Genetic Analysis of the Colicin V Secretion Pathway

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ABSTRACT

Colicin V (ColV) is peptide antibiotic secreted by *Escherichia coli* through a dedicated exporter composed of three proteins, CvaA, CvaB, and TolC. ColV secretion is independent of the *E. coli* general secretory pathway (Sec) but requires an N-terminal export signal specific for the CvaAB/TolC exporter. ColV secretion was characterized using genetic and biochemical methods. When the ColV N-terminal extension is replaced with the OmpA signal sequence, the Sec system can localize ColV to the periplasm. Periplasmic ColV is lethal to cells lacking the ColV immunity protein, Cvi. Based on this result, a genetic assay was designed to monitor for the presence of periplasmic ColV during normal CvaAB/TolC mediated secretion. Results indicate that low levels of ColV may be present in the periplasm during secretion. Precursor and mature ColV were also characterized from the wild-type system and in various exporter mutant backgrounds using immunoprecipitation. ColV processing is rapid in wild-type cells, and CvaA and CvaB are critical for processing to occur. In contrast, processing occurs normally, albeit more slowly, in a TolC mutant.

THE bacterial ABC-export systems comprise a large family of proteins involved in the export of a wide range of proteins, peptides, and other small molecules from prokaryotic organisms (HIGGINS 1992; FATH and KOLTER 1993). The best characterized of these export systems is the HlyBD/TolC system, which is responsible for the secretion of the α-hemolysin (HlyA) protein from *Escherichia coli* (see HOLLAND *et al.* 1990; HUGHES *et al.* 1992; FATH and KOLTER 1993 for recent reviews). A great deal of work has gone into characterizing the secretion pathway for HlyA. Several labs have carried out biochemical studies looking at HlyA localization during secretion by the HlyBD/TolC system (GRAY *et al.* 1986, 1989; FELMLEE and WELCH 1988; KORONAKIS *et al.* 1989; OROPEZA-WEKERLE *et al.* 1990; THOMAS *et al.* 1992). These studies show that secretion of HlyA out of *E. coli* occurs without a detectable periplasmic intermediate. They also show that HlyA localizes to the cytoplasm and the inner membrane when HlyB, HlyD, HlyBD or TolC export proteins are absent. HlyA is the only ABC-protein substrate for which a detailed secretion pathway has been worked out. As such, HlyA serves as the prototype for this superfamily. But, until additional secretion systems are similarly characterized, it will be difficult to say with certainty that the details of HlyA secretion reflect general principles of this superfamily.

We have studied the genetics and molecular biology of another molecule that is secreted by a bacterial ABC-export system: the peptide antibiotic colicin V (ColV). ColV is an 88 amino acid polypeptide encoded by many different large, low-copy number plasmids from *E. coli* (GILSON *et al.* 1987, 1990; FATH *et al.* 1992, 1994).

The ColV operon is composed of four genes: the *cvaC* structural gene, the *cvi* immunity gene, and the two export genes, *cvaA* and *cvaB*. ColV is synthesized as a 103 amino acid primary translation product (pre-ColV), which is processed and secreted out of producing cells (FATH *et al.* 1994; HÅVARSTEIN *et al.* 1994). Extracellular secretion requires the action of three proteins: CvaA, CvaB, and TolC (GILSON *et al.* 1990). CvaB is structurally and functionally related to the ABC-exporter HlyB, and CvaA is structurally and functionally related to HlyD (FATH *et al.* 1991). The third ColV export protein is the outer membrane protein TolC. Both the CvaAB and the HlyBD export systems require the TolC protein to facilitate extracellular secretion of their respective protein substrates (GILSON *et al.* 1990; WANDERSMAN and DELEPELAIRE 1990).

Uptake of ColV by the target cells requires the presence of the outer membrane receptor Cir and the proteins involved in Cir-mediated uptake—TonB, ExbB, and ExbD (DAVIES and REEVES 1975a; POSTLE 1990). There is an evidence that ColV kills sensitive cells by inserting into their inner membrane thus disrupting their membrane potential (YANG and KONISKY 1984). The presence of the immunity protein, Cvi, is sufficient to fully protect a target cell from the bactericidal activity of ColV (YANG and KONISKY 1984; FATH *et al.* 1992).

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In this study, we have carried out experiments to characterize the pathway of ColV secretion and activity. We demonstrate that ColV is only bactericidal when provided from the periplasmic face of the inner membrane. We also show that ColV can be exported to the periplasm by the Sec-dependent secretion pathway when fused to the OmpA signal sequence, but does not cross the outer membrane. We used these results to devise a genetic assay for monitoring for the presence of periplasmic ColV. The results of this assay indicate that while ColV does not accumulate in the periplasm, a small amount of ColV appears to be present in that compartment during secretion. The secretion pathway was further characterized using immunoprecipitation to identify precursor and mature ColV in the wild-type system and in various exporter mutant backgrounds. Last, two strains were constructed that contained mutations in the C-terminal cysteine residues of ColV. These mutants were secreted efficiently but lacked all bactericidal activity.

MATERIALS AND METHODS

Media and culture conditions: Except where noted, LB was used as both the liquid and solid growth medium (MILLER 1972). Antibiotics were used at the following final concentrations (in μg/ml): ampicillin (Ap), 150; chloramphenicol (Cm), 30; kanamycin (Km), 50; spectinomycin (Sp), 100; and tetracycline (Tc), 15.

Bacterial strains and plasmids: The strains and plasmids used in this study are listed in Table 1. The *tolC* phenotype of ZK796 was confirmed using a detergent sensitivity assay (DAVIES and REEVES 1975b). All plasmid constructions were made by standard restriction enzyme and PCR methodologies. Details of these constructions as well as physical and genetic maps of the plasmids can be obtained from the corresponding author.

Manipulation of DNA: Isolation of plasmid DNA, transformation of bacteria, restriction enzyme digestions, and other routine DNA manipulations were performed using standard procedures (AUSUBEI *et al.* 1987). P1 transductions were carried out as described (MILLER 1972). The mini-Tn*10-kan* transpositions were carried out as described (FATH *et al.* 1989).

Colicin V assays: A halo assay was used to detect extracellular ColV from growing cells as described (FATH *et al.* 1991). When assaying ColV activity from pHLZ101, M63 glucose plates were supplemented with IPTG (isopropylthiogalactoside) at 40 μg/ml.

Gradient plate assay: An IPTG gradient plate was used to assay for the growth of strain KS300 transformed with the OmpA-ColV fusion plasmid, pHLZ101, and the *lacI*^{Q} bearing plasmid, pMS421. The transformation mix was spread on an LB plate with Sp and Km. A filter disc was placed at the center of the plate, and 30 μl of 100 mM IPTG were spotted on the filter disc. This plate was incubated overnight at 37°, and the morphology and the distribution of colonies was recorded.

Transformation assay: A genetic assay was designed to monitor for the presence of periplasmic ColV based on the toxic effect of periplasmic ColV. The assay was carried out using three plasmids: pHK22–6 (Cm^{r}; CvaA^{+}, CvaB^{+}, and Cvi^{+}), pMJF116 (Cm^{r}; CvaA^{+}, CvaB^{+}, and Cvi^{-}), and pHK890-WT (Ap^{r}; CvaC^{+}). For each assay, the strain being analyzed was first transformed with either pHK22–6 or pMJF116 and grown on LB Cm plates. Then each strain was transformed with 500 ng of pHK890-WT plasmid DNA and plated on LB Ap Cm. Each transformation mix was assayed for its ability to form colonies on the selective medium. Similar results were obtained if strains were transformed with pHK890-WT first and pHK22–6 or pMJF116 second. Three isogenic strains were used as hosts for this assay: MC4100 (Cir^{+}, TolC^{+}), JK838 (Cir^{-}, TolC^{+}), and ZK796 (Cir^{+}, TolC^{-}).

Labeling, immunoprecipitation and SDS-PAGE: The ColV producing strain MC4100 (pHK11) was grown overnight at 37° in M63 and 0.2% glucose (MILLER 1972) plus Ap. The overnight culture was diluted 1:40 in the same medium containing 50 μg/ml of each amino acid except methionine.The cells were grown to OD_{600} of 0.2–0.3, and inducer (0.1 mM IPTG for inducing P_{lacUV5} or 0.1 mM 2,2′-dipyridyl for inducing the ColV promoter, which is iron regulated (CHEHADE and BRAUN 1988; GILSON *et al.* 1990) was added for 1 hr. Proteins were pulse labeled with 30 μCi/ml [^{35}S] methionine. For short pulse labeling (10 sec) cells were labeled and stopped by addition of ice-cold TCA (trichloroacetic acid) to a final concentration of 10%. Precipitated proteins were collected by centrifugation, washed with ice-cold acetone, dried and dissolved in 50 μl STEB (1% SDS, 10 mM Tris.HCl pH 8.0, 1 mM EDTA and 5% mercaptoethanol). Longer pulses labeling (2 min) was stopped by adding 500 μl of stop solution (0.24% NaN3, 0.6 mg/ml spectinomycin). The bacteria were pelleted by centrifugation for 4 min at 4° and the supernatant was separated from the pellet. Supernatant (400 μl) was used directly for immunoprecipitation. Pelleted cells were first boiled 2 min in 800 μl of chilled KI buffer (50 mM TrisHCl pH 8.0, 150 mM NaCl, 2% Triton X-100, 1 mM EDTA) and 400 μl of the sample were transferred for later immunoprecipitation. Immunoprecipitation was performed as described previously (HARLOW and LANE 1988). After immunoprecipitation, each sample was resuspended in 35 μl of loading buffer (LAEMMLI 1970) and 10 μl were loaded and electrophoresed on a 20% SDS-polyacrylamide gel optimized for resolving small proteins (THOMAS and KORNBERG 1978).

RESULTS

Intracellular ColV is not bactericidal to the producing cell: pHK890-WT is a high-copy pBR322 derivative that contains the intact *cvaC* gene driven by P_{lacUV5} but lacks *cvaA*, *cvaB*, and *cvi*. Strains that carry pHK890-WT produce cytoplasmic ColV with no deleterious effects. The lack of deleterious effects could be explained in part by degradation of the cytoplasmic ColV produced from this construct (see below, Figure 4, lane 6). However, it is clear that some ColV accumulates in the cytoplasm because lysate from strains harboring this construct contain ColV activity (data not shown). In addition, these strains produce extracellular ColV when they also carry pHK22–6, a compatible *cvaA*$^{+}$, *cvaB*$^{+}$, *cvi*$^{+}$, and *cvaC*$^{-}$ plasmid (GILSON *et al.* 1987). pHK890-WT was constructed in and can be maintained in strains that lack *cvi*, the ColV immunity gene (Table 2). Thus, the action of the immunity protein is not required when ColV remains in the cytoplasm.

Secretion of an OmpA-ColV fusion protein by the Sec-dependent pathway: Some proteins appear to be inherently incompatible with the Sec-dependent export pathway, *i.e.*, they cannot be secreted when fused to functional signal sequences (LEE *et al.* 1989; KENNY *et al.* 1991). The ABC-export systems used by HlyA, ColV

TABLE 1

Strains and plasmids used in this study

Strains	Genotype	Reference
MC4100	Δ*lacU169 araΔ139 rpsL150 thi-1 recA1 deoC7 flbB5301 ptsF25 relA1*	CASADABAN (1976)
71-18	Δ*(lac-proAB) thi-1 supE/F' lacI*^{Q} Δ(*lacZ*)M15	YANISCH-PERRON *et al.* (1985)
MC1000	Δ*lacX74 rpsL galU galK araD139* Δ(ara-leu)7687	
KS300	MC1000 Leu^{+} Ara^{+} Δ*phoA-PvuII recA1*	STRAUCH and BECKWITH (1988)
DH5αF'	F'*/endA1 hsdR17*($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA*(Nal^{r}) *relA1* Δ*(lacZYA-argF)U169 deoR* [φ80dlacΔ(*lacZ*)M15]	HANAHAN (1983)
JK838	MC4100 *cir*	GRIGGS and KONISKY (1989)
ZK434	KS300 (pLY21)	This work
ZK503	MC4100 (pHK11)	GILSON *et al.* (1987)
ZK508	MC4100 (pKH11-5)	This work
ZK509	MC4100 (pHK11-6)	This work
ZK796	MC4100 *tolC*::Tn*10*	WANDERSMAN and DELEPELAIRE (1990)
Zk1322	DH5αF' (pLZBC102A, pHK22-6)	This work
ZK1344	DH5αF' (pLZBC91A, pHK22-6)	This work

Plasmids	Description	Drug markers	Reference
pHK11	pBR322 with *cvaABC* and *cvi*	Ap^{r}	GILSON *et al.* (1987)
pHK22	pACYC184 with *cvaABC* and *cvi*	Cm^{r}	GILSON *et al.* (1987)
pHK11-1	pHK11 *cvaC*::Tn*5*	Ap^{r}	GILSON *et al.* (1987)
pHK11-5	pHK11 *cvaA*::Tn*5*	Ap^{r}	GILSON *et al.* (1987)
pHK11-6	pHK11 *cvaB*::Tn*5*	Ap^{r}	GILSON *et al.* (1987)
pHK22-6	pHK22 *cvaC*::Tn*5*	Cm^{r}	GILSON *et al.* (1987)
pLY21	pACYC184 with *cvaC* and *cvi*	Cm^{r}	GILSON *et al.* (1987)
p*cvi*	pACYC184 with *cvi*	Cm^{r}	This work
pMJF116	pACYC184 with *cvaAB*	Cm^{r}	This work
pMS421	pSC101 with *lacI*^{Q}	Sp^{r}	GRANA *et al.* (1988)
pHK890-WT	pBR322 with *cvaC* driven by *lac*UV5 promoter	Ap^{r}	This work
pHLZ101	pBR322 with *ompA* signal sequence and *cvaC* driven by *lac*UV5 promoter	Km^{r}	This work
pHLZ79	pBR322 with *ompA* signal sequence and *cvaC* with frame-shift in 2nd codon of *cvaC* driven by *lac*UV5 promoter	Km^{r}	This work
pLZBC91A	pBluescript with *cvaC* mutation C91A	Ap^{r}	This work
pLZBC102A	pBluescript with *cvaC* mutation C102A	Ap^{r}	This work

Ap^{r}, ampicillin; Cm^{r}, chloramphenical; Sp^{r}, spectinomycin; and Km^{r}, kanamycin.

and many other proteins serve as an alternative way by which proteins can be secreted from bacterial cells (FATH and KOLTER 1993). To date, no protein or peptide normally secreted by an ABC-export system has been fused to a Sec-dependent signal sequence and analyzed for export competence. We wanted to test whether or not one such ABC-exporter protein substrate, ColV, was compatible with the Sec-dependent secretion pathway.

Plasmid pHLZ101 contains a P_{lacUV5}-regulated *ompA* signal sequence fused directly upstream of the *cvaC* segment encoding the mature 88 aa ColV protein (Fig-

TABLE 2

Summary of transformation results

Plasmids	Relative level of transformants[a]	Cell characteristics
pBR322	++++	Healthy
pHK890-WT	++++	Healthy, smaller
pHLZ101	−	None
pHLZ101 + p*cvi*	++++	Healthy
pHLZ101 + pMS421	++	Small, sickly
pHLZ101 + pMS421 with IPTG	−	None

[a] Qualitative estimate for the number of transformants/micrograms DNA.

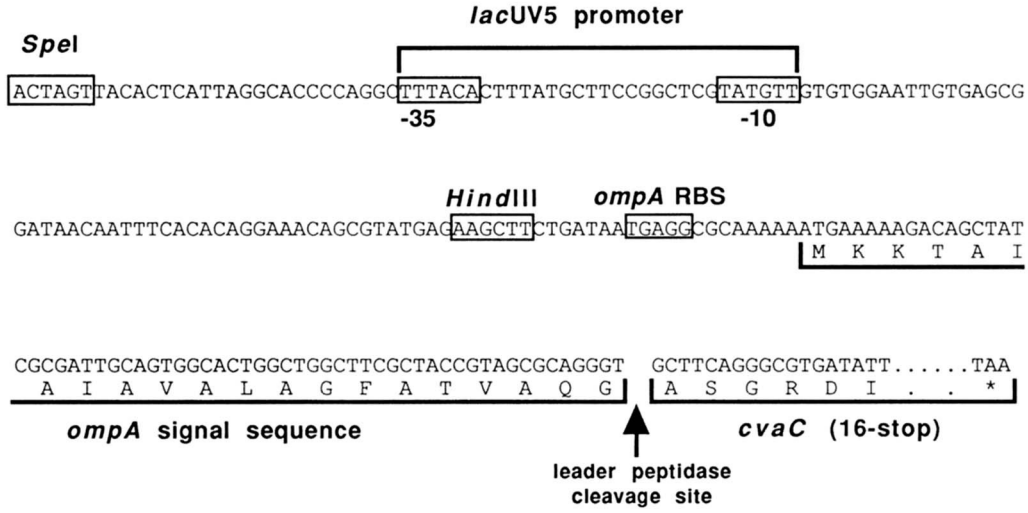

FIGURE 1.—Sequence of the OmpA-ColV fusion junction in pHLZ101. DNA sequences around the junction of *ompA* signal sequence and *cvaC* gene fusion are shown. The amino acid sequence is represented using one-letter code. See text for details.

ure 1). pHLZ101 expresses an OmpA-ColV fusion protein that should result in Sec-dependent export of the ColV protein across the inner membrane. Cells lacking LacI repressor could not be transformed with pHLZ101 unless they also carried p*cvi* (see Table 2). This indicates that the OmpA-ColV fusion is transported across the inner membrane by the Sec pathway and that the resulting ColV is bactericidal. In addition, strains harboring pHLZ101 and p*cvi* do not produce extracellular ColV regardless of whether or not they contain CvaA and CvaB exporters.

Expression of the OmpA-ColV fusion from pHLZ101 could be regulated using $lacI^Q$ (carried on pMS421) and IPTG. pHLZ101 could be introduced into KS300 (pMS421) even in the absence of p*cvi*. However, the colonies were tiny, translucent and sickly (Figure 2A). If IPTG was added to the transformation plates, no colonies were obtained. To characterize this phenomenon further, a transformation mix containing KS300 (pHLZ101, pMS421) was plated on an IPTG gradient plate. On this plate, a ring of colonies was seen at the edge of the plate where the concentration of IPTG was the lowest, but no colonies were found near the center of the plate, colony size increased with the decrease of IPTG concentration (Figure 2B). No such effect was seen when an inactive OmpA-ColV frameshift mutant (pHLZ79) was assayed the same way (Figure 2C). We conclude that expression of the OmpA-ColV fusion results in accumulation of active ColV in the periplasm and that this accumulation is inhibitory for growth in the absence of p*cvi*.

These results show that active ColV can be secreted through the inner membrane when fused to the OmpA signal sequence and that cell growth in the absence of *cvi* is inhibited when there is ColV in the periplasm. They also show that ColV secreted by the Sec-dependent system is not able to cross the outer membrane. Thus, the ColV dedicated export system is necessary to facilitate the translocation of ColV across both membranes.

Monitoring periplasmic ColV during CvaAB-medi-

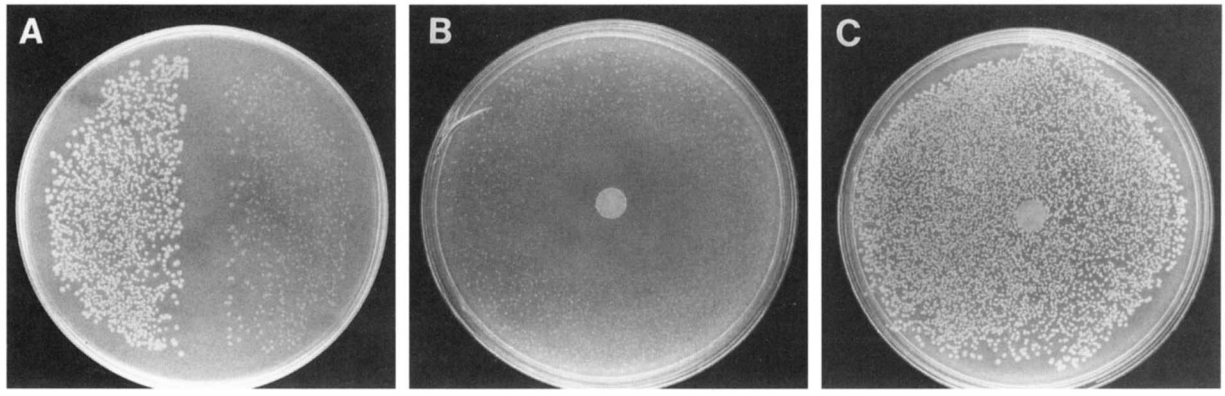

FIGURE 2.—Transformation assay results for OmpA-ColV fusion constructions. Growth patterns and colony morphology of transformants on a LB plate (A) or IPTG gradient plates (B and C) comparing strains expressing ColV fused to the OmpA signal sequence (A, right side, and B) and an inactive frameshift ColV mutant (A, left side, and C). In B and C the strains carry the $lacI^Q$ plasmid pMS421.

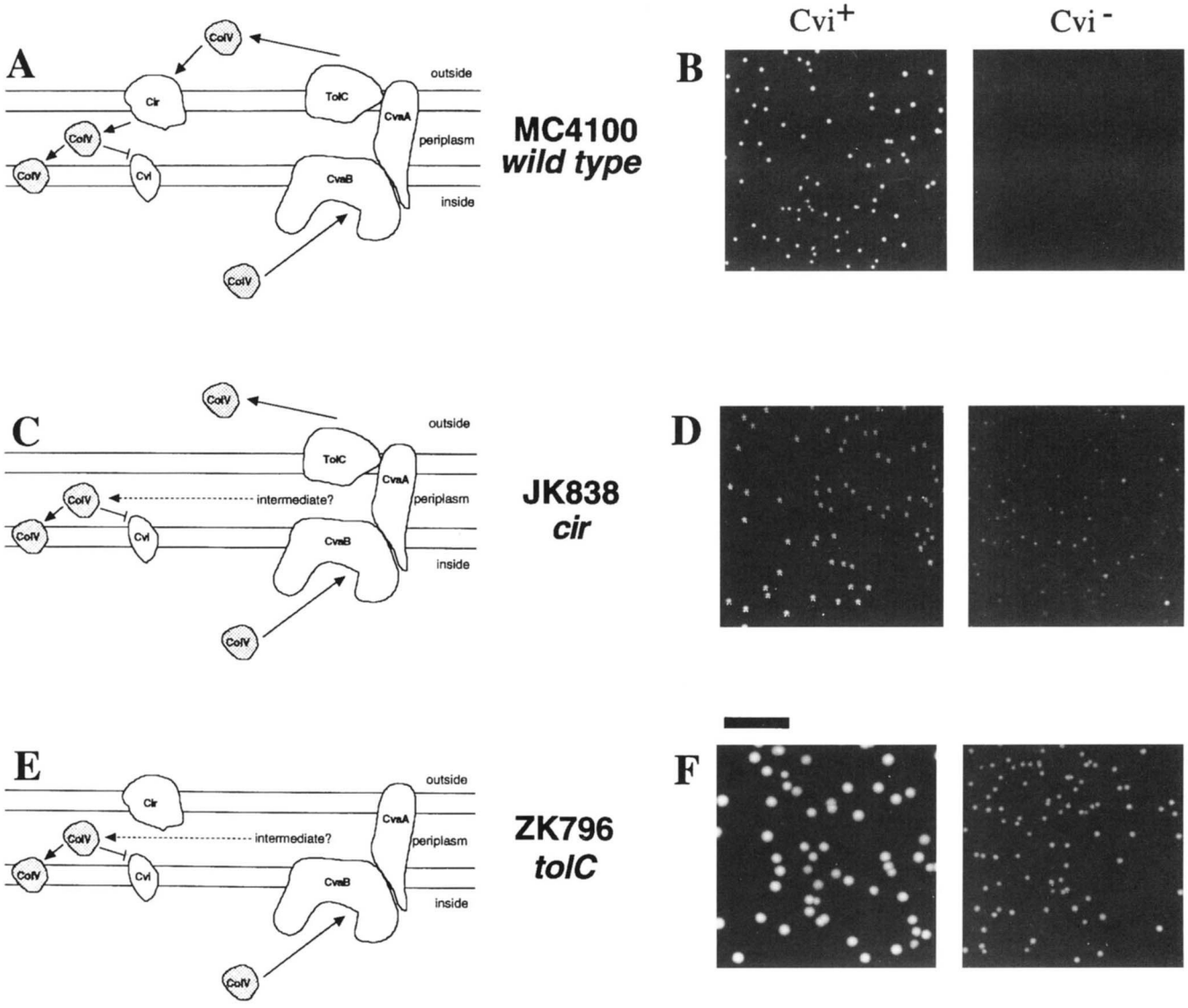

FIGURE 3.—Transformation assay to monitor for periplasmic ColV during secretion. (A) Model for ColV secretion from wild-type MC4100 cells; (B) MC4100 transformation assay results; (C) model for ColV secretion from Cir$^-$ JK838 cells; (D) JK838 transformation assay results; (E) Model for ColV secretion from TolC$^-$ ZK796 cells; (F) ZK796 transformation assay results. See text for details. Scale bar, 6 mm.

ated secretion: We were interested in testing whether there is a detectable periplasmic ColV intermediate during normal CvaAB/TolC-mediated secretion. Using some of the specific features of the ColV system, a transformation assay was designed to monitor for the presence of periplasmic ColV. The assay relies on two observations: ColV is taken up by any cell that is Cir$^+$, including the producer cell (DAVIES and REEVES 1975a), and periplasmic ColV is toxic to any cell that lacks Cvi (see above).

We set out to test the hypothesis that there is no ColV in the periplasm during the secretion process, but that there is periplasmic ColV after uptake through the Cir receptor. If this hypothesis is correct, then strains that are CvaA$^+$, CvaB$^+$, TolC$^+$, CvaC$^+$, and Cir$^+$ should only be viable when they are Cvi$^+$. This is diagrammed in Figure 3A.

As a control experiment, a CvaC$^+$ plasmid was introduced into strains that were CvaA$^+$, CvaB$^+$, TolC$^+$, Cir$^+$ and either Cvi$^-$ (using plasmid pMJF116) or Cvi$^+$ (using plasmid pHK22–6). Because a Cir$^+$ strain was used, ColV could come into the periplasm from at least one route: uptake through the Cir receptor (Figure 3A). Thus every ColV producing cell should have ColV in its periplasm. In this control experiment, we expected CvaC$^+$ transformants from the Cvi$^+$ strain but not from the Cvi$^-$ strain. Figure 3B shows the results of this experiment. As expected, Cir$^+$ cells take up ColV into their periplasm and must be Cvi$^+$ to grow.

If the Cir receptor is removed, then ColV can no longer enter into the periplasm via the uptake pathway. Thus, in a Cir$^-$ strain background, strains that are CvaA$^+$, CvaB$^+$, TolC$^+$, and CvaC$^+$ should require the presence of Cvi only if there is periplasmic ColV during

secretion. This is diagrammed in Figure 3C. If there is no periplasmic ColV during the secretion process, then a Cir$^-$ strain which is CvaA$^+$, CvaB$^+$, TolC$^+$, and CvaC$^+$ should grow well, even when the strain is Cvi$^-$. A CvaC$^+$ plasmid was introduced into Cir$^-$, CvaA$^+$, CvaB$^+$, TolC$^+$strains that were either Cvi$^+$ or Cvi$^-$. Figure 3D shows the results of this experiment. Transformant colonies were obtained for both strains. However, the colonies from the Cvi$^-$ strain were smaller, sickly and translucent, while the colonies from the Cvi$^+$ strain appeared normal. This growth inhibition of the Cvi$^-$ transformants is probably due to the presence of low levels of ColV in the periplasm. The Cvi$^-$ transformants do make extracellular ColV but they fail to form colonies upon restreaking. We interpret these results to mean that, while large amounts of ColV do not accumulate in the periplasm during secretion, there is some level of ColV that can access the periplasmic space and then insert into the cytoplasmic membrane. The possibility that some ColV reenters the cell via a second uptake pathway or through residual Cir due to leakiness is unlikely because the Cir$^-$ mutant used was resistant to the highest level of extracellular ColV (data not shown). In preliminary fractionation experiments, the amount of ColV activity present in the periplasm was below detectable levels (FATH 1993).

The transformation assay was also used to monitor for periplasmic ColV in cells that lack TolC. Using the method described, a CvaC$^+$ plasmid was introduced into Cir$^+$, CvaA$^+$, CvaB$^+$, TolC$^-$ strains that were either Cvi$^+$ and Cvi$^-$. If the *tolC* mutation results in accumulation of periplasmic ColV, then no transformants should be obtained (Figure 3E). The results are shown in Figure 3F. Transformants were obtained from both Cvi$^-$ and Cvi$^+$ strains. Even though the Cvi$^-$ transformants were smaller than the Cvi$^+$ transformants, they grew well upon restreaking. This indicates that in the TolC$^-$ strain there is less ColV in the periplasm than in the TolC$^+$ Cir$^-$ strain (Figure 3D).

Secretion and N-terminal processing of ColV: The ColV primary translation product undergoes processing to remove the N-terminal 15 aa residues (FATH *et al.* 1994). To further dissect the secretion pathway, we assayed N-terminal processing using pulse labeling and immunoprecipitation.

First, processing was monitored in cells carrying the wild-type ColV system. In a 10-sec labeling experiment, the majority of labeled ColV from a mixture of cell extract and supernatant was already in the mature form, and the precursor form (pre-ColV) was barely detectable (Figure 4, lane 3). With longer labeling times (2 min), the labeled ColV was found only in the mature form (Figure 4, lane 2). These results indicate that the processing of ColV occurs rapidly in wild-type cells.

The N-terminal processing was next tested in a variety of mutant strains. These included strains that lacked CvaAB (ZK434), CvaA (ZK508), CvaB (ZK509), or TolC

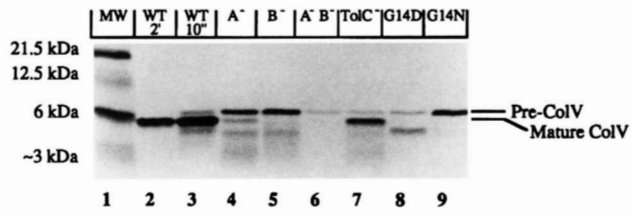

FIGURE 4.—Processing of ColV: kinetics and mutant analysis. SDS-PAGE gels show immunoprecipitated products from various ColV derivatives. Lane 1, molecular weight standards; lane 2, 2-min pulse-labeled ColV; lane 3, 10-sec pulse-labeled ColV; lane 4, 2-min pulse-labeled ColV from *cvaA* mutant strain; lane 5, 2-min pulse-labeled ColV from *cvaB* mutant strain; lane 6, 2-min pulse-labeled ColV from *cvaAB* double mutant; lane 7, 2-min pulse-labeled ColV from *tolC* mutant strain; lane 8, 2-min pulse-labeled ColV from *cvaC* G14D mutant strain; lane 9, 2-min pulse-labeled ColV from *cvaC* G14N mutant strain.

(ZK796). When both CvaA and CvaB were absent, ColV could only be detected in the precursor form (Figure 4, lane 6). In addition, the overall level of labeled ColV was much lower compared with the other strains tested. This suggests that pre-ColV is unstable in the absence of CvaA and CvaB. This is consistent with previous data showing that internal ColV activity is unstable in export deficient strains (FATH *et al.* 1992).

In the CvaB$^-$ strain, the labeled ColV was almost entirely found as pre-ColV (Figure 4, lane 5). In the CvaA$^-$ strain, the labeled ColV was also predominantly in the precursor form, but some processed ColV could be detected (Figure 4, lane 4). Both the CvaB$^-$ and CvaA$^-$ strains had no detectable extracellular ColV activity. It is interesting that some processing occurs in the CvaA$^-$ strain because it suggests that CvaB is sufficient for processing. These results show that the CvaAB exporter is necessary for processing to occur, and CvaB appears to have a more critical role than CvaA. They also show that N-terminal processing is an intermediate step in secretion and that other steps are required after processing to complete the secretion process.

The effect of a *tolC* mutation on ColV processing was tested next. In the TolC$^-$ strain, most of the labeled ColV is processed, and only a small amount of pre-ColV is present (Figure 4, lane 7). Processing in the TolC mutant appears to be similar to the wild type except slightly slower.

Last, processing of ColV was tested in strains that carry missense mutations in the ColV N-terminal export signal (GILSON *et al.* 1990). Two mutations, G14D and G14N, were constructed previously and shown to have internal ColV activity but have greatly reduced extracellular activity (GILSON *et al.* 1990; FATH *et al.* 1991). The two mutants are differentially affected in processing (Figure 4, lanes 8 and 9). While G14N is not processed at all, G14D appears to be incorrectly processed to yield a smaller band. Similar incorrect processing may be occurring for wild-type ColV in the CvaB and CvaA mu-

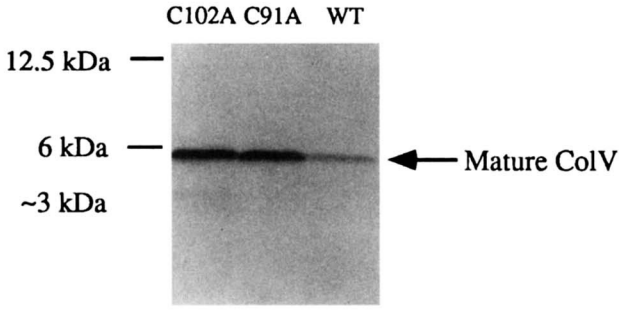

FIGURE 5.—Extracellular secretion of ColV Cys to Ala mutants. SDS-PAGE showing immunoprecipitated *cvaC* Cys to Ala mutant proteins. Lane 1, 2-min pulse-labeled supernatant from *cvaC* C102A; lane 2, 2-min pulse-labeled supernatant from *cvaC* C92A mutant strain; lane 3, 2-min pulse-labeled supernatant from wild-type ColV.

tants (Figure 4, lanes 4 and 5) and even to a very small extent in the presence of the wild-type exporter (Figure 4, lane 3, 10-sec pulse). This indicates that the size and charge of the residues near the cleavage site of pre-ColV are critical for proper processing and extracellular secretion.

Point mutations in the C-terminal cysteines abolish ColV activity but do not interfere with secretion. There are two cysteine residues at positions 91 and 102 of pre-ColV, which may play a role in ColV secretion and/or activity. To test this hypothesis, these residues were individually changed to alanine using site-directed mutagenesis, and the mutants were characterized. Each mutant was completely inactive for ColV activity. But, each mutant was processed and secreted normally from the cell in the presence of CvaAB, and immunoprecipitation experiments show that these mutant ColV proteins are the same molecular weight as wild-type ColV (Figure 5). This was confirmed by Western analysis of concentrated culture supernatants (data not shown). We previously showed that ColV-1, a C-terminal deletion derivative of ColV, could be secreted normally but had lost all biological activity (FATH *et al.* 1994). These point mutants have the same properties and further support the hypothesis that the C-terminal region of ColV is critical for function but not involved in secretion.

DISCUSSION

In this study, we have carried out a number of related experiments to characterize the ColV system and its secretion by the Sec-dependent pathway and by its natural exporters, CvaAB/TolC. Our results show that the action of Cvi is not required when ColV remains localized to the cytoplasm. This demonstrates that ColV can only exhibit its bactericidal activity when presented from outside the inner membrane. Similar results have been observed for the colicin M protein, but that colicin has a different mode of action, it inhibits peptidoglycan synthesis (HARKNESS and BRAUN 1990). We also showed that the Sec-dependent pathway can partially substitute for the ColV exporter to secrete ColV across the inner membrane. Thus, there are no structures present in ColV incompatible with the Sec export machinery. However, to export ColV from the cytoplasm all the way into the medium, the dedicated export system is necessary.

We have made use of the genetic properties of the ColV system to devise a novel genetic assay to monitor for periplasmic ColV. Results from this genetic assay indicate that a small amount of ColV is present in the periplasm during secretion. This is the first evidence suggesting a periplasmic intermediate in the secretion of substrates through an ABC-exporter. The results also suggest that the biochemical analysis used for HlyA may not be as sensitive as the genetic assay described here. Biochemical fractionation methods have limits on how well they can separate subcellular components and are thus incapable of detecting small amounts of periplasmic intermediates. Our genetic assay does not depend on the biochemical isolation of protein, but on the bactericidal activity of ColV and thus may provide a more sensitive indicator of periplasmic ColV. Because ColV appears to form channels in lipid bilayers (FATH 1993), even a single ColV molecule may be enough to inhibit the growth of a sensitive cell (DAVIDSON *et al.* 1984).

The immunoprecipitation results show that processing of ColV in the wild-type system occurs rapidly and is dependent on the CvaAB exporter. CvaB is required for both N-terminal processing and extracellular secretion. CvaA is also required for secretion and is important, though not essential, for processing. In a TolC$^-$ background, nearly all of the ColV is processed. Thus, ColV processing occurs after interaction with CvaAB but before interaction with TolC, most likely in the inner membrane or within the CvaAB export complex. Some possible mechanisms for ColV N-terminal processing were discussed previously (FATH *et al.* 1994). More recently, it was demonstrated that the N-terminal processing of the related bacteriocin, lactococcin G, is meditated by its cognate ABC exporter LagD (HÅVARSTEIN *et al.* 1995).

The HlyA pathway can be divided into early and late steps based on differences in energetic requirements (KORONAKIS *et al.* 1991). The ColV pathway can also be divided into early and late steps based on a separate criterion: the processing of pre-ColV that occurs during secretion. Thus, while the ColV system has much in common with HlyA, its unique features make it a valuable model for understanding the mechanisms of ABC-protein mediated export.

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