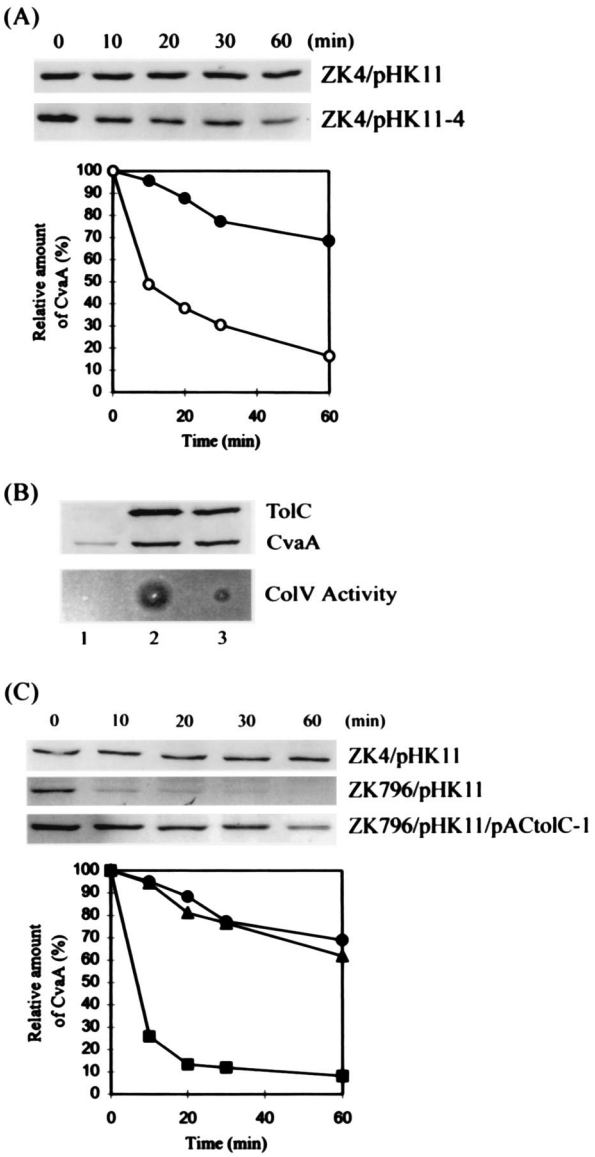

FIG. 2. Effects of the presence and absence of CvaB and TolC on the CvaA protein stability. (A) CvaB effect. ZK4 cells containing pHK11 (closed circles) and pHK11-4 (CvaB$^-$; open circles) were induced with 2,2′-dipyridyl for 30 min. To stop the new protein synthesis, chloramphenicol (200 μg per ml) was added, and the cells were taken at the time points indicated; 25 μg of total cell extracts was used for SDS-PAGE (10% gel) followed by immunoblotting. (B) CvaA and TolC expression. Amounts of CvaA and TolC, and extracellular ColV activity were measured in ZK796/pHK11 (lane 1), ZK796/pHK11/pAC*tolC* (lane 2), and ZK796/pHK11/pAC*tolC*-1 (lane 3) cells. The immunoblot membrane sheet was incubated in the mixture of anti-CvaA and anti-TolC antibodies. (C) TolC effect. ZK4/pHK11 (circles), ZK796/pHK11 (squares), and ZK796/pHK11/pAC*tolC*-1 (triangles) cells were treated as in panel A except that kanamycin (200 μg per ml) was added to stop new protein synthesis and 20 μg of each sample was used. CvaA amounts in both graphs were slightly adjusted and normalized by the CvaA/EF-G ratios. The data were obtained from the averages of two independent experiments.

Effects of CvaA or TolC on CvaB stability. The effects of CvaA or TolC on CvaB stability were similarly examined (Fig. 3). As expected, CvaB was less stable in the absence of either CvaA or TolC. Interestingly, the instability of CvaB was more severe when CvaA was absent than when TolC was absent.

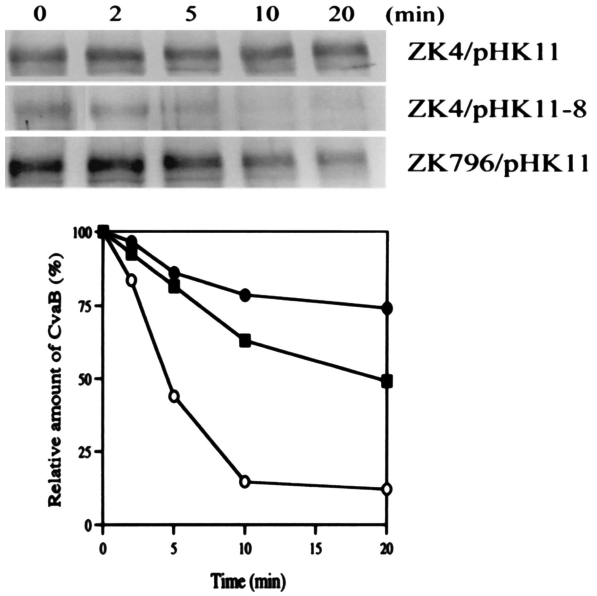

FIG. 3. Effect of the presence and absence of CvaA and TolC on CvaB protein stability. ZK4/pHK11 (closed circles), ZK4/pHK11-8 (CvaA$^-$; open circles) and ZK796/pHK11 (TolC; squares) cells were induced, and new protein synthesis was stopped as in Fig. 2A. Total cell membranes were prepared (see Materials and Methods) and dissolved in the presence of 4% SDS for 30 min at 37°C; 30 μg of each sample was used for high-Tris SDS-PAGE (10% gel) followed by immunoblotting against anti-CvaB antibodies. The data were the averages of two independent experiments.

However, the stability of CvaA was more reduced by the absence of TolC than that of CvaB (Fig. 2A and C). Since most residues of the C-terminal end of CvaA stretch to the periplasm (33) and are believed to attach to the outer membrane (8), it is possible that TolC, which resides in the outer membrane, may play a more important role in stabilizing CvaA than CvaB. On the other hand, as a member of the ABC transporters, CvaB is in the inner membrane and is believed to interact with CvaA but not with TolC directly. Thus, CvaA may be more important than TolC for the stability of CvaB.

CvaA connects CvaB and TolC. Previously, it was proposed that as a member of the MFP family, CvaA may fuse the inner and outer membranes (8). However, no direct evidence has been reported so far concerning proteins in the MFP family. To determine the hypothetical structural role of CvaA, i.e., that it may connect the inner and the outer membrane via interactions with both CvaB and TolC, cross-linking experiments were performed. Using formaldehyde under optimal conditions determined experimentally, we carried out in vivo cross-linking and detected CvaA, CvaB, and TolC by immunoblotting. Total membranes from ZK4 and ZK4/pHK11 were analyzed with anti-CvaA, anti-CvaB, and anti-TolC antibodies. As expected, only TolC appeared in the ZK4 wild-type cells (Fig. 4A, lane 3), and CvaA and CvaB were detected only in the presence of plasmid pHK11 (Fig. 4B, lanes 1 and 2). Previously, it was reported that the TolC protein, which resides in the outer membrane, had two forms (29), which we confirmed by using N-terminal protein sequencing (data not shown) and immunoblotting (Fig. 4A and B). In addition, several other bands were detected by TolC antibodies (Fig. 4A, lane 3). One of around 150 kDa (Fig. 4, asterisk), which was reported previously (29), appeared to be a TolC trimer by its size. The other three to four bands appeared at between 70 and 80 kDa (Fig. 4, bracketed) and might be complexes of TolC with other

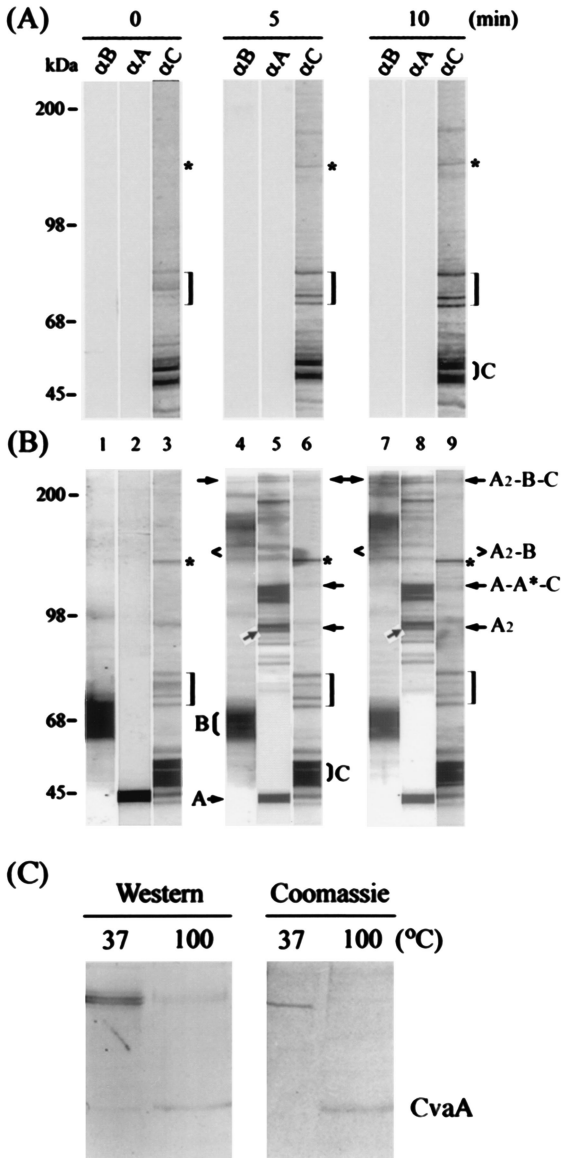

FIG. 4. Immunoblots of formaldehyde-cross-linked membrane proteins. Membrane fractions of ZK4 (A) and ZK4/pHK11 (B) cells without cross-linking (lanes 1 to 3) or with 1% formaldehyde for 5 min (lanes 4 to 6) and 10 min (lanes 7 to 9) of cross-linking were subjected to high-Tris SDS-PAGE (10% gel). Samples in lanes 1 to 3, 4 to 6, and 7 to 9 were from the same sample wells, and the membrane sheets were sliced into three pieces for Western blotting with three different antibodies. After development, the membrane sheets were lined up against markers. Anti-CvaB (lanes 1, 4, and 7), anti-CvaA (lanes 2, 5, and 8), and anti-TolC (lanes 3, 6, and 9) antibodies were used, and putative complexes are indicated. (A) CvaA; (B) CvaB; (C) TolC. Possible TolC trimers are marked by asterisks; nonspecific TolC-containing bands are bracketed (see text). (C) Putative CvaA dimer. To extract the cross-linked CvaA complex (B, lane 8, boxed arrow), the band was excised from the wet gel and soaked in 0.1 M sodium bicarbonate (pH 8.2) overnight. The extracted sample was concentrated with Millipore ULTRAFREE-MC filter units and incubated at 37°C or boiled to retain or to cleave the cross-linking, respectively.

unknown proteins. Since we treated membranes at 37°C to retain cross-linking rather than boiling before SDS-PAGE, the TolC that interacted tightly with other proteins could not be fully dissociated. In fact, all of these TolC-interacting non-CvaAB bands disappeared when samples were heated at 100°C (data not shown).

We observed additional bands which were identified by the

anti-CvaA, anti-CvaB, and anti-TolC antibodies (Fig. 4B, lanes 4 to 9) in the presence of formaldehyde for either 5 or 10 min. A CvaA dimer-like band was detected at around 90 kDa (Fig. 4B, arrows in lanes 5 and 8). When this band was excised, boiled to cleave cross-linking, and analyzed by SDS-PAGE, only CvaA was detected by both Western blotting and Coomassie staining (Fig. 4C) which suggests that this band may be a CvaA dimer or a CvaA monomer which interacted with other, similar-size proteins. In addition, other bands of between 68 and 98 kDa were shown only against anti-CvaA antibodies; thus, CvaA* (~27 kDa), which stabilizes CvaA (23), may also interact with CvaA. Two bands against anti-CvaA antibodies were detected at around 120 to 130 kDa (Fig. 4B, lanes 5 and 8). The lower band, which appeared only with anti-CvaA antibodies, may be a CvaA*-CvaA-CvaA complex. However, the upper band, which was also shown with anti-TolC antibodies, is consistent with a CvaA*-CvaA-TolC complex by size (Fig. 4B, lanes 5, 6, 8, and 9). One band of around 100 kDa cross-reacted with both anti-CvaA and CvaB antibodies, suggesting that CvaA* may interact with CvaB (Fig. 4B). Bands that appeared at around 150 to 160 kDa, which were also matched by both anti-CvaA and CvaB antibodies, may be a CvaA-CvaA-CvaB complex, as judged by size (doublet probably due to CvaB which showed a diffused band) (Fig. 4B, lanes 1, 4, and 7). A band above 200 kDa was detected by all three antibodies and is presumably a CvaA-CvaA-CvaB-TolC complex. These complexes, though the exact stoichiometry is not certain, appeared to have a CvaA-CvaA backbone by size. Interestingly, no matching band except the CvaA-CvaB-TolC band was detected with both anti-CvaB and anti-TolC antibodies. These results imply that both CvaB and TolC interact with CvaA but do not directly interact with each other.

Taken together, these results indicate that as a member of the MFP family, CvaA interacts directly with both CvaB and TolC to connect the inner and the outer membranes and suggest that the interactions of these components form the ColV secretory complex.

DISCUSSION

The extracellular secretion of ColV requires at least three proteins, CvaA, CvaB, and TolC (17). CvaB and TolC reside in the inner and outer membranes, respectively. CvaA, which anchors in the inner membrane and may stretch to the outer membrane, is believed to connect these two proteins (17, 29, 33). In this study, we examined the interactions of these proteins in the ColV secretion system and verified the hypothesized structural role of MFPs (8) by using a cross-linking reagent. When either CvaA or CvaB was depleted by Tn*5* insertion, the amount of CvaB or CvaA, respectively, was also reduced, and both proteins were not stable in the absence of TolC. The instability of CvaA was greater in the absence of TolC than CvaB (Fig. 2). Since most of CvaA is in the periplasm (33) and may interact with TolC through its C-terminal hydrophobic region which is highly conserved among the proteins in the MFP family (8), it is possible that depletion of TolC causes detachment of CvaA from the outer membrane. This detachment probably induces a drastic change in the CvaA conformation, such that it becomes more susceptible to proteolytic digestion. On the other hand, the depletion of CvaB may not induce a substantial structural change in CvaA which is stabilized by its N-terminal transmembrane hydrophobic domain in the inner membrane as well as the anchoring by TolC in the outer membrane. Thus, there is more stable CvaA protein in the CvaB-depleted cells than in the TolC-depleted cells. However, the instability of CvaB was much more severe in the absence of CvaA than in the absence of TolC (Fig. 3). As an ABC transporter, CvaB has six transmembrane domains and may directly interact with CvaA but not with TolC; thus, the depletion of CvaA may lead to the easy degradation of CvaB. The direct interactions of CvaA with both CvaB and TolC proteins were confirmed by in vivo cross-linking. However, there is no clear evidence of direct interaction between CvaB and TolC.

As a minor outer membrane protein, TolC has been shown to have a major effect on the outer membrane of *E. coli* (28). The *tolC* mutants that lack OmpF protein exhibit an altered sensitivity pattern against bacteriophage, become hypersensitive to detergents, certain antibiotics, and dyes, and also become tolerant to colicin E1 (6, 18, 28, 30). In addition, the secretion of some proteins such as colicin V, α-hemolysin, protease SM, and the heat-stable ST_B enterotoxin was TolC dependent in *E. coli* (14, 17, 25, 37). These observations, however, are all based on knockout null mutants, and thus the protein could have other secondary effects (e.g., outer membrane conformation or iron uptake). Under our conditions, the amounts of CvaA and CvaB were reduced in TolC-depleted cells (Fig. 1A, lane 5) without affecting their synthesis, suggesting the structurally stabilizing effect of TolC on the CvaA and CvaB proteins. To clarify the functional effect of TolC on ColV secretion, we generated a point mutant of *tolC* by random mutagenesis. In this L193V mutant, the amounts of CvaA and CvaB were determined to be within normal levels, but the mutant is severely defective in ColV secretion. These findings indicate the structural and functional importance of TolC on ColV secretion; it is required not only to stabilize the transporters but also to secrete ColV. These results demonstrate that TolC is directly involved in the secretion of exported proteins.

Using biochemical methods, we detected no free ColV in the periplasm during ColV secretion, and when either CvaA, CvaB, or TolC was depleted, no free ColV was detected in the periplasm or extracellular medium (12). These results are consistent with the notion that these three essential proteins may form a complex to secrete ColV directly from the cytoplasm to the extracellular medium without any detectable periplasmic intermediates, as also in the case of the α-hemolysin secretion system (38). (However, it has also been reported that, on the basis of a genetic assay, low levels of ColV may be present in the periplasm during secretion [40].) Recently, the ordered association of the three components of other ABC exporters was determined by substrate affinity chromatography, and complex formation was promoted by the binding of substrates which possess uncleavable C-terminal signal sequences (26). It is not known whether the assembly of ColV secretory proteins is also ordered. However, our stability data suggest that the components of ColV exporters probably form a stable complex in either the presence or absence of substrate.

Based on the stability and cross-linking results, and other ABC transporter models (11, 36), we proposed a model for ColV secretion system as shown in Fig. 5. Though the exact stoichiometry of the complex is not known, two CvaAs may form a channel-like path via bridging with CvaB and TolC, thus allowing the ColV to pass without any periplasmic intermediate. TolC is depicted as a trimer, since a TolC band was detected at around 150 kDa and is consistent with previous observations (29). CvaA*, an in-frame protein of *cvaA* which lacks the N-terminal hydrophobic region of CvaA, was proposed to reside in the cytoplasm (10, 17) and may support the ColV secretory complex on the cytoplasmic side by interaction with either CvaA, CvaB, or both (23). Although the exact mechanism of ColV secretion is not clear, it is tempting to speculate that ColV, which may be processed by the N-termi-

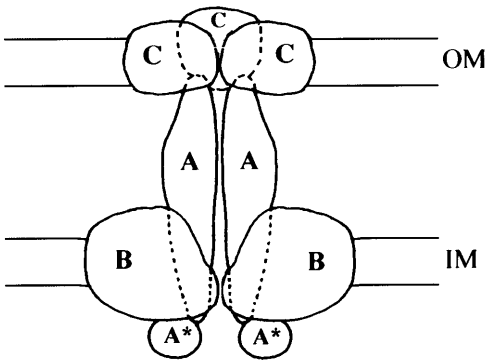

FIG. 5. Proposed model of ColV secretory complex. The schematic model is based on the results of stability and in vivo cross-linking. A, CvaA; B, CvaB; C, TolC; A*, CvaA*. IM, inner membrane; OM, outer membrane.

nal catalytic domain of the ABC transporter CvaB (21), is inserted into the channel of the export complex formed by CvaA then pushed out directly to the extracellular space by the energy of nucleotide hydrolysis that is carried out by the C-terminal ATP-binding domain of CvaB (41, 42). Further work is required to clarify this hypothesis.

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