The leader peptide of colicin V shares consensus sequences with leader peptides that are common among peptide bacteriocins produced by Gram-positive bacteria

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Colicin V is a ribosomally synthesized antimicrobial peptide produced by *Escherichia coli*. Four recently characterized genes, arranged in two convergent operons on the plasmid pColV-K30, are required for colicin V synthesis, export and immunity. We report the purification and N-terminal amino acid sequencing of the colicin V protein. Our results demonstrate that the colicin V primary translation product, which consists of 103 amino acids, is proteolytically processed. A leader peptide, consisting of 15 amino acid residues, is removed from the N-terminus during maturation of colicin V. This leader peptide is not related to the N-terminal signal sequences which direct proteins across the cytoplasmic membrane via the Sec pathway. The molecular mass of colicin V, obtained by mass spectrometry analysis, showed that the peptide consists of only unmodified amino acids. The deduced amino acid sequence of the leader peptide was highly homologous to the N-terminal extensions found in non-lantibiotic, peptide bacteriocins produced by Gram-positive bacteria. These findings strongly indicate that colicin V belongs to a family of small peptide bacteriocins that have been found previously only among the Gram-positive lactic acid bacteria.

Keywords: colicin V, bacteriocin, microcin

INTRODUCTION

Colicin V is a bacteriocin which kills sensitive cells by damaging their cytoplasmic membrane (Yang & Konisky, 1984). It is produced by several strains of *Escherichia coli*, and also by some other members of the family *Enterobacteriaceae* (Waters & Crosa, 1991). These bacteria also define the activity range for the bacteriocin. Colicin V displays several properties which distinguish it from other colicins described. Colicin V is of small size (less than 10 kDa), its synthesis is not SOS inducible and its secretion requires a set of dedicated export proteins. Other colicins have molecular masses ranging from 27 to 80 kDa, and they utilize specific lysis proteins for their release from the cell (Hershman & Helinski, 1967; Gilson *et al.*, 1987, 1990; Waters & Crosa, 1991). It has therefore been suggested that colicin V instead should be classified as a microcin, a class of post-translationally modified antimicrobial polypeptides produced by some strains of *E. coli* (Yang & Konisky, 1984). However, we think colicin V has more in common with so-called lantibiotic and non-lantibiotic bacteriocins from Gram-positive bac-

teria. The lantibiotic and non-lantibiotic peptide bacteriocins (in the following called lantibiotics and non-lantibiotics) are two families of small, ribosomally synthesized bacteriocins, whose antimicrobial effect is due to their capacity to cause leakage in the cytoplasmic membrane of sensitive cells (Freund & Jung, 1992; Kok *et al.*, 1993; Klaenhammer, 1993). In contrast to the non-lantibiotic bacteriocins, lantibiotics are post-translationally modified and contain unusual amino acids such as dihydroalanine, dihydrobutyrine, lanthionine and β -methyllanthionine.

Four genes involved in colicin V synthesis, export and immunity have been cloned and sequenced (Frick *et al.*, 1981; Gilson *et al.*, 1987, 1990). These genes are arranged in two convergent operons. One operon contains the genes encoding colicin V (*cvaC*) and the immunity protein (*cvi*). The other operon contains *cvaA* and *cvaB*, the genes encoding the proteins required for transport of colicin V across the cytoplasmic membrane. In addition, secretion of colicin V from *E. coli* requires TolC, the product of an unlinked chromosomal gene, needed for transport of

colicin V across the outer membrane (Gilson *et al.*, 1990). Many proteins secreted from bacteria use the signal peptide dependent general export pathway, which comprises the *sec* gene products and the signal peptidases. However, several bacteriocins such as colicin V, lantibiotics and non-lantibiotics, are secreted by a signal peptide independent pathway (Gilmore *et al.*, 1990; Engelke *et al.*, 1992; Klein *et al.*, 1992; Marugg *et al.*, 1992; Schnell *et al.*, 1992; Stoddard *et al.*, 1992). Secretion of these proteins is mediated by members of a large family of bacterial transporters termed ABC transporters (Higgins, 1992). The most characteristic feature of the ABC transporter proteins is a highly conserved ATP-binding cassette that probably couples ATP hydrolysis to the transport process.

Lantibiotic and non-lantibiotic peptide bacteriocins are synthesized as precursor proteins. Their N-terminal leader peptides are cleaved off concomitant with or after secretion of the bacteriocins (Holo *et al.*, 1991; Freund & Jung, 1992; Klaenhammer, 1993). Homology analysis revealed that the sequence of the 15 N-terminal amino acids of the primary translation product of colicin V has significant similarity to the leader peptides of non-lantibiotics and some lantibiotics. We therefore set out to purify colicin V in order to determine if its primary translation product undergoes N-terminal processing.

METHODS

Bacterial strains, plasmids and media. Two different strains that produce colicin V were used in the purification of the bacteriocin. One was *E. coli* ATCC 14763, which is a natural colicin V producer. The other strain was *E. coli* MC4:00 harbouring pHK22, a derivative of pACYC184 containing a 9.4 kb fragment which encodes both colicin V operons (Gilson *et al.*, 1987, 1990). *E. coli* MH1 (Casadaban & Cohen, 1980) was used as the colicin V-sensitive indicator organism in bacteriocin assays. The amount of colicin V produced by MC4100(pHK22), as judged by the size of the growth inhibition zones in an overlay assay, was comparable to the amount produced by ATCC 14763. The strains were propagated in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) and incubated at 37 °C. The strain harbouring pHK22 was grown in LB medium containing 20 µg chloramphenicol ml⁻¹. To increase colicin V production 0.2 mM 2,2'-dipyridyl (Sigma) was added to the medium (Chehade & Braun, 1988).

Bacteriocin assay. Colicin V activity was assayed using microtitre plates as described by Holo *et al.* (1991). Twofold dilutions of colicin V fractions were made with 100 µl LB medium in each well. Then 100 µl of an overnight culture of the indicator strain, diluted 1:100 in LB medium, was added to each well to give a final volume of 200 µl. The LB medium used in the microtitre plate assay was supplemented with 0.1% (v/v) Tween 80. The microtitre plate cultures were incubated at 37 °C for 3–6 h and growth of the indicator organism was measured spectrophotometrically at 600 nm using a Dynatech Microplate Reader. One unit of bacteriocin activity (BU) was arbitrarily defined as the amount of bacteriocin required to inhibit growth by 50% (50% of the optical density of the control without bacteriocin). Bacteriocin activity was also determined using a soft agar overlay assay in which bacterial cultures were patched on LB agar and incubated at 37 °C for 18–24 h. The plates were

then overlaid with soft agar (0.8%) seeded with indicator cells at the dilution mentioned above. Bacteriocin production appeared as clearly defined zones of inhibition surrounding the producing bacterial colony.

Colicin V purification. All purification steps were carried out at room temperature. The bacteriocin was purified from 1-litre cultures of *E. coli* strains ATCC 14763 or MC4100(pHK22). Overnight cultures were diluted 1:100 in LB medium containing 0.2 mM 2,2'-dipyridyl and grown at 37 °C under vigorous agitation for 8–12 h. The cells were removed by centrifugation at 4000 g for 20 min at 4 °C, and 600 g ammonium sulphate was added per litre of supernatant. The protein precipitate was left at 4 °C for at least 2 h and pelleted by centrifugation at 7000 g for 30 min. The pellet was dissolved in 100 ml buffer A (10 mM sodium phosphate buffer, pH 5.0). The solution was transferred to an Erlenmeyer flask, and 15 g amberlite XAD-16 (Supelco) was added. The flask was placed on a rotary shaker at low speed for 1–2 h. Unbound material was removed by filtration. The amberlite resin was washed three times with 200 ml 40% ethanol. Each time the Erlenmeyer flask was left shaking for 30 min. After the last washing step the amberlite resin was transferred to a column and bacteriocin bound to the resin was eluted with 50 ml 70% 2-propanol containing 10 mM acetic acid, pH 2 (adjusted with concentrated HCl). The volume of the fraction was reduced to 25 ml or less by using a vacuum centrifuge (Heto VR-1). The fraction was diluted tenfold in buffer A and applied to a 12 ml SP Sepharose Fast Flow cation-exchange column (Pharmacia) equilibrated with the same buffer. The column was washed extensively with buffer A and eluted in 30 ml buffer A containing 1 M NaCl. This 30 ml fraction was applied directly to a C₂/C₁₈ reverse-phase column (PepRPC HR 5/5; Pharmacia) equilibrated with water containing 0.1% trifluoroacetic acid (TFA). Bound bacteriocin was eluted with a gradient of 2-propanol (0–100%) containing 0.1% TFA (Fig. 1). The most active fractions were combined, diluted tenfold in water and rechromatographed.

Mass spectrometry, amino acid composition and sequencing. Mass spectrometry analysis of colicin V was performed by Dr Stephen Bayne at Novo Nordisk A/S (Gentofte, Denmark) on a PE Sciex API 1 electrospray mass spectrometer. To determine the amino acid composition of colicin V, the purified protein was hydrolysed and analysed on an amino acid analyser as described earlier (Nieto Lozano *et al.*, 1992). The amino acid sequence was determined by Edman degradation using an Applied Biosystems 477A automatic sequencer with an on-line 120A phenylthiohydantoin amino acid analyser (Cornwell *et al.*, 1988). Amino acid analysis and sequencing were performed by Dr Knut Sletten at the University of Oslo.

SDS-PAGE. SDS-PAGE was carried out using PhastGel high-density gels, and the PhastSystem (Pharmacia) according to the manufacturer's recommendations. The molecular mass standard consisted of five peptides ranging from 2500 to 17000 Da (Pharmacia).

RESULTS AND DISCUSSION

Solubilization and purification of colicin V

The structural gene of colicin V was shown by Gilson *et al.* (1990) to encode a protein of 103 amino acids. Complete characterization of the bacteriocin, however, has been hindered because purified colicin V has not been available for analysis. It is generally believed that the difficulties associated with the isolation of colicin V are due to its low

Table 1. Purification of colicin V

Purification stage	Vol. (ml)	Total A_{280} *	$10^{-6} \times$ Total activity (BU)	Specific activity†	Increase in specific activity	Yield (%)
Culture supernatant	1000	15470	12.8	8.3×10^2	1	100
Ammonium sulphate precipitation	100	2836	10.3	3.6×10^3	4.4	80
Amberlite XAD-16	50	479	5.1	1.1×10^4	13	40
SP Sepharose	30	31	3	9.7×10^4	117	23
Reverse-phase chromatography	1	0.6	1.6	2.68×10^6	3240	13

* Total A_{280} is the A_{280} multiplied by the volume in ml.

† Specific activity is bacteriocin units (BU) divided by the A_{280} .

production and instability (Frick *et al.*, 1981; Chehade & Braun, 1988). Colicin V production by *E. coli* strains ATCC 14763 and MC4100(pHK22) was easily detected using a soft agar overlay assay with *E. coli* strain MH1 as indicator. The inhibition zones surrounding the colonies producing colicin V had diameters of 1–2 cm. By using a microtitre plate assay we attempted to detect colicin V activity in sterile filtered growth medium, but very little or no activity was found. This may be due to a lower colicin V production in liquid culture, or that colicin V is somehow inactivated by binding to other components in the medium and/or lost by binding to surfaces in flasks and microtitre plates. Since the structural gene of colicin V encodes a protein which is very hydrophobic in its N-terminal half, we reasoned that addition of Tween-80 to the supernatant might increase the solubility of colicin V. Indeed, by using 0.1% Tween-80 in the microtitre plate assay, the bacteriocin titre increased 100–200-fold. To obtain reliable results from the microtitre plate assays it was important not to incubate the plates at 37 °C for more than 3–6 h. If the microtitre plates were incubated too long, there was growth in all wells. This was probably due to the growth of spontaneously resistant cells, since colonies of resistant cells were always seen inside growth inhibition zones in agar diffusion assays. It has been shown previously that colicin V production is regulated by iron via the repressor encoded by the *fur* gene, and that iron limitation would increase colicin V production three- to fourfold (Chehade & Braun, 1988). To stimulate colicin V production we therefore added 0.2 mM 2,2'-dipyridyl, an iron chelator, to the growth medium. This increased colicin V production four- to sixfold, which is in good agreement with the results obtained by Chehade & Braun (1988).

The purification scheme developed for isolating colicin V is outlined in Table 1. The purification step involving amberlite XAD-16 had a poor yield, and only gave a small increase in specific activity. However, if this step was omitted, the bacteriocin would not bind to the cation

Fig. 1. Reverse-phase chromatography of colicin V. The bacteriocin was eluted by a gradient of 2-propanol (---) in 0.1% TFA.

exchanger. After rechromatography on the reverse-phase column, the colicin V absorbance peak usually appeared homogeneous (Fig. 1), but sometimes contained a shoulder. In both cases analysis of the colicin V peak(s) by SDS-PAGE and subsequent staining with Coomassie brilliant blue revealed only one band, demonstrating that colicin V had been purified to near homogeneity (results not shown). Colicin V was purified with a yield of about 0.5 mg from 1 l culture broth. Assuming that the activity of the bacteriocin was not reduced during purification, *E. coli* strain ATCC 14763 produced approximately 4 mg colicin V l⁻¹ when 0.2 mM 2,2'-dipyridyl was added to the medium, and about 1 mg l⁻¹ without this iron chelator. The concentration of colicin V that inhibited growth of

Fig. 2. The deduced amino acid sequence of colicin V. The amino acid sequence of purified colicin V determined by Edman degradation is in bold type. The bacteriocin processing site is indicated by the vertical arrow.

the indicator organism by 50% was determined to be about 1 ng ml^{-1} . Our results show that the bacteriocin is stable and is produced in ample amounts for purification and characterization.

Purified colicin V lacks the 15 N-terminal amino acids of the predicted primary product

Partial amino acid sequences for colicin V, purified from *E. coli* strains ATCC 14763 and MC4100(pHK22) were determined by Edman degradation. The sequence of the 47 N-terminal amino acid residues of colicin V (strain ATCC 14763), is shown in bold type in Fig. 2. When compared to the primary translation product of *cvaC* (Gilson *et al.*, 1990), it was found that 15 amino acids are missing at the N-terminus. This finding proves that colicin V is synthesized as a precursor peptide, and that 15 amino acids are removed from its N-terminus during maturation. Otherwise, the colicin V sequence obtained by Edman sequencing was almost identical to that deduced from the nucleotide sequence of *cvaC* (Fig. 2). The exception was the amino acid residue in position 56, which was determined to be a glycine by Edman degradation. The nucleotide sequence of *cvaC*, however, predicts a histidine residue in this position. Amino acid composition data and mass spectral measurements (see below) support the DNA sequence data, indicating that the glycine residue found in position 56 was the result of a peptide sequencing error.

The sequence of the nine N-terminal amino acid residues of colicin V, purified from *E. coli* MC4100(pHK22), was also determined. It turned out to be the same as the corresponding sequence from strain ATCC 14763, demonstrating that colicin V isolated from strain MC4100(pHK22) has been processed identically to colicin V from the natural producer strain. This proves that an enzyme

capable of carrying out correct processing of the colicin V precursor protein is present in strain MC4100(pHK22). It is possible that this enzyme is a chromosomally encoded hitherto unknown leader peptidase constitutively expressed in *E. coli* MC4100. Alternatively, it is also possible that the gene encoding the leader peptidase was introduced into strain MC4100 after transformation with plasmid pHK22. Further studies are necessary to clarify these matters.

Active colicin V probably contains a disulphide bridge

The primary translation product of colicin V is a protein of 103 amino acids, and during maturation 15 amino acids are removed from its N-terminus. Hence the mature colicin V consists of a maximum 88 amino acids. By SDS gel electrophoresis the molecular mass of purified colicin V was estimated to be 4500–5000 Da (results not shown). This result indicates that colicin V is a peptide consisting of 40–50 amino acids. On the other hand, judging from the amino acid composition data (results not shown), colicin V is a peptide of about 86 amino acid residues. To obtain a better estimate of the molecular mass of the bacteriocin, mass spectral measurements of purified colicin V using electrospray ionization was performed. A mean molecular mass of 8732.9 ± 0.8 Da was found. The molecular mass of colicin V, based on the nucleotide sequence of Gilson *et al.* (1990), was estimated to be 8735.7 Da for the 88 C-terminal amino acid residues. This suggests that the primary translation product of colicin V is processed by removal of a 15 amino acid leader peptide, but does not undergo post-translational modifications. The minor discrepancy between the measured and calculated molecular mass of colicin V can be explained if the parent bacteriocin contains a disulphide bridge between the two cysteines at positions 91 and 102 (Fig. 2). In this connection it is interesting to note that addition of 5 mM dithiothreitol to the microtitre plate bacteriocin assay completely abolished colicin V activity, suggesting that this putative disulphide bridge may be essential for bacteriocin activity.

Colicin V and the non-lantibiotics belong to the same family of bacteriocins

In Fig. 3 an alignment of the amino acid sequences of 21 bacteriocin leader peptides is shown. Most of the aligned leader peptides belong to the non-lantibiotic bacteriocins from lactic acid bacteria. In addition are the leader peptide of CvaC (colicin V) and the leader peptides of the lantibiotics Cyl L1 (*Enterococcus faecalis* hemolysin/bacteriocin), LcnDR1 (lactococcin DR), ScnA (streptococcin A-FF22), and SalA (salivaricin A). The sequence alignment reveals a distinct pattern with both positionally conserved amino acids and conservative substitutions. Only one amino acid is fully conserved, namely the glycine residue in position -2. The glycine in position -1 is also highly conserved, but in the leader peptides of LcnDR1 and ScnA, alanine is found next to the processing site instead of glycine. The putative leader peptides of

LcnN and Cyl L1 may have a serine in position -1, but this remains to be shown since the processing sites indicated for these bacteriocins are based solely on sequence similarity. As noted by Fremaux *et al.* (1993), hydrophobic amino acids almost without exception occupy positions -4, -7, -12 and -15, whereas hydrophilic amino acids dominate in between these hydrophobic ones, especially in positions -8, -9 and -12 (Fig. 3). The following seven amino acids are found to be conserved in more than half of the aligned leader peptides: glycine in position -1, glycine in position -2, isoleucine in position -4, leucine in position -7, glutamic acid in position -8, serine in position -11, and leucine in position -12. This gives the following consensus sequence L(-12), S(-11), X, X, E(-8), L(-7), X, X, I(-4), X, G(-2), G(-1). All non-lantibiotics known to date have leader peptides of this type, which we have termed the double-glycine type of leader peptide. In addition the following properties are shared among all non-lantibiotic bacteriocins: (i) they are small, heat-stable antimicrobial peptides, (ii) they are ribosomally synthesized as precursor peptides, (iii) they induce cell death

Several lantibiotics, such as nisin, subtilin, epidermin, gallidermin and Pep5, have leader peptides which all have the consensus proteolytic cleavage site: X(-4), X(-3), P(-2), X(-1), X(+1), X(+2), where residue X(-3) is negatively charged or polar, residue X(-1) is positively charged or polar, and residues X(-4), X(-2), and X(+1) are hydrophobic (Jung, 1991). It has been demonstrated that this type of leader peptide, which is unrelated to the double-glycine type found among non-lantibiotics, is cleaved off by a subtilisin-like serine protease after secretion of the lantibiotic precursor protein (van der Meer *et al.*, 1993). Nevertheless, some lantibiotics, namely Cyl L1, LcnDR1, SalA and ScnA, undoubtedly have leader peptides of the double-glycine type (Fig. 3). This finding indicates that some lantibiotics

and non-lantibiotics may be derived from a common ancestor.

Amino acid sequence comparison of the available bacteriocin transporters show that they all share significant sequence homology. The ABC transporters of the non-lantibiotics CvaC (Gilson *et al.*, 1990), LcnA (Stoddard *et al.*, 1992), PedA (Marugg *et al.*, 1992) and LagA/LagB (L. S. Håvarstein, J. Nissen-Meyer & I. F. Nes, unpublished results) all consist of about 700 amino acids. The corresponding transporters of the lantibiotics Cyl L1 (Segarra, 1992) and LcnDR1 (EMBL: U04057) are of the same size. However, the ABC transporters of the lantibiotics nisin (Engelke *et al.*, 1992), subtilin (Klein *et al.*, 1992), and epidermin (Schnell *et al.*, 1992), which do not have a leader peptide of the double-glycine type, are at least 100 amino acids shorter. This is due to an N-terminal extension of 100–150 amino acids, which is present only in the largest ABC transporters. Since this conserved domain is present in all ABC transporters exporting bacteriocins with the double-glycine type of leader peptide, it probably plays an important role in the secretion of these proteins.

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