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The DNA Replication Inhibitor Microcin B17 is a Forty-Three-Amino-Acid Protein Containing Sixty Percent Glycine

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ABSTRACT **Microcin B17 is a low-molecular-weight protein that inhibits DNA replication in a number of enteric bacteria. It is produced by bacterial strains which harbor a 70-kilobase plasmid called pMccB17. Four plasmid genes (named *mcbABCD*) are required for its production. The product of the *mcbA* gene was identified by labelling minicells. The *mcbA* gene product was slightly larger when a mutation in any of the other three production genes was present. This indicates that these genes are involved in processing the primary *mcbA* product to yield the active molecule. The *mcbA* gene product predicted from the nucleotide sequence has 69 amino acids including 28 glycine residues. Microcin B17 was extracted from the cells by boiling in 100 mM acetic acid, 1 mM EDTA, and purified to homogeneity in a single step by high-performance liquid chromatography through a C18 column. The N-terminal amino acid sequence and amino acid composition demonstrated that *mcbA* is the structural gene for microcin B17. The active molecule is a processed product lacking the first 26 N-terminal residues. The 43 remaining residues include 26 glycines. While microcin B17 is an exported protein, the cleaved N-terminal peptide does not have the characteristic properties of a "signal sequence," which suggests that it is secreted by a mechanism different from that used by most secreted proteins of *E. coli*.**

Key words: microcins, peptide antibiotic, protein processing, HPLC, protein secretion

INTRODUCTION

The studies on the relationship between structure and function of proteins aim to achieve an understanding of the role played by each component amino acid.[1] Small proteins are thus particularly well suited for these types of studies. We are interested in understanding the relationship between structure and function of a small protein called microcin B17 (MccB17) which inhibits DNA replication in enteric bacteria. Microcins constitute a diverse group of low-molecular-weight (<5,000 Daltons) antibiotic substances produced by many diffferent isolates of enteric bacteria.[3,6] MccB17 is the prototype of the group B microcins. Like most other microcins, it is plasmid-encoded and is active on a large number of Gram(−) enteric bacteria.[3] The wild-type plasmid responsible for the production of and immunity to MccB17 is a 70-kilobase (kb) incFII plasmid called pMccB17.[5] A region of 6.3 kb of this plasmid contains the genes essential for MccB17 production and immunity.[8,31] This region has been cloned and genetic studies have shown that at least four genes, called *mcbABCD*, are necessary for the production of an active protein.[32] Initial studies with crude preparations of MccB17 indicated that it was a slightly hydrophobic protein of about 4000 Daltons (M. Herrero, Ph.D. thesis, Universidad Complutense de Madrid). By using these preparations it was possible to show that MccB17 has a bactericidal effect on *E. coli*.[12,13] The primary effect of treating *E. coli* with the antibiotic is immediate cessation of DNA replication. Cells then massively degrade their DNA and die.

From its synthesis within the producer cell to its interference with the replication machinery in the sensitive cell, MccB17 must interact specifically with a number of different proteins. Such proteins include those involved in (1) intracellular post-translational processing, (2) secretion outside the cell, (3) recognition and uptake by the sensitive cell, (4) DNA replication, and (5) the MccB17 immunity system. Yet all the structural information necessary to accomplish these tasks must be contained in a rather short sequence of amino acids. A good understanding of the role of each amino acid in these steps will aid the general understanding of several biological processes. In this paper we present the purification and initial biochemical and genetic characterization of the MccB17 protein. These results provide the basis from which we can start detailed studies on the structure-function relationships of MccB17.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The minicell-producing strain P678-54T^{-2} and MC4100 *recA*56[32] were from our laboratory collection. Plasmids pMM102, pSS81, pSS11, pSS15, and pSS18 have been described.[31,32] pMM102 contains most of pBR322 with a 5.2-kb *Bam*HI to *Eco*RI insert coding for the production of MccB17 (see Fig. 2). The

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pSS plasmids contain insertion mutations in each of the four genes known to be essential for MccB17 production. pMM102 is defective in immunity and strains harboring it grow poorly. Therefore we also used two other plasmids which produce MccB17 and which have a normal immunity: pMM39 and pIF1. Both of these plasmids have the 6.3-kb *Bam*HI to *Bgl* II fragment of DNA shown in Figure 2. In pMM39, pBR322 is the cloning vector while pIF1 contains pACYC184. They were constructed by M.C. Garrido and F. Moreno[8] (unpublished results).

Minicell Experiments

Minicells were prepared as described previously.[18] Purified minicells were resuspended at an OD_{600} of 0.5 in M63 salts[21] supplemented with 0.2% glucose, 1 μg/ml thiamine, 1 mM $MgSO_4$, and 2 μg/ml of all amino acids except cysteine. Minicells were then incubated with shaking at 37°C for 20 minutes. This was followed by a one-minute pulse with 20 μCi/ml ^{35}S-cysteine (Amersham Corporation, SJ.232, 1170 Curies/mmole). In pulse-chase experiments, cold cysteine was added to 100 μg/ml. One-milliliter samples were transferred to tubes containing 100 μl 100% (w/v) trichloroacetic acid (TCA), mixed, and kept on ice for 10 minutes. Precipitates were pelleted and resuspended in 20 μl of 10 mM sodium phosphate, pH 7.2, 7 M urea, 1% SDS, 1% 2-mercaptoethanol, and 0.05% bromphenol blue. Samples were loaded on 15% polyacrylamide gels (bis:acrylamide, 0.8:30) containing 0.1 M sodium phosphate, pH 7.2, 0.1% SDS, and 6 M urea. Running buffer was 0.1 M sodium phosphate, pH 7.2, and 0.1% SDS. Gels were 30 cm long and 0.4 mm thick; they were run at 125 volts for 12 hr, fixed in 10% TCA, dried onto filter paper, and exposed on Kodak XAR film for four days. Prestained molecular weight standards were purchased from Bethesda Research Laboratories.

DNA Sequence Analysis

The sequence of the *mcbA* gene presented here was obtained by using the chain termination method.[30] It was obtained as part of a project in which a 3.8-kb fragment of DNA containing all MccB17 production genes[31] was sequenced (R. Kolter et al., manuscript in preparation). Random clones into M13mp8 were obtained by sonicating the DNA and ligating into the *Sma* I site.[36] The phage were propagated in strain 71-18.[19]

MccB17 Assay

Antibiotic activity was determined by the critical dilution method.[17] Five microliters of serial dilutions were spotted on a freshly seeded lawn of MC4100 *recA*56 cells on an M63 glucose minimal agar plate. A *recA* strain was used because of its increased sensitivity to MccB17.[13] Plates were incubated overnight at 37°C. The number of units in 5 μl is defined as the reciprocal of the highest dilution which still gives a clear halo of growth inhibition.

High-Performance Liquid Chromatography (HPLC)

Methanol and acetonitrile were purchased from Burdick and Jackson Laboratories (Muskegon, MI). HPLC/Spectro grade trifluoroacetic acid (TFA) was from Pierce Chemical Co. (Rockford, IL). The C18 column (10 mm × 25 cm) was from Vydac (Hesperia, CA). Chromatography was performed by using a Waters Associates HPLC system, model 510 (two pumps) controlled by a model 680 automated gradient controller. The effluent was monitored at 254 or 280 nm with a model 440 absorbance detector or at 214 nm with a model 481 absorbance detector. The mobile phase was 0.1% TFA and the mobile-phase modifier was acetonitrile containing 0.1% TFA. The flow rate was 2 ml/min.

Amino Acid Composition and Sequence Analyses

For amino acid analysis, samples were sealed under vacuum in Pyrex tubes with 6 N HCl and heated at 110°C for 24 hours. After lyophilization, the residue was reacted in 20 μl of ethanol:water:triethylamine: phenylisothiocyanate (7:1:1:1) at room temperature for 20 minutes. Samples were lyophilized again and stored at −20°C. The resulting mixture of phenylthiocarbanyl-amino acids was resolved on a Hewlett-Packard 1084B HPLC by using an Altex-ODS 3 μm column. A gradient program was used with 0.14 M sodium acetate pH 6.4, 500 μl triethylamine/l as buffer A, and acetonitrile:water (3:2) as buffer B.

Sequencing was performed on an Applied Biosystems model 470A protein sequencer. Phenylthiohydantoin-amino acid separations and determinations were performed on a Hewlett-Packard 1090 HPLC with a 1040-diode-array detector. The column was a DuPont Zorbax ODS 4.6 mm × 25 cm. These analyses were done at the Harvard Microchemistry Facility by William S. Lane and David W. Andrews.

Electron Microscopy

Negative staining was done by a miniaturization of a method described previously[34]; 50–100-μl samples were used. All specimens were prepared and examined in duplicate—one using 0.5% uranyl acetate and one using 0.5% uranyl formate as the negative stain. Both micrographs shown used uranyl formate. The micrographs were taken at 50,000× magnification on a JOEL 100B operated at 80 kV with 50-μm condensor and objective apertures.

RESULTS

Identification of the *mcbA* Gene Product

The MccB17 production and immunity region from plasmid pMccB17 has been cloned into pBR322.[31] Mutations which abolish MccB17 production (Mcc$^-$) were obtained in both plasmids and complementation studies were carried out.[32] These experiments showed that at least four genes, *mcbABCD*, were necessary for production of MccB17. The *mcbA* complementa-

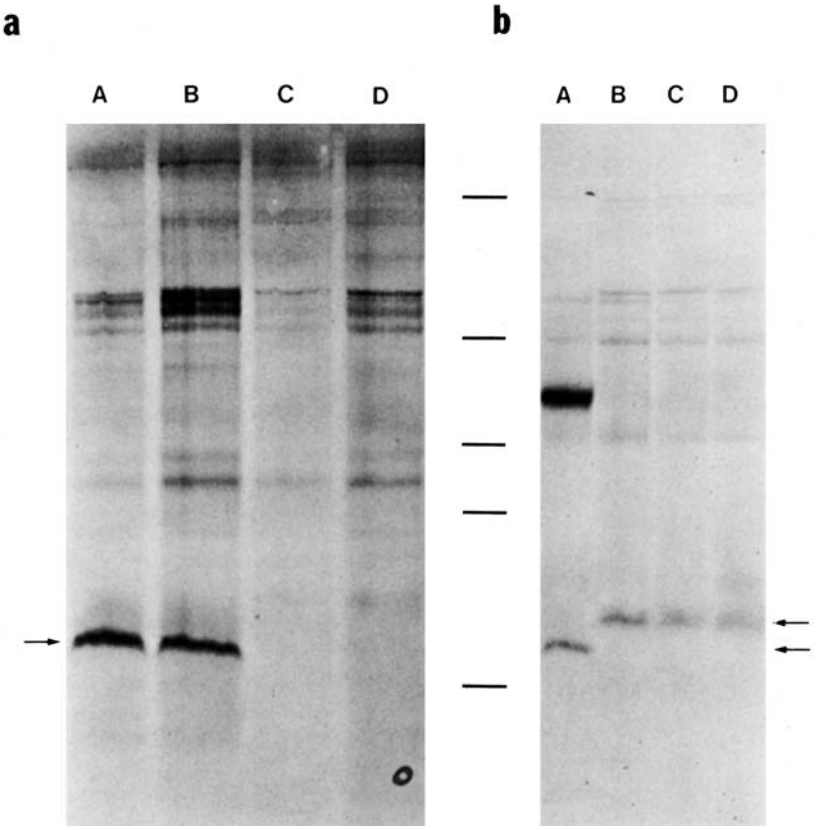

Fig. 1. Identification of the *mcbA* gene product. Minicells were prepared, labelled, and run on polyacrylamide gels as described in Materials and Methods. The horizontal lines denote the position of migration of prestained molecular weight standards, from *top to bottom:* ovalbumin, 43,000; chymotrypsin, 25,700; lactoglobulin, 18,400; lysozyme, 14,300; and bovine trypsin inhibitor, 6,200. a) The *mcbA* gene product of a Mcc$^+$ plasmid (pMM102) and its disappearance in a *mcbA*$^-$ mutant (pSS81). The arrow denotes the position of the *mcbA* product. Lanes A: pMM102, one-minute pulse; B: pMM102, 15-minute chase; C: pSS81, one-minute pulse; D: pSS81, 15-minute chase. b) A larger *mcbA* product is made when mutations in *mcbB*, *mcbC*, or *mcbD*, are present (pSS11, pSS15, and pSS18, respectively). The arrows denote the position of the forms of the *mcbA* product. All lanes are one-minute pulses. Lanes A: pIF1; B: pSS11; C: pSS15; D: pSS18. The heavy band migrating between 18,400 and 25,700 in lane A is chloramphenicol acetyl transferase, which is produced by pIF1 but not by any of the other strains.

tion group spanned a region of about 300 base pairs (bp). The small size of this complementation group suggested that it could be the structural gene for MccB17. To identify the protein product of this gene we introduced MccB17-producing plasmids and Mcc$^-$ derivatives into the minicell-producing strain P678-54T$^-$.[2] Purified minicells were pulsed with 20 μCi/ml ^{35}S-cysteine for one minute; samples were removed and the label was chased with excess unlabelled cysteine for fifteen minutes. Samples were treated and analyzed by electrophoresis as described in Materials and Methods. Figure 1a shows the results of labelling minicells bearing a MccB17-producing plasmid (pMM102) in lanes A and B and an *mcbA*$^-$ derivative of that plasmid (pSS81) in lanes C and D. The prominent band which migrates just behind the 6,200-Dalton standard disappears with the introduction of the *mcbA*$^-$ mutation. Figure 1b shows the results of a one-minute pulse of 20 μCi/ml ^{35}S cysteine on minicells harboring another MccB17-producing plasmid (pIF1, lane A) and Mcc$^-$ derivatives of pMM102 with mutations in *mcbB* (pSS11, lane B), *mcbC* (pSS15, lane C), and *mcbD* (pSS18, lane D). In every case a slightly larger *mcbA* product than that found in MccB17$^+$ plasmids was observed. The results presented in Figure 1 indicate that the *mcbA* gene product is stable and that it is cleaved in the presence of functional *mcbB*, *mcbC*, and *mcbD* products. Since no cleavage is observed in mutants lacking any one of these products, they must be acting in steps prior to cleavage or in the cleavage step itself. The protein products of these genes (*mcbBCD*) have been identified and those results will be presented elsewhere (R. Kolter et al., manuscript in preparation). The gel system used here to identify the *mcbA* gene product does not resolve these large products well.

DNA Sequence of the *mcbA* Gene and Predicted Amino Acid Sequence of the *mcbA* Gene Product

To analyze further the *mcbA* gene we determined its nucleotide sequence. The sequence information was obtained as part of a larger project in which the

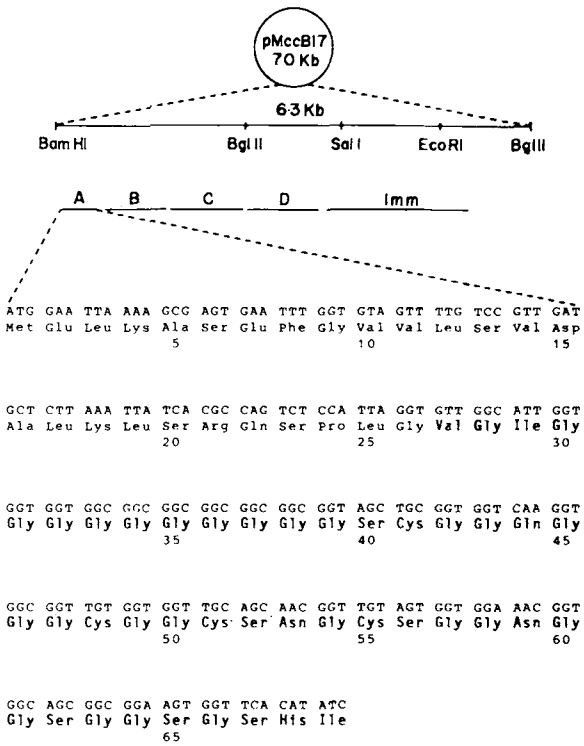

Fig. 2. The physical and genetic map of the *mcb* region from plasmid pMccB17 and the sequence of the *mcbA* gene with its predicted amino acid sequence. The boldface amino acids from position 27 to the end represent the amino acids found in the mature form of MccB17.

entire production region (*mcbABCD*) was sequenced. Those full results will be presented elsewhere (R. Kolter et al., manuscript in preparation). The sequence of the *mcbA* gene is presented here because of its relevance to subsequent results. Figure 2 shows a schematic of the MccB17 production and immunity region, the sequence of the *mcbA* gene, and the predicted amino acid sequence for the *mcbA* gene product. The most striking feature of this 69-amino-acid sequence is its high glycine content. Also, after the arginine at position 21 there are no residues which could be cleaved by either trypsin or chymotrypsin. It had been previously noted that the MccB17 activity was resistant to treatment with these proteases (M. Herrero, Ph.D. thesis, Universidad Complutense de Madrid). This finding suggested that the *mcbA* gene was indeed the structural gene for MccB17 and that the portion lost by cleavage was the N-terminal end. Consistent with this observation was our inability to label the *mcbA* gene product in minicells by using ^{35}S-methionine (results not shown), since the only methionine is the N-terminal residue. However, the direct identification of the structural gene for MccB17 required the amino acid sequence of purified MccB17.

Purification of MccB17

Previous experiments had shown that MccB17 antibiotic activity increased in late logarithmic phase and was maximal 2 hr after the onset of stationary phase.[11] We inoculated liquid media (M63 glucose)

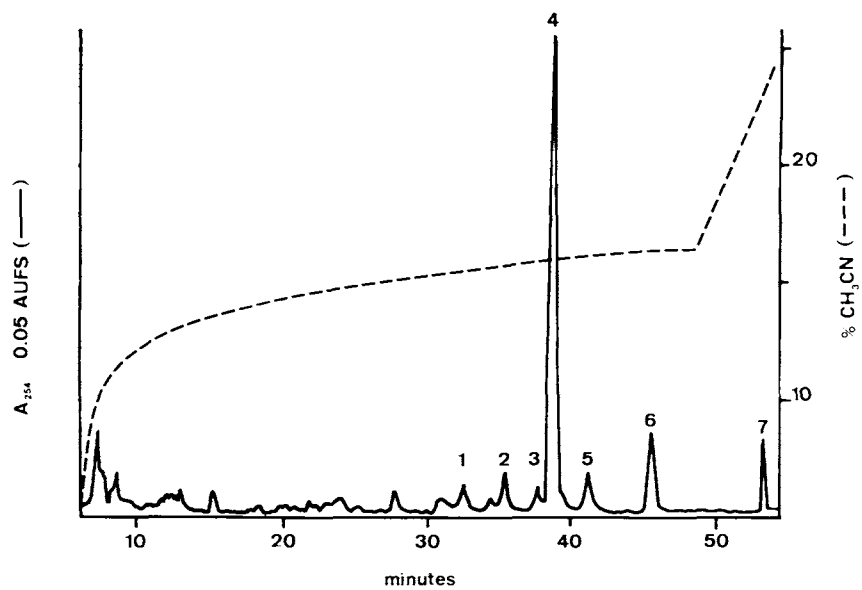

Fig. 3. Elution profile of a MccB17 preparation chromotographed through C18 in an HPLC apparatus. This preparation had been prechromatographed through a Sep-Pak cartridge as described in the text. Peaks 1–3 are not active; peaks 4–7 show antibiotic activity. AUFS: absorbance units full scale.

TABLE I. Purification of MccB17*

	Units† ($\times 10^{-3}$)	Protein‡ (mg)	Specific activity ($U \times 10^{-3}$/mg)	Recovery (units) (%)
FI**	415	—	—	100
FII**	242	30.27	8	58
HPLC peaks				
4	170	1.53	111	
5	13	0.13	100	
6	26	0.27	96	
7	5	0.07	71	
Total HPLC	214	2.00	—	52

*From an initial culture volume of 360 ml.
†Determined by the method of critical dilution.[17]
‡For FII and peak 4 the mass was determined by weighing the dry residue; the weight of peaks 5–7 was calculated from the area of the peak from HPLC relative to peak 4.
**See text for definitions of FI and FII.

with an overnight culture of a producing strain, usually MC4100 *recA*56 (pMM39), and grew the cells 2 hr into stationary phase. Cells were harvested by centrifugation and the supernatant was discarded because very little activity could be recovered from unconcentrated supernatants. Most of the activity remained associated with the cells. The cell pellet was resuspended in 1/150 volume of 100 mM acetic acid, 1 mM EDTA, and the suspension was boiled for 10 minutes. Cells and debris were removed by a centrifugation step (12,000g for 10 minutes). The supernatant, called FI, was then dried in a vacuum at 90°C. The residue was resuspended in 1/3,000 of the original culture volume of 100% TFA. This fraction was called FII and loaded onto a C18 column on an HPLC apparatus. The column was rinsed with 8% acetonitrile in 0.1% TFA and eluted with an 8–23% gradient of acetonitrile in 0.1% TFA. To avoid the presence of some contaminants which bind weakly to C18, the FI was sometimes prechromatographed in a C18 Pre-Pak cartridge and eluted with 45% acetonitrile in 0.1% TFA prior to drying. A typical HPLC elution profile is shown in Figure 3. When extracts prepared in the same way from cells harboring any Mcc^- plasmid were chromatographed through C18 in the HPLC system, no peaks were observed after the initial flow-through material (results not shown). This indicated that all the peaks seen in the profile in Figure 3 are related to mature MccB17. Table I shows the purification table with the specific activities of the various peaks. Peaks 4–7 show antibiotic activity. It should be noted that the quantification of the antibiotic units is done by the critical dilution method and is thus subject to considerable error.[17] To calculate the specific activity we determined the weight of the material after drying. This also allowed us to obtain a value for the extinction coefficient of MccB17 at several wavelengths. The protein has a peak at 245 nm with an extinction coefficient of $5 \times 10^{-3}\ M^{-1}$. This peak probably is due to absorption from disulfide bonds although it is somewhat stronger than expected.[23]

Analysis of Purified MccB17: *mcbA* is the Structural Gene for MccB17

We subjected several of the peaks obtained from the HPLC C18 chromatography (peaks 1, 2, 4, and 6 in Fig. 3) to partial N-terminal amino acid sequence analysis and partial amino acid composition analysis as described in Materials and Methods. In every case the sequence of the first 11 N-terminal residues was

Val-Gly-Ile-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly

This sequence matches the residues predicted from the sequence of the *mcbA* gene at positions 27–37 (see Fig 2). This indicates that the processing step(s) removes the first 26 amino acid residues from the precursor and the active MccB17 contains 43 amino acids. The molecular weight of the amino acid chain is 3,255. This contrasts with the migration of the *mcbA* gene product which is just behind the 6,200-Dalton standard (see Fig. 1). However, small proteins can migrate abnormally in SDS-polyacrylamide gels.

The results of the amino acid composition analysis are presented in Table II. By and large the results are in good agreement with the composition predicted from the nucleotide sequence. In particular, the absence of aromatic residues, lysine, arginine, and methionine, and the presence of a single histidine, are corroborated. Because the samples were not carboxymethylated, the cysteine content could not be determined. One notable deviation from the predicted composition is serine. From the DNA sequence six serines were predicted; yet only two or three serines were obtained in the analysis. We cannot explain this discrepancy. Some of the serines could be modified. We performed some experiments to test this possibil-

Table II. Amino acid composition of MccB17*

	Predicted from DNA sequence	Peak No. 1	2	4	6
Gly	26	27	27	26	27
His	1	1	1	1	1
Val	1	1	1	1	2
Ile	2	2	2	2	2
Glx	1	1	1	1	1
Ser	6	3	2	2	2
Asx	2	0.3	0.7	0.9	0.3
Cys	4	ND	ND	ND	ND

*The molar amounts were determined by setting histidine equal to one. ND, not determined.

ity. MccB17 cannot be labelled with $^{32}PO_4$ in vivo and is thus not likely to be phosphorylated. In addition, we did not detect lipid or carbohydrate in gas chromatography after methanolysis of purified MccB17 (results not shown). The low values obtained for asparagine also contrast with the expected value of two. Here again we do not know the reason for the discrepancy.

Electron Microscopy of Purified MccB17

During initial attempts at crystallization of MccB17, we prepared negatively stained samples from the various active peaks from the HPLC profile for viewing in the electron microscope. Figure 4 shows some typical micrographs. Highly ordered and extremely long filaments often resembling twisted ribbons up to 450 Å across and several microns long were readily apparent in samples from all the peaks. Many of the structures observed appeared to be helical. These long filaments were observed at low pH and tended to be shorter at higher pH (not shown).

DISCUSSION

We have chosen to study the antibiotic substance microcin B17 (MccB17) because the structural information for several specific interactions is coded within a short chain of amino acids. These interactions include steps leading to its production and release from the cell and recognition and killing of sensitive cells by inhibition of DNA replication.[12,13] In this paper we presented a procedure for the purification of MccB17. This procedure entails a single C18 chromatographic step in HPLC of a boiled extract of producer cells. The yields are high, about 6 mg/liter of culture and the activity is very stable. These features should make further structural analyses relatively easy.

Seven peaks of absorbance are detected in the HPLC profile (Fig. 3) but they are probably all isoforms of MccB17. While peaks 1–3 did not show antibiotic activity, peaks 1, 2, 4, and 6 were shown to be related by amino acid sequence and composition. From those results and DNA sequencing of the structural gene for MccB17 we have determined the primary structure of the protein. The 43-amino-acid chain contains 26 glycine residues. The DNA sequence also predicts the presence of six serines, yet the amino acid analysis revealed only two or three (Table II). The exact determination of serine residues is difficult because of degradation but losses are generally within 10–20% of total serine content.[14] It is possible that some of the serines in MccB17 are modified. However, we have shown that MccB17 does not contain phosphate, carbohydrate, or lipid.

What are the differences which result in the seven isoforms of MccB17? We have some evidence indicating that the two major peaks seen in the HPLC profile (peaks 4 and 6 in Fig. 3) represent two interconvertible forms. When MccB17 is resuspended in 100% TFA before loading onto the C18 column, the relative proportions of peaks 4 and 6 are 85% and 15%, respectively (see Fig. 3). But when samples are loaded in pH 8 buffer, the proportions switch to 10% peak 4 and 90% peak 6 (results not shown). At intermediate pH values the proportions are also intermediate. There seems to be a transition point at about pH 6. This is interesting because histidine, which has a pK_a of 6.2 in small model compounds,[33] is the only charged side-chain residue of the protein. Thus peaks 4 and 6 could represent two structures which interconvert depending on the protonation state of this residue.

The formation of long filaments which can be visualized in the electron microscope is also pH dependent (see Fig. 4). The presence of these filaments is an indication that MccB17 can assume highly ordered quaternary structure. Whether these filaments form in vivo remains unknown. We are presently attempting to characterize these filaments by electron microscopy and to obtain crystals of MccB17.

The data presented in this paper demonstrate that the antibiotic MccB17 is the processed product of the *mcbA* gene. Three other gene products, McbB, McbC, and McbD, are required to process the primary translation product into a mature and active molecule. This processing event appears to be rapid since after a one-minute pulse no precursor is observed in minicells producing the antibiotic. This rapid cleavage and loss of an N-terminal peptide is similar to what is observed for most secreted proteins in *E. coli*.[4,16] Yet the 26 residues which are cleaved do not have the typical charge distribution and hydrophobic residue content of other signal sequences,[9,20,25] nor do they resemble the sequence cleaved in lipoproteins.[15,22] It thus appears that MccB17 is secreted by still another mechanism. In the case of the secretion of colicins, it has been suggested that the high glycine content of their N-terminal end plays an important role in their transport across the inner membrane.[26,28,35,39] However, for the colicins to be released, the action of a lysis gene product is also needed.[7,24,27,29] There is no indication that the production and release of MccB17 require such a product, and there is no similar lysis

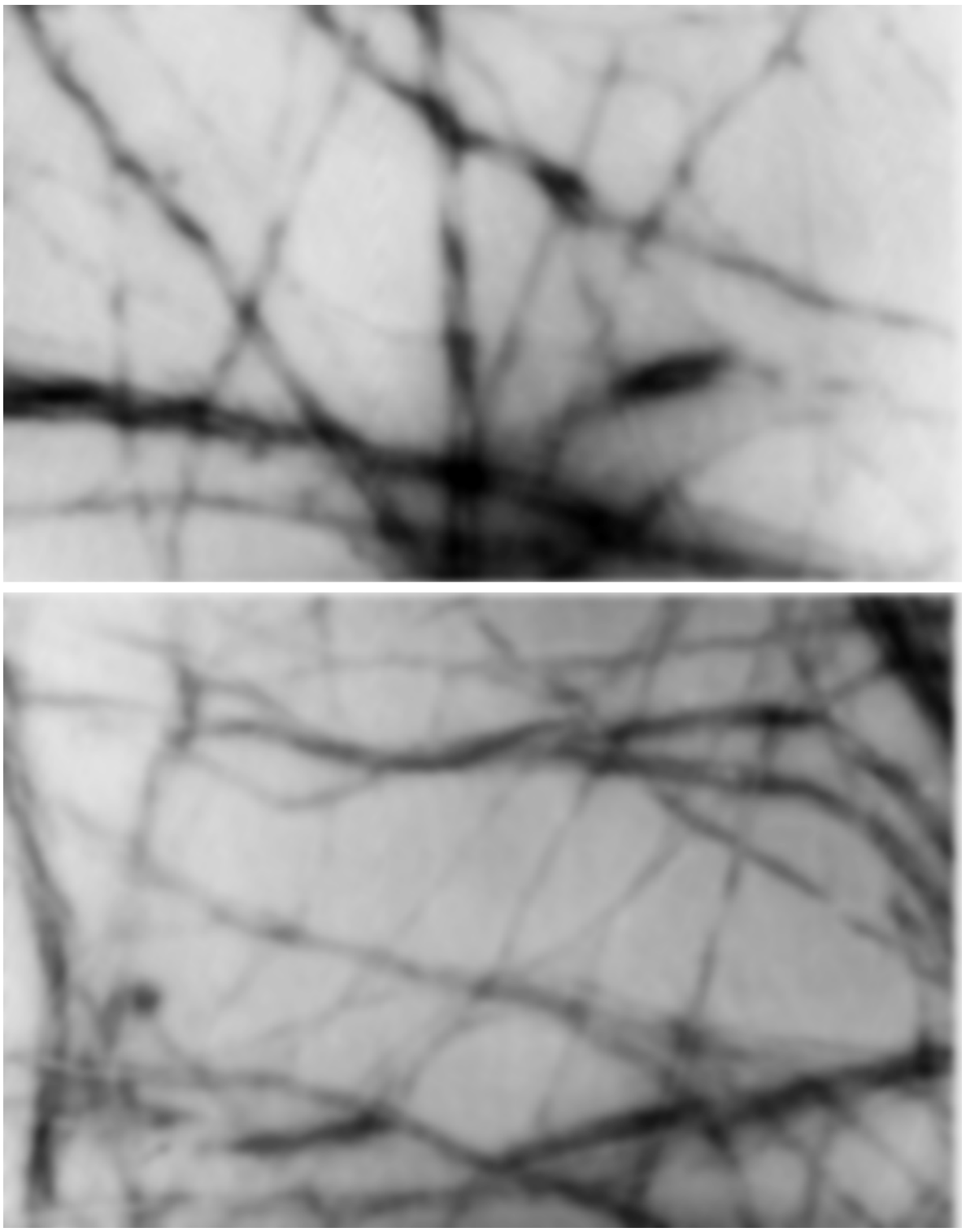

Fig. 4. Electron micrographs of negatively stained MccB17 purified by HPLC. Note "twisted-ribbon" appearance of aggregated fibers (arrow). Mag. = ×100,000.

gene in the *mcb* region (R. Kolter et al., manuscript in preparation). Any one of the three other *mcb* production genes that are required for processing of McbA could be a specific peptidase which cleaves the N-terminus. The sequence which is cleaved, Ser-Pro-Leu-Gly/Val-Gly-Ile-Gly (see Fig. 2), contains a proline at position −3 which is not acceptable in a signal sequence cleavage site.[37,38] Yet it is possible that the *mcbBCD* products act prior to cleavage to modify the precursor such that it may be cleaved by a cellular peptidase. Three chromosomal loci involved in MccB17 production have been identified.[6] One of these is the *ompR* locus whose product activates the transcription of the *mcb* genes.[6,10] The other two genes do not affect this transcription (C. Hernández-Chico, Ph.D. thesis, Universidad Complutense de Madrid). The products of these two genes are candidates for processing enzymes. Other experiments to determine the exact pathway of MccB17 maturation as well as to understand the mechanism by which it kills sensitive cells are in progress in our laboratories. In particular, we have generated a large collection of point mutations in the *mcbA* gene. We are presently studying their effects on each step of MccB17 production and action.

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REFERENCES

1. Ackers, G.K., Smith, F.R. Effects of site-specific amino acid modification on protein interactions and biological function. Ann. Rev. Biochem. 54:597–629, 1985.
2. Adler, H.I., Fisher, W.D., Cohen, A., Hardigree, A.A. Miniature *Escherichia coli* cells deficient in DNA. Proc. Natl. Acad. Sci. U.S.A. 57:321–326, 1967.
3. Asencio, C., Pérez-Díaz, J.C., Martínez, M.C., Baquero, F. A new family of low molecular weight antibiotics from Enterobacteria. Biochem. Biophys. Res. Commun. 69:7–14, 1976.
4. Bankaitis, V.A., Rasmussen, B.A., Bassford, P.J. Intragenic suppressor mutations that restore export of maltose binding protein with a truncated signal peptide. Cell 37:243–252, 1984.
5. Baquero, F., Bouanchaud, D., Martinez-Perez, M.C., Fernandez, C. Microcin plasmids: A group of extrachromosomal elements coding for low-molecular-weight antibiotics in *Escherichia coli*. J. Bacteriol. 135:342–347, 1978.
6. Baquero, F., Moreno, F. The microcins. FEMS Microbiol. Lett. 23:117–124, 1984.
7. Cole, S.T., Saint-Joanis, B., Pugsley, A.P. Molecular characterization of the colicin E2 operon and identification of its products. Mol. Gen. Genet. 198:465–472, 1985.
8. Garrido, M.C., San Millán, J.L., Moreno, F. A novel plasmid vector allowing positive selection for cloned fragments. FEMS Microbiol. Lett. 34:261–264, 1986.
9. Hall, M.N., Silhavy, T.J. Genetic analysis of the major outer membrane proteins of *Escherichia coli*. Ann. Rev. Genet. 15:91–142, 1981.
10. Hernández-Chico, C., Herrero, M., Rejas, M., San Millán, J.L., Moreno, F. Gene *ompR* and regulation of microcin 17 and colicin E2 syntheses. J. Bacteriol. 152:897–900, 1982.
11. Hernández-Chico, C., San Millán, J.L., Kolter, R., Moreno, F. Growth phase and OmpR regulation of transcription of microcin B17 genes. J. Bacteriol. 167:1058–1065, 1986.
12. Herrero, M., Kolter, R., Moreno, F. Effects of microcin B17 on microcin B17-immune cells. J. Gen. Microbiol. 132:403–410, 1986.
13. Herrero, M., Moreno, F. Microcin B17 blocks DNA replication and induces the SOS system in *Escherichia coli*. J. Gen. Microbiol. 132:393–402, 1986.
14. Hill, R.L. Hydrolysis of proteins. Adv. Protein Chem. 20:37–107, 1965.
15. Inukai, M., Ghrayeb, J., Nakamura, K., Inouye, M. Apolipoprotein, an intermediate in the processing of the major lipoprotein of *Escherichia coli* outer membrane. J. Biol. Chem. 259:757–760, 1984.
16. Josefsson, L.-G., Randall, L.L. Different exported proteins in *E. coli* show differences in the temporal mode of processing in vivo. Cell 25:151–157, 1981.
17. Mayr-Harting, A., Hedges, A.J., Berkeley, R.C.W. Methods for studying bacteriocins. Methods Microbiol. 7A:315–422, 1972.
18. Meagher, R.B., Tait, R.C., Betlach, M., Boyer, H.W. Protein expression in *E. coli* minicells by recombinant plasmids. Cell 10:521–536, 1977.
19. Messing, J., Gronenborn, B., Muller-Hill, B., Hofschneider, P.H. Filamentous coliphage M13 as a cloning vehicle: Insertion of a Hind II fragment of the lac regulatory region in M13 replicative form in vitro. Proc. Natl. Acad. Sci. U.S.A. 74:3642–3646, 1977.
20. Michaelis, S., Beckwith, J. Mechanism of incorporation of cell envelope proteins in *Escherichia coli*. Ann. Rev. Microbiol. 36:435–465, 1982.
21. Miller, J.H. "Experiments in Molecular Genetics." Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1972.
22. Neilsen, J.B.K., Lampen, J.O. Membrane-bound penicillinases in Gram-positive bacteria. J. Biol. Chem. 257:4490–4495, 1982.
23. Otey, M.C., Greenstein, J.P. Studies of polycysteine peptides and proteins. II. Apparent dissociation constants and ultraviolet and infrared absorption spectra of isomeric cystinylcystein peptides. Arch. Biochem. Biophys. 53:501–513, 1954.
24. Oudega, B., Stegehuis, F., van Tiel-Menkveld, G.J., de-Graaf, F.K. Protein H encoded by plasmid CloDF13 is involved in excretion of cloacin DF13. J. Bacteriol. 150:1115–1121, 1982.
25. Perlman, D., Halvorson, H.O. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J. Mol. Biol. 167:391–409, 1983.
26. Puglsey, A.P. The ins and outs of colicins. Part I: Production and translocation across membranes. Microbiol. Sci. 1:168–175, 1984.
27. Pugsley, A.P., Schwartz, M. Expression of a gene in a 400-base-pair fragment of colicin plasmid ColE2-P9 is sufficient to cause host cell lysis. J. Bacteriol. 156:109–114, 1983.
28. Pugsley, A.P., Schwartz, M. Colicin E2 release: Lysis, leakage or secretion? Possible role of a phospholipase. EMBO J. 3:2393–2397, 1984.
29. Sabik, J.F., Suit, J.L., Luria, S.E. *cea-kil* operon of the ColE1 plasmid. J. Bacteriol. 153:1479–1485, 1983.
30. Sanger, F., Nicklen, S., Coulson, A.R. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74:5463–5467, 1977.
31. San Millán, J.L., Hernández-Chico, C., Pereda, P., Moreno, F. Cloning and mapping of the genetic determinants of microcin B17 production and immunity. J. Bacteriol. 163:275–281, 1985.
32. San Millán, J.L., Kolter, R., Moreno, F. Plasmid genes required for microcin B17 production. J. Bacteriol. 163:1016–1020, 1985.

33. Tanford, C. The interpretation of hydrogen ion titration curves of proteins. Adv. Protein Chem. 17:69–165, 1962.
34. Valentine, R.C., Green, N.M. Electron microscopy of an antibody-hapten complex. J. Mol. Biol. 27:615–617, 1967.
35. van den Elzen, P.J.M., Walters, H.H.B., Veltkamp, E., Nijkamp, H.J.J. Molecular structure and function of the bacteriocin gene and bacteriocin protein of plasmid CloDF13. Nucleic Acid Res. 11:2465–2477, 1983.
36. Vieira, J., Messing, J. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259–268, 1982.
37. von Heijne, G. Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. 133:17–21, 1983.
38. von Heijne, G. How signal sequences maintain cleavage specificity. J. Mol. Biol. 173:243–251, 1984.
39. Yamada, M., Ebina, Y., Miyata, T., Nakazawa, T., Nakazawa, A. Nucleotide sequence of the structural gene for colicin E1 and predicted sequence of the protein. Proc. Natl. Acad. Sci. U.S.A. 79:2827–2831, 1982.