Purification and Characterization of Colicin V from *Escherichia coli* Culture Supernatants†

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ABSTRACT: The peptide antibiotic, colicin V (ColV), has been purified and characterized from *Escherichia coli* culture supernatants by precipitation with trichloroacetic acid (TCA) and high-performance liquid chromatography (HPLC). Polyacrylamide gel electrophoresis (PAGE) and Western analysis identifies ColV as a polypeptide with an apparent molecular mass of 5.8 kDa. The protein identified remains biologically active after purification and SDS–PAGE. A mutant form of ColV, ColV-1, removes the carboxy-terminal 21 amino acids and replaces them with eight heterologous residues. The ColV-1 mutant is also secreted into the extracellular medium, demonstrating that the carboxy-terminal 21 amino acids are not required for secretion by the dedicated ColV export system, CvaAB/TolC. N-Terminal amino acid sequencing shows that the primary translation product of *cvaC*, the ColV structural gene, is processed to remove the N-terminal 15 amino acids. The cleavage site is preceded by the sequence Ser-Gly-Gly, making it a potential substrate for leader peptidase. The ColV leader sequence has many characteristics in common with the amino-terminal leader sequences of the lactococcins, lactacins, and pediocins from Gram-positive bacteria. Mass spectroscopy of purified ColV shows that it has a mass of 8741.0 amu, consistent with the mass of the unmodified 88 amino acid polypeptide. The purification scheme provides a rapid and simple way to obtain ColV for further biochemical analysis.

Colicin V (ColV)[1] is a ribosomally synthesized peptide antibiotic encoded in large, low-copy-number plasmids of *Escherichia coli* and other closely related Enterobacteriaceae (Fath et al., 1992; Kolter & Moreno, 1992). ColV inhibits cellular growth by disrupting the membrane potential of target cells, probably by inserting into their inner membrane (Yang & Konisky, 1984). Four plasmid genes were cloned and sequenced and shown to be required for ColV production, secretion, and immunity (Gilson et al., 1987, 1990). The *cvaC* gene encodes a 103 amino acid ColV precursor (pre-ColV), *cvaA* and *cvaB* encode components of a dedicated secretion system required for the extracellular secretion of the colicin, and *cvi* encodes a 78 amino acid protein that provides immunity against the bactericidal activity of ColV.

Several genetic approaches have been used to characterize the ColV system. *cvaC–phoA* gene fusions and point mutations in *cvaC* were used to localize the export domain in pre-ColV to the N-terminal 39 amino acids (Gilson et al., 1990). Functional complementation studies showed that ColV could be secreted by heterologous secretion systems such as the α-hemolysin and *Erwinia* protease exporters (Fath et al., 1991). But, to date, no structural characterization of the ColV protein has been reported.

ColV structure is of interest because it is a peptide antibiotic secreted by a dedicated ABC-export system (Higgins, 1992; Fath & Kolter, 1993). Many ribosomally synthesized peptide antibiotics undergo posttranslational modifications (Jung, 1991; Kolter & Moreno, 1992), and several proteins secreted by dedicated ABC exporters are known to be posttranslationally modified (Delepelaire & Wandersman, 1989; Issartel et al., 1991). The α-hemolysin protein is modified by the addition of a fatty acid moiety, and the *Erwinia* proteases undergo N-terminal processing even though they have C-terminal secretion signals. Thus, posttranslational modifications of ColV could potentially explain its use of a dedicated exporter for its secretion.

In this report, we describe the purification of ColV from *E. coli* culture supernatants and several biochemical analyses that have been carried out on the purified protein. We show that biologically active ColV runs on SDS–PAGE with an apparent molecular mass of 5.8 kDa and that the pre-ColV is processed to remove the N-terminal 15 amino acids, resulting in an 88 amino acid mature peptide. A truncated ColV derivative, ColV-1, is also secreted into the extracellular medium, showing that the C-terminal region of ColV is not required for extracellular secretion. Mass spectroscopy shows that the purified ColV protein has an observed molecular weight of 8741.0, consistent with the predicted molecular mass of the unmodified 88 amino acid mature ColV peptide. ColV appears to be most similar in sequence and structure to the lactococcins and other class II peptide bacteriocins from Gram-positive lactic acid bacteria (Klaenhammer, 1993).

MATERIALS AND METHODS

Media and Culture Conditions. E. coli strains harboring ColV plasmids were grown in tryptone broth (TB) (Miller, 1972). ColV production was induced by addition of the iron chelator 2,2′-dipyridyl (Sigma) at 0.1 mM since the expression of the ColV genes is repressed by excess iron. Except where

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[1] Abbreviations: ColV, colicin V; TCA, trichloroacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TB, tryptone broth; KLH, keyhole limpet hemocyanin; MALDI-TOF, matrix-assisted laser-desorption ionization time-of-flight.

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Table 1: Bacterial Strains and Plasmids Used in This Study

strain or plasmid	genotype or description	ref
strains		
MC4100	*ΔlacU169 araD139 rpsL150 thi recA1 deoC7 flbB5301 ptsF25 relA1*	Casadaban, 1976
71-18	*Δ(lac-proAB) thi supE/F' lacI^q lacZΔM15*	Yanisch-Perron et al., 1985
plasmids		
pHK11	pBR322 containing the cloned ColV operons *cvaAB* and *cvi/cvaC*; Ap^R	Gilson et al., 1987
pHK22	pACYC184 containing the cloned ColV operons *cvaAB* and *cvi/cvaC*; Cm^R	Gilson et al., 1987
pHK11-1	pHK11 with Tn5 inserted into the 82nd codon of *cvaC*; Ap^R Km^R	Gilson et al., 1987
pLY21	a deletion derivative of pHK22 that is *cvaC$^+$*, *cvi$^+$*; Cm^R	Gilson et al., 1990
pLY11	a deletion derivative of pHK11 that is *cvaA$^+$*, *cvaB$^+$*; Ap^R	Gilson et al., 1990

noted, antibiotics were used at the following final concentrations: ampicillin, 150 μg/mL; chloramphenicol, 20 μg/mL; kanamycin, 25 μg/mL.

Bacterial Strains and Plasmids. The *E. coli* strain used throughout these experiments was MC4100. Plasmid pHK11 contains the ColV operon cloned into pBR322 (Gilson et al., 1987). pHK11-1 is pHK11 with a Tn*5* insertion in *cvaC* (Gilson et al., 1987). pLY11 is a derivative of pHK11-1 deleted for *cvaC* and *cvi* (Gilson et al., 1990). pLY21 is a derivative of pHK22 (the ColV operon in pACYC184) that contains *cvaC* and *cvi* but which lacks the export proteins *cvaAB* (Gilson et al., 1990). See Table 1 for more details.

Generation of Antibodies to a ColV-Derived Synthetic Peptide. A synthetic peptide was generated that contained residues 41–68 predicted from the *cvaC* nucleotide sequence. The sequence used was as follows: CAGGVAGGAIYDY-ASTHKPNPAMSPSGLG. The peptide was sent to East Acres Biologicals (Southbridge, MA), where it was conjugated to the carrier protein KLH (keyhole limpet hemocyanin) and used for generating antibodies. Preimmune and immune antisera were obtained and used for immunological detection of ColV by Western blot. The polyclonal antibodies obtained were specific for ColV (see Results) and inactivated ColV bactericidal activity.

TCA Precipitations. Strains carrying ColV derivatives were grown overnight in TB with 0.1 mM dipyridyl and antibiotics, diluted 1:10 in fresh medium, and grown for 3 h to late log phase. Supernatants were removed, filter sterilized, and treated with trichloroacetic acid (TCA) at a final concentration of 10%. Samples were incubated on ice for 45 min and then centrifuged at 30000*g* for 15 min. Supernatant was removed and the precipitate was resuspended in 1/225th of the original volume of 3.5 M Tris base (pH 9.5) to neutralize the TCA. Bioassays were done on samples before and after precipitation to determine yield.

ColV Bioassays. ColV activity from culture supernatants was quantitated using the critical dilution method (Mayr-Harting et al., 1972). Twofold serial dilutions of supernatants (in 10-μL aliquots) were spotted onto a lawn of sensitive 71-18, and the highest dilution which still showed bactericidal activity was recorded as the critical dilution. ColV activity present in gels after SDS–PAGE was monitored by soaking the gel in M63 glucose for 1–2 h and then placing the gel onto an M63 glucose plate. The gel was then overlayed with *E. coli* strain 71-18 in H-top agar (Miller, 1972) and incubated overnight. The region of the gel containing active ColV is determined by looking for inhibition of growth.

SDS–Polyacrylamide Gel Electrophoresis. TCA-precipitated samples were diluted with an equal volume of 2× Laemmli buffer (Laemmli, 1970), heated at 42 °C for 15 min, and then loaded on a 20% SDS–polyacrylamide gel optimized for small proteins (Thomas & Kornberg, 1978). The separating gel had a final composition of 20% acrylamide, 0.1% bis(acrylamide), 0.1% SDS, and 0.5 M Tris-HCl, pH 8.8. The stacking gel had a final composition of 5% acrylamide, 0.25% bis(acrylamide), 0.1% SDS, and 0.12 M Tris HCl, pH 6.8.

Membrane Transfer and Western Blots. Proteins were transferred from polyacrylamide gels onto modified PVDF membranes (ProBlot, Applied Biosystems) for Western blot and N-terminal sequencing analyses using methods described in the ProBlot instruction sheets. Western analyses were carried out using standard techniques (Ausubel et al., 1987).

N-Terminal Amino Acid Sequencing. ProBlot membrane containing the ColV protein band stained with Coomassie Brilliant Blue was cut out and sequenced using a 477A automated sequencer (Applied Biosystems) and following the protocol of Tempst and Riviere (1989).

High-Performance Liquid Chromatography (HPLC). The C-18 column (0.46 × 25 cm) was from Vydac. The column was run at constant temperature (36 °C) and constant flow rate (0.5 mL/min). Solvents used were as follows: A = 0.6% trifluoroacetic acid; B = 0.054% trifluoroacetic acid/80% acetonitrile. The gradient conditions were as follows: 5% B for 5 min; 5–50% B in 22.5 min; 50–100% B in 12.5 min. Absorbance was monitored at λ 214 and 277 nm.

Mass Spectroscopy. Peptides were analyzed by matrix-assisted laser-desorption time-of-flight mass spectroscopy (MALDI-TOF) (Chait & Kent, 1992; Scoble et al., 1993) using a Fisons/VG TofSpec instrument. Samples or standards were mixed with equal volumes of a saturated solution of α-cyano-4-hydroxycinnamic acid in 0.05% trifluoroacetic acid and 50% acetonitrile directly on targets, dried, and analyzed in positive ion mode. The instrument was calibrated with mixtures of gramicidin S (1142.0 Da) and bovine insulin (5734.5 Da) immediately before collecting data for peptide samples. Thirty spectra of each sample were collected and signal-averaged before analysis.

RESULTS

Active ColV Can Be Obtained from Culture Supernatants by TCA Precipitation. Using the protocol described above, we were able to obtain highly concentrated supernatant fractions after precipitation in 10% TCA and resuspension in Tris base. Fractions were obtained from supernatants of strains carrying the wild-type ColV genes on plasmid pHK11 and found to have high levels of bactericidal activity (see Figure 1). Levels of ColV activity were quantitated using the critical dilution method (Mayr-Harting et al., 1972). Typically, ColV supernatants had a critical dilution of 64, while concentrated ColV fractions had a critical dilution of 7200. These data show that ColV activity could be concentrated 225 times by TCA precipitation with approximately 50% yield.

Similar methods were used to precipitate and concentrate culture supernatants from three strains carrying various mutations in the ColV genes: pHK11-1 (*cvaC*::Tn*5*), pLY11 (Δ*cvaC*, Δ*cvi*), and pLY21 (Δ*cvaAB*). These samples were

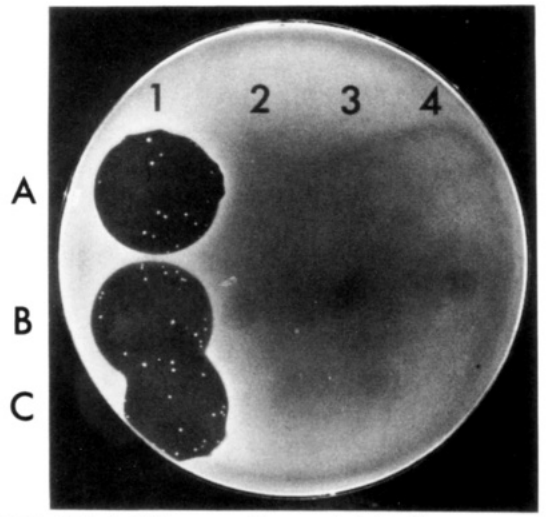

FIGURE 1: Colicin V bioassays of concentrated culture supernatants from various colicin V derivatives. Column 1, pHK11 (*cvaC* wild type); column 2, pHK11-1 (*cvaC*::Tn*5*); column 3, pLY11 (Δ*cvaC*, Δ*cvi*); column 4, pLY21 (Δ*cvaAB*). Row A, supernatant precipitated with 10% TCA and resuspended in Tris base 225× concentrated; row B, concentrated supernatants mixed with an equal volume of 2× Laemmli buffer; row C, a 1:20 dilution of samples from row B.

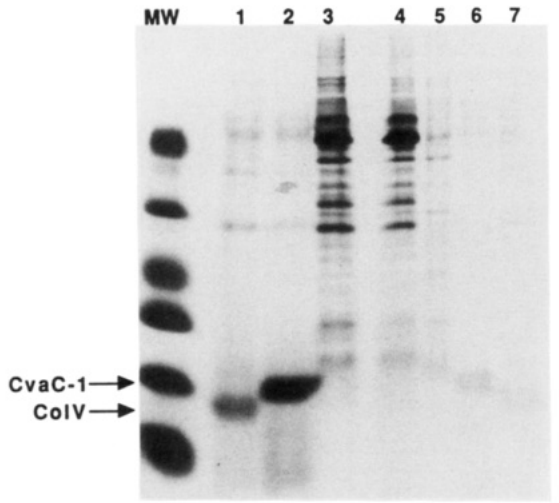

FIGURE 2: Coomassie-stained gel containing culture supernatants from various colicin V derivatives. MW: molecular mass standards from top to bottom are 45.5, 29.5, 18.7, 15.5, 5.9, and 2.9 kDa. Lanes 1–3 contain supernatant preparations from one experiment. Lanes 4–7 contain preparations from an independent experiment. Concentrated supernatants are from pHK11 (lanes 1 and 7), pHK11-1 (lanes 2 and 6), pLY11 (lanes 3 and 4), and pLY21 (lane 5).

all found to have no bactericidal activity on assay plates, demonstrating that the activity observed from pHK11 culture supernatants is indeed the colicin (Figure 1).

SDS–PAGE Analysis of Culture Supernatants. Concentrated culture supernatants from the four strains were diluted with equal volumes of 2× Laemmli buffer for SDS–PAGE. The ColV in this buffer remained biologically active (Figure 1). Samples were run on a 20% acrylamide gel and stained with Coomassie Brilliant Blue. Results of two independent preparations are shown in Figure 2. Supernatants from cultures containing pHK11 (lanes 1 and 7) show a prominent band with an apparent molecular mass of 5.8 kDa corresponding to ColV, as well as a few higher molecular mass bands. Surprisingly, a prominent band with an apparent molecular mass of 7.3 kDa was detected in supernatants from strains carrying pHK11-1, which contains the *cvaC* gene inactivated by insertion of Tn*5* (lanes 2 and 6). This suggests that an inactive ColV product is made and secreted from cells

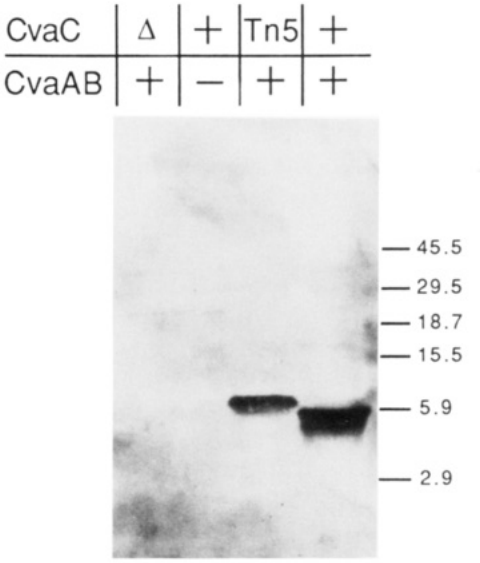

FIGURE 3: Western blot of ColV culture supernatants. Concentrated supernatants are from pLY11 (lane 1), pLY21 (lane 2), pHK11-1 (lane 3), and pHK11 (lane 4).

carrying pHK11-1. This protein, called ColV-1, is discussed in more detail below. Supernatants from strains expressing only *cvaAB* from pLY11 (lanes 3 and 4) did not contain proteins the size of ColV but did contain a surprisingly large amount of proteins not present in supernatant fractions from pHK11 and pHK11-1. Most prominent was a protein that runs at 47.9 kDa, which could be the CvaA protein overexpressed from pLY11. We do not yet know the identity of these proteins, but it may be possible to purify some of them and determine their N-terminal sequence. This would allow us to determine if they are ColV or plasmid related. Lastly, supernatant from strains carrying pLY21 (expressing only *cvaC* and *cvi*) was characterized (lane 5), and it also did not contain protein bands corresponding in size to ColV.

Western Blot of Gel Containing ColV Supernatant Fractions. To identify ColV-related protein bands, culture supernatant fractions were run on 20% SDS–PAGE gels, and the proteins were transferred onto ProBlot. The membranes were blotted with polyclonal anti-ColV-peptide antibodies. Both preimmune and immune antisera were tested. Preimmune antiserum did not cross-react with any low-molecular-mass proteins (data not shown). In contrast, the immune antisera specifically cross-reacted with the 5.8-kDa band from pHK11 supernatants and with the 7.3-kDa band from pHK11-1 supernatants (Figure 3). This immunologically identifies ColV as the 5.8-kDa peptide seen on the Coomassie-stained gel and also demonstrates that ColV-1 is a secreted ColV derivative. No protein bands from pLY11 or pLY21 lanes cross-reacted with the anti-ColV antisera, demonstrating that there is no extracellular ColV in a *cvaC* deletion (pLY11) or in a strain lacking the *cvaAB* export genes (pLY21) (Figure 3, first two lanes). This also shows that the higher molecular mass proteins observed in culture supernatants from Figure 2 are not ColV-related.

Bactericidal Activity of ColV Run on SDS–PAGE. Since ColV activity was observed in the supernatant samples even in the presence of Laemmli buffer, we thought that it might be possible to observe the bactericidal activity of ColV even after SDS–PAGE. This could be accomplished by running concentrated culture supernatants from pHK11 on 20% SDS–PAGE, then placing the gel on an M63-glucose agar plate and overlaying the gel with H-top agar containing the indicator strain 71-18. If ColV remained active, then a zone of growth

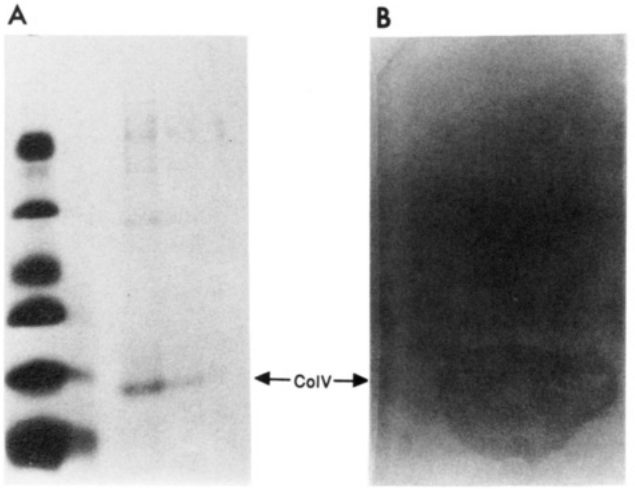

FIGURE 4: SDS–PAGE of supernatants containing ColV. The left gel (A) was stained with Coomassie Brilliant Blue. The right gel (B) was overlaid with indicator strain 71-18 to show the area of growth inhibition.

inhibition should appear around the area of the gel where the ColV migrated. This could be used as a way to correlate proteins on the gel with ColV activity (Bhunia et al., 1987).

A gel was run with two sets of concentrated pHK11 supernatants. One half of the gel was stained with Coomassie Brilliant Blue, and the other half was overlayed onto a bioassay plate with 71-18 and incubated overnight. The results are shown in Figure 4. A large zone of growth inhibition appeared at the bottom of the bioassay gel corresponding to the region of the gel where ColV was observed to run. Thus, the ColV observed by Coomassie staining and Western analysis was also biologically active.

pHK11-1 Produces a Truncated Form of ColV That Is Secreted into the Medium. The original pHK11-1 mutant was isolated as a Tn*5* insertion which abolished cell-associated and extracellular ColV activity (Gilson et al., 1987). The insertion mutation was subsequently sequenced, and the Tn*5* was found to interrupt the 82nd codon of *cvaC*. In this biochemical analysis, pHK11-1 supernatant was intended to be used as a ColV$^-$ negative control. Thus, ColV-1 is produced and secreted into the medium, and this mutant protein can be detected by the polyclonal antibody (Figure 3).

The predicted sequence differences between wild-type ColV and ColV-1 proteins are shown in Figure 5. Specifically, the C-terminal 21 amino acids of ColV are removed and replaced with a heterologous 8 amino acid C-terminal tail. The predicted and observed molecular weights of the two proteins are also shown in Figure 5. The observed mobility of ColV-1 matches well with the predicted MW. But surprisingly, wild-type ColV, which is predicted to run as an 8.7-kDa protein, migrates with an apparent molecular mass of only 5.8 kDa, 33% smaller than predicted. The aberrant mobility of the ColV as compared to ColV-1 suggested that ColV might contain posttranslational modifications resulting in a more compact final structure and causing the protein to run faster on SDS–PAGE. Alternatively, wild-type ColV may not be fully denatured by SDS. At least one other peptide antibiotic, lactacin F, shows similar aberrant mobility on SDS–PAGE (Muriana & Klaenhammer, 1991).

N-Terminal Amino Acid Sequence Analysis of ColV. Previous results with the ColV-PhoA fusion protein suggested that ColV may undergo N-terminal processing in the presence of CvaAB (Gilson et al., 1990). Once the ColV band was identified on SDS–PAGE and Western blot, it was possible

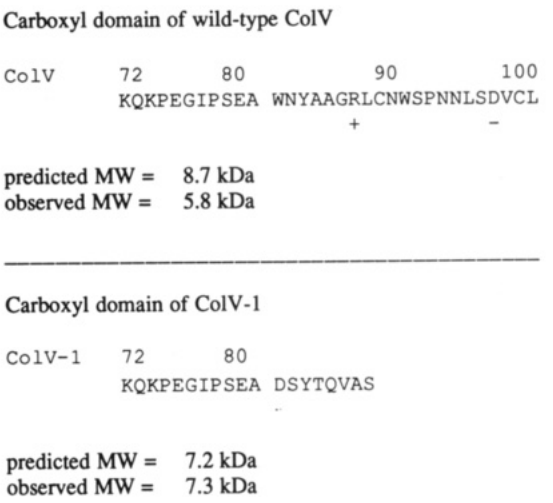

FIGURE 5: Comparison of the C-terminal regions of wild-type ColV and the truncated form found to be secreted from cells carrying plasmid pHK11-1. The predicted molecular weights are based on sequence analysis of the two genes and take into account the fact that the N-terminal 15 amino acids of pre-ColV are processed and are not present in the mature proteins (see Figure 6).

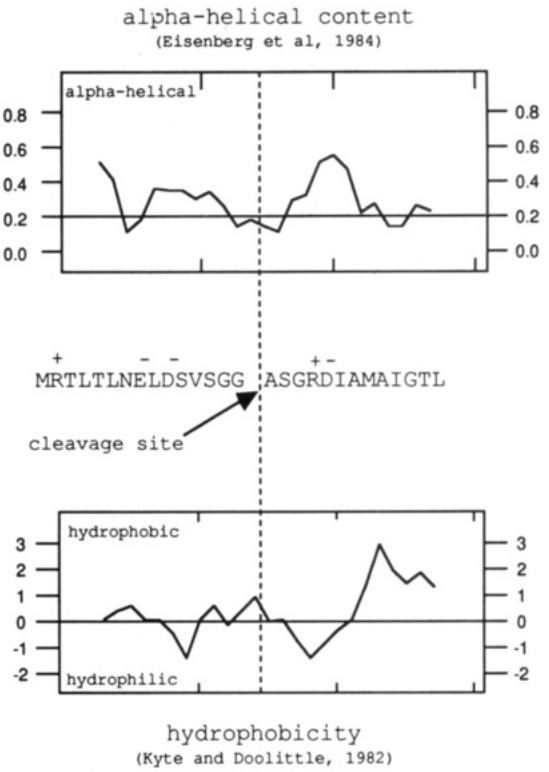

FIGURE 6: Site of N-terminal processing in pre-ColV and predicted α-helical content and hydrophobicity of the region.

to test this hypothesis directly. The ColV band was transferred onto ProBlot, cut out, and then subjected to N-terminal sequence analysis. The sequence of the first 13 amino acids of the protein band was determined, and the results of this analysis are shown in Figure 6. The N-terminal amino acid sequence demonstrates that the primary translation product of *cvaC* is a precursor, pre-ColV, that is processed to remove the first 15 amino acids. Thus, the processed ColV molecule is an 88 amino acid polypeptide with a predicted MW of 8.7 kDa. In addition, the processed ColV mutant, ColV-1, should be 75 amino acids long with a predicted molecular mass of 7.2 kDa (see Figure 5).

There are several interesting features about the site where proteolytic cleavage occurs. Secondary structure analysis was

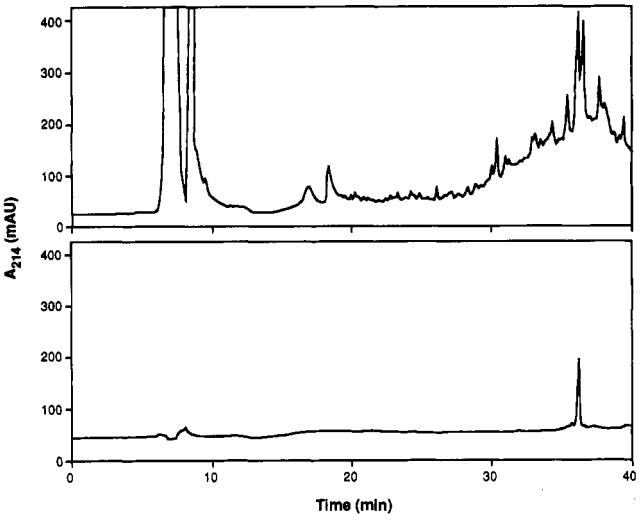

FIGURE 7: Reverse-phase HPLC elution profiles of a ColV preparation. The top profile is of TCA-precipitated ColV. ColV activity elutes between 36 and 37 min. The bottom profile was obtained by immediately developing the column with the same gradient but without injecting further sample. Again, ColV activity elutes between 36 and 37 min.

carried out on the N-terminal region of pre-ColV using the methods of Chou and Fasman (1978) and of Eisenberg et al. (1984) (see Figure 6). This analysis suggests that the leader sequence residues 1–12 are mostly α-helical and are followed by a large β-turn that spans from amino acids 13 to 19. Thus, the cleavage site is probably in a turn region, making it accessible to a peptidase. Notably, the pre-ColV leader sequence has no observable similarities with the N-terminal extensions found on the *Erwinia* protease zymogens (Figure 9) (Delepelaire & Wandersman, 1989). In addition, the leader sequence has a net negative charge, making it a very poor substrate for the Sec-dependent export system (Figure 6).

The pre-ColV leader sequence shares a common predicted secondary structure with the leader sequences of many other peptide antibiotics (Kolter & Moreno, 1992). It has the most in common with a subset of these, the leader sequences from the lactococcins (Klaenhammer et al., 1992; Klaenhammer, 1993). Colicin V, lactococcins A, B, and M, lactacins A and F, and pediocin PA-1 all have leader sequences 15–21 residues long which are cleaved immediately following the sequence Gly-Gly.

Further Purification of ColV through HPLC. ColV was further purified from concentrated culture supernatant using C-18 reverse-phase HPLC. During purification, two major peaks elute at a concentration of about 40% acetonitrile (Figure 7, top). The fraction containing these peaks contains ColV activity. Interestingly, activity is not limited to this fraction, but is also seen at lower levels in all samples after this one.

After the first HPLC run, when the column was developed with the same gradient but without injecting more sample, a single large peak was observed (Figure 7, bottom). The peak appeared at the same position as one of the peaks associated with ColV activity in the first run and contained about 40% of the ColV activity found in the first HPLC run. It appears that active ColV adsorbs to C-18 columns, and a large fraction of this protein remains bound even after the first elution. This provides a simple method for purifying biologically active ColV to homogeneity since the profile from the second HPLC run shows only one peak.

Mass Spectroscopy. Using the HPLC-purified ColV protein, it was possible to test if ColV undergoes posttranslational modifications beyond N-terminal processing. Several

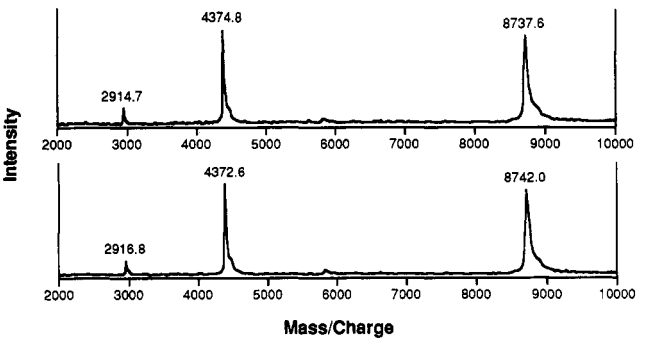

FIGURE 8: Mass spectrometric analysis of ColV. The top spectrum shows the raw data. The bottom spectrum shows the data after smoothing and averaging. The mass of 8742.0 Da identified by the peak in the bottom spectrum corresponds to the observed mass of ColV, 8741.0 plus 1.0 Da for the proton added to the molecule during analysis. The species at 4372.6 and 2916.8 Da correspond to doubly and triply protonated ColV, respectively.

lines of evidence suggested that it might. Many peptide antibiotics contain posttranslational modifications (Kolter & Moreno, 1992), and the aberrant mobility of ColV on SDS–PAGE suggested an unusually compact structure. We could determine if chemical groups have been added or removed by analyzing ColV using mass spectroscopy and comparing the observed mass with the predicted mass.

HPLC-purified ColV was analyzed by mass spectroscopy, and a typical spectrum in shown in Figure 8. This result shows that ColV has a mass of 8741.0 ± 17.5 Da. The calculated mass of the 88 residues present in ColV is 8735.8 Da, well within the range of the experimentally determined value. These results strongly suggest that ColV is not modified by the covalent addition of fatty acid groups or other large moieties like other ABC-protein substrates. However, the mass accuracy of the MALDI-TOF method cannot rule out low-mass modifications such as methylation.

DISCUSSION

Purification of ColV. The protocol described for ColV purification provides a rapid and simple way to obtain relatively high amounts of active colicin V from culture supernatants. Quantitation of protein concentration by Bradford assay showed that pure protein (Figure 7, bottom) had a concentration of about 50 μg/mL (6 nmol/mL). This fraction had a critical dilution of about 100, resulting in an estimated specific activity of 200 antibiotic units/μg of protein.

N-Terminal Processing. The N-terminal sequencing results show that extracellular ColV is processed to remove the N-terminal 15 amino acids from the primary translation product. Previous experiments had shown that ColV-PhoA fusions changed mobility in the presence of CvaAB and suggested that ColV underwent N-terminal processing concomitant with export (Gilson et al., 1990). Taken together, these two results suggest that the N-terminal processing requires the presence of the CvaAB exporters. Several testable models could be made to explain CvaAB-dependent N-terminal processing: (1) ColV could be processed autocatalytically, but only after secretion. It has been shown that another ABC-protein substrate, *Erwinia* protease B, has its N-terminal leader sequence removed autocatalytically (Delepelaire & Wandersman, 1989). (2) The CvaAB exporters could have a "leader-peptidase" activity and cleave ColV as it is transported. There is no evidence that CvaAB has protease activity, but this possibility cannot be ruled out. (3) A cellular peptidase might carry out the N-terminal processing. One

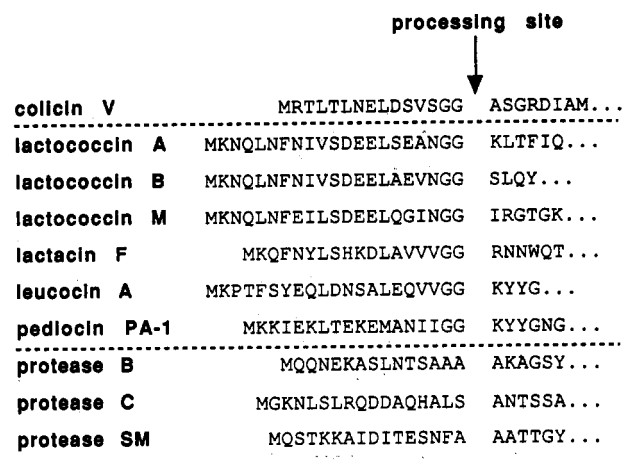

FIGURE 9: Comparison of the cleaved leader sequences from colicin V, the lactococcins, and the proteases (Kolter & Moreno, 1992; Klaenhammer et al., 1992; Delepelaire & Wandersman, 1989). Colicin V shares several features with the lactococcin family (see text).

candidate peptidase is leader peptidase which cleaves following the sequence S-X-S↓, where S could be any small uncharged amino acid (Gly, Ala, Ser) and X is any amino acid (Dalbey, 1991). The ColV cleavage site, Ser-Gly-Gly↓, meets this criterion. Leader peptidase usually cleaves proteins after translocation by the Sec pathway, but it is possible for leader peptidase to act independently of Sec to cleave other translocated molecules such as the M13 procoat protein (Dalbey, 1991). Recently though, it was shown that mutations in the Gly at position −2 of lactacin F abolish lacticin activity (Fremaux & Klaenhammer, 1993). This result argues against a role of leader peptidase, at least in lactic acid bacteria. Experiments are underway in our laboratory to distinguish among the possible models.

The ColV C-Terminal Region Is Not Necessary for Secretion. Previously, *cvaC–phoA* fusions had been used to localize the export signals in ColV to the N-terminal 39 amino acids of the protein (Gilson et al., 1990). However, the resulting fusion proteins were not secreted into the medium and remained embedded in a protease-resistant membrane fraction (Gilson et al., 1990). Thus, the possibility remained that there were some signals in the C-terminal region of ColV that were required for complete extracellular secretion. This possibility was supported by the observation that several of the other ABC-protein substrates (α-hemolysin and *Erwinia* proteases) utilize C-terminal export signals (Fath & Kolter, 1993). The fact that ColV-1 is secreted demonstrates that the C-terminus of ColV is not required for its extracellular secretion. It also shows that heterologous peptide sequences can be substituted at the C-terminus of ColV and that these peptides can be secreted by the CvaAB/TolC export system.

Similarity of Cleavage Sites of ColV and the Lactococcins. The cleavage sites for ColV and several other peptide antibiotics and ABC-protein substrates were compared, and the results are shown in Figure 9. ColV was found to share many features with the lactococcins and related bacteriocins found in Gram-positive bacteria (Klaenhammer, 1993). These features include the following: (a) a cleaved leader sequence 15–21 amino acids in length; (b) the N-terminal sequence: Met-(Lys/Arg); (c) a predicted α-helical structure over most of the leader sequence; (d) a negatively charged region in the middle of the leader sequence; (e) a predicted β-turn that begins 1–3 amino acids before the cleavage site; (f) a cleavage site that follows a Gly-Gly pair; (g) the processed products in all these cases are small protein bacteriocins (43–88 amino acids in length) which function by increasing membrane permeability in target cells (Kolter & Moreno, 1992). Interestingly, ColV represents the first example of such a toxin produced by Gram-negative bacteria.

Posttranslational Modification. Several lines of evidence had suggested that ColV might contain some type of posttranslational modification. However, the mass spectroscopy results are most consistent with the absence of any posttranslational modification beyond the N-terminal processing. Mass spectroscopy does not address the possibility that other molecules may interact noncovalently with the mature ColV polypeptide. We have observed that TCA-precipitated supernatants that contain active ColV are more reddish-orange in color than negative control supernatants. This has prompted us to consider the possibility that ColV might be a metalloprotein. A candidate metal ion is iron, which is known to result in a reddish-brown color when bound to proteins (Wong et al., 1977). This is intriguing because the ColV genes are under iron regulation, and ColV uptake is mediated by Cir. Cir is also the outer membrane receptor for ferrated β-lactams (Critchley et al., 1991) and is thought to be a receptor for other iron-containing molecules. The two cysteine residues in ColV could serve as part of an iron binding site in the ColV C-terminal domain. We are currently investigating this possibility.

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