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Microcin 25, a Novel Antimicrobial Peptide Produced by *Escherichia coli*

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Microcin 25, a peptide antibiotic excreted by an *Escherichia coli* strain isolated from human feces, was purified to homogeneity and characterized. Composition analysis and data from gel filtration indicated that microcin 25 may contain 20 amino acid residues. It has a blocked amino-terminal end. Microcin synthesis and immunity are plasmid determined, and the antibiotic was produced in minimal medium when the cultures entered the stationary phase of growth. The peptide appears to interfere with cell division, since susceptible cells filamented when exposed to it. This response does not seem to be mediated by the SOS system.

Microcins are a miscellaneous group of low-molecular-weight antibiotics produced by a number of enterobacteria, mostly *Escherichia coli* strains (2). Several features distinguish them from the more widely studied colicins. Microcins are considerably smaller, their synthesis is neither lethal nor controlled by the SOS regulatory circuit, and synthesis takes place during the stationary phase of growth (with the possible exception of microcin E492 from *Klebsiella pneumoniae*, the production of which starts in the exponential phase) (3). Moreover, colicins are bactericidal proteins, whereas microcins may exhibit bactericidal or bacteriostatic modes of action. At least for the most extensively characterized microcins, B17 and C7, the genetic systems involved in their production and immunity are considerably more complex than the contiguous arrangement of three genes (synthesis, immunity, and release via lysis) which is a common feature of colicin-producing plasmids (16, 31, 34). Like those of colicins, the genetic determinants for microcins seem to be invariably plasmid borne. However, Laviña et al. have recently described a novel member of the family, microcin H47, which is chromosomally encoded (24).

Microcins are a promising model system for the study of peptide transport mechanisms and the regulation of gene expression in nonproliferating cells. Studies of them may also contribute to a deeper understanding of the structure-function relationships of antimicrobial peptides.

This article introduces the study of a novel peptide antibiotic which is excreted into the culture medium by a fecal strain of *E. coli*. We have called it microcin 25 (Mcc25). The purification, initial biochemical characterization, and some properties of Mcc25 are reported. Also, we present results suggesting that it can act as a cell division inhibitor.

MATERIALS AND METHODS

Bacterial strains. The bacteria used in this work are listed in Table 1. All strains are derivatives of *E. coli* K-12 except for AY25, the natural *E. coli* producer of Mcc25, and AY29, its nonproducing plasmid-cured derivative. Strain AY25 was isolated in our laboratory from the feces of a newborn infant, as described previously (3).

Media. LB rich and M63 minimal media have been described by Miller (29). Minimal medium was supplemented with 0.2% of the specified carbon source, thiamine (1 μg/ml), and, when appropriate, L-amino acids (20 μg/ml) or casein acid hydrolysate (0.1%). Solid medium contained 1.2% agar.

Microcin purification. Two 1-liter Erlenmeyer flasks, each containing 400 ml of medium M63-glucose, were inoculated with 4 ml of an overnight culture of *E. coli* AY25. The flasks were incubated in a rotary shaker for 36 h at 37°C, and the cells were removed by centrifugation at 12,000 × *g* for 20 min. All subsequent operations were performed at room temperature. Supernatants were combined, mixed with activated charcoal (1 mg/ml), and vigorously stirred in a magnetic mixer for 30 min. The suspension was then filtered under vacuum on a Buchner funnel containing a layer of Hyflo Super Cel (Fisher Scientific Co.), 0.5 cm thick. The filtrate was discarded, and the cake was resuspended in 100 ml of 40% acetone, stirred for 20 min, and filtered again. The pigmented filtrate was discarded, and the mixture of charcoal and Hyflo was resuspended in 20 ml of 80% acetone and poured into a column. The flowthrough obtained during column packing was saved, as it contained eluted microcin. Elution of the antibiotic was completed by passing an additional 80 ml of the same solvent. The eluate was evaporated to dryness on a heating plate at 40°C under a stream of air. The dark brown residuum was redissolved in 3 ml of *n*-propanol and held overnight at −20°C. Pigmented insolubles were removed by centrifugation, and the supernatant was applied to a preparative silica gel H thin-layer chromatography (TLC) plate (20 by 20 cm) and run in chloroform–methanol–25% NH_4OH (2:1:1, vol/vol/vol).

After the chromatogram had been developed, antibiotic activity was detected by scraping off portions of silica at 1-cm intervals and placing them directly on the surface of an LB plate with a freshly seeded lawn of indicator AB1133 cells. After 4 to 5 h of incubation at 37°C, halos of growth inhibition were visible around the antibiotic-containing mounds. The zone containing active material (R_f = 0.88) was scraped from the chromatoplate, mixed with an equal amount of Hyflo, resuspended in 20 to 30 ml of 70% methanol, and poured into a column. An additional 50 ml of the same solvent was passed. The eluate was concentrated to 2 ml. Further purification was achieved by reversed-phase high-performance liquid chromatography (HPLC) on a Waters apparatus. The concentrate was applied to a μBondapak

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TABLE 1. *E. coli* strains and plasmid used

Strain or plasmid	Genotype or description[a]	Source or reference[b]
E. coli		
AB1133	F⁻ *thr-1 ara-14 leuB6 lacY1 Δ(gpt-proA)62 supE44 galK2 λ⁻ rac hisG4 rfbD1 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1*	CGSC
BM21	F⁻ *gyrA* (λ⁺)	F. Moreno
RYC816	BM21 *recA56 srl*::Tn*10*	F. Moreno
HB101	F⁻ *hsdS20* (r_B^- m_B^-) *recA13 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44 ara-14* (λ⁻)	6
DH5α	F⁻ φ80d*lacZ* M15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	BRL
MC4100	F⁻ *araD* Δ(*argF-lac*)*U169 rpsL thi relA flbB deoC pstF rbsR*	9
AY25	Natural isolate, Mcc25⁺ Imm25⁺	This work
AY29	AY25 cured of plasmid pTUC100, Mcc25⁻ Imm25⁻	This work
SBG101	MC4100(pTUC100)	This work
AB259	*supQ80 λ⁻ relA1 spoT1 thi-1*	CGSG
Plasmid pTUC100	Wild type, Mcc25⁺ Imm25⁺	This work

[a] Mcc25⁺, microcin 25 producer; Imm25⁺, immune to Mcc25.
[b] CGSC, *E. coli* Genetic Stock Center; BRL, Bethesda Research Laboratories.

C_{18} column (3.9 mm inner diameter by 30 cm) from Waters. The mobile phase consisted of methanol-water-glacial acetic acid (125:75:5, vol/vol/vol) at a flow rate of 0.8 ml/min. The A_{254} was monitored. Active fractions were pooled and concentrated to 1 ml. This material was recycled with methanol-water (125:75, vol/vol) as the solvent system. Microcin emerged as a sharp peak with a retention time of 12 min. The peak was collected and stored at −20°C.

Amino acid analysis. In order to ascertain the purity of the material obtained from the C_{18} chromatography, a sample was subjected to HPLC analysis on an RP-300 (C_8) column (2.1 mm inner diameter by 30 mm). Elution was carried out with a 10 to 60% gradient of acetonitrile in 0.1% trifluoroacetic acid, and the A_{215} of the effluent was monitored. The antibiotic behaved homogeneously in this system as well, appearing as a sharp peak. A sample of this peak was used for amino acid analysis on an Applied Biosystems model 420A amino acid analyzer. For the determination of cysteine as cysteic acid, a vacuum-dried sample was oxidized with performic acid (35).

Assay of antibiotic activity and sensitivity tests. Microcin activity was determined by the critical dilution method (28). Ten-microliter samples of serial twofold dilutions in sterile water were spotted on an M63-glucose-agar plate enriched with casein enzymatic hydrolysate. After the drops had dried into the plate, it was layered with 3 ml of soft agar containing about 10^7 indicator cells. *E. coli* AB1133 was used routinely as the indicator strain in microcin activity assays. The plate was incubated overnight at 37°C and examined for growth inhibition. One unit of inhibitor is defined as the quantity present in the 10-μl sample of the last dilution which gave a clear spot. Microcin activity is expressed either as antibiotic units (AU) per milliliter or as micrograms per milliliter. The specific activity was about 100 AU/μg.

Sensitivity to microcin was determined by a cross-streaking assay, as follows. A few colonies of strain AY25 were suspended in sterile water to give about 10^8 cells per ml. Two drops of this suspension was placed near the edge of an M63 plate and allowed to run in parallel down the length of the plate. The plate was incubated at 37°C for 24 h. Fresh colonies of the clones to be tested were then cross-streaked inwards from the edge of the plate. After another day of incubation, only resistant cells grew up to the heavy central strip of producer cells. When a more reliable comparison of the sensitivities of different strains was desired, we used a spot-on-lawn test or determined the MIC of microcin for each strain. For the spot test, doubling dilutions of a microcin preparation were spotted (10 μl) on freshly seeded lawns of the strains to be tested. After overnight incubation, the plates were examined for different degrees of inhibition; the higher the last dilution which produced a clear spot, the more sensitive the strain tested.

MICs were measured by the twofold serial dilution assay (23). Exponentially growing cultures of the test strains (optical density at 600 nm [OD_{600}], 0.4 to 0.5) were suitably diluted in the medium chosen for the test, and 0.25-ml volumes (about 10^3 cells) were pipetted into tubes containing twofold serial dilutions (0.25 ml) of antibiotic. Turbidity was read after 24 h of incubation at 37°C.

Gel filtration. For gel filtration, a 0.1-ml sample (12,000 AU) of HPLC-purified Mcc25 was dried at 37°C, redissolved in 0.4 ml of 1 M acetic acid, and applied to a Sephadex G25 column (1.5 by 30 cm) equilibrated with 1 M acetic acid. The column was eluted with this solvent at a flow rate of 0.25 ml/min. One-milliliter fractions were collected.

Plasmid DNA manipulations. Plasmid DNA was prepared from overnight LB cultures by the alkaline lysis method of Birnboim and Doly (4). Transformation with plasmid DNA was done as described by Dagert and Ehrlich (10).

Curing conditions. Cells (10^3 to 10^4) from a logarithmically growing culture of strain AY25 were inoculated into a series of tubes containing nutrient broth and increasing concentrations of ethidium bromide (from 10 to 120 μg/ml). After 24 h of incubation, the last tube which showed turbidity was selected, and dilutions were plated on LB agar. Individual colonies were tested for loss of microcin production and immunity.

Induction of filaments by Mcc25 and recovery of septation. Tubes containing 4 ml of M63-glucose medium were inoculated with 10^6 cells from stationary-phase cultures of the different strains. Microcin was added at the MIC for each strain. After incubation for 24 h, the filamented cultures were passed through membrane filters (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.), washed once with medium without antibiotic, and suspended in the same volume of LB medium at 37°C. Recovery of septation was then monitored microscopically. In some experiments, filaments were inoculated on slides coated with nutrient agar and incubated at 37°C in a humid atmosphere until they developed into microcolonies.

Prophage induction. Induction of bacteriophage lambda was studied as follows. A culture of strain BM21(λ) growing exponentially in LB medium (OD_{600}, 2×10^8) was split into two parts. One of them received microcin at 0.16 μg/ml (which is double the MIC for this strain), and the other served as a control. Cultures were aerated at 37°C for 30 min, diluted 1:1,000 in fresh LB, and incubated with shaking for 2 h. Samples were taken immediately after dilution and at the end of the incubation to determine the number of CFU and PFU. MC4100 was used as the lambda indicator strain.

RESULTS

Mcc25 production and immunity are plasmid determined. *E. coli* AY25 was found to be resistant to ampicillin but sensitive to other conventional antibiotics. Physical analysis revealed five plasmids. The ampicillin resistance marker was located on one of them, since ampicillin resistance could be transferred by transformation to strain DH5α, with an efficiency of 10^5 transformants per μg of AY25 DNA. Transformants did not become microcin producers; however, this did not exclude the possibility that some of the microcin genetic determinants were encoded by this plasmid.

Plasmid-curing experiments with ethidium bromide allowed us to isolate microcin-nonproducing (Mcc^-) variants with high efficiency (99%). Supernatants from cultures of these cured strains showed no detectable antibiotic activity, and loss of microcin production was concurrent with loss of immunity to the antibiotic. Gel electrophoresis analysis showed that cured strains had lost a plasmid of about 60 kb.

To confirm that the $Mcc25^+$ phenotype was plasmid determined, plasmid DNA was extracted from strain AY25 and used to transform strain DH5α. The transformation mixture was plated on microcin-containing (800 AU/ml) LB medium. The source of microcin was a concentrate of the supernatant of a stationary-phase culture of AY25 in M63-glucose. Transformants were selected on the basis of the immunity conferred by the plasmid. Selection was hampered by the high frequency of mutation to Mcc25 resistance (10^{-5}). Actually, 98% of the clones were not transformants but resistant mutants. However, we succeeded in isolating a few microcin-producing clones. Plasmid DNA analysis showed that they harbored a single plasmid of about 60 kb, which was called pTUC100. The plasmid was subsequently transferred by transformation to strain MC4100, where it was stably maintained. Production of Mcc25 by strains transformed with pTUC100 was comparable to that of the original isolate.

Strains carrying the wild-type plasmid encoding the genetic determinants for Mcc25 production and immunity were fully sensitive to microcins B17, C7, E492, and H47 and to colicin V, a small peptide which is now regarded as a microcin.

We have been unable to detect conjugal transfer of pTUC100 from either AY25 (the natural host) or SBG101 (MC4100 transformed with pTUC100) to several *E. coli* K-12 strains (DH5α, MC4100, and AB1133) even after selecting for Mcc25 immunity.

The Mcc25 genetic system has been cloned into pBR325, and the resulting plasmid directed overproduction of Mcc25. From this construct, a fragment determining only immunity to the antibiotic was subcloned into pBR322 (33a).

Purification. The first attempts to detect inhibitory activity in filter-sterilized culture supernatants of the producer strain AY25 proved unsuccessful. We thought it likely that the antibiotic was being adsorbed to the nitrocellulose membrane filters used (GSWP 02500; Millipore Corp.). In fact, when supernatants were chloroform sterilized, autoclaved, or assayed on plates supplemented with 100 μg of streptomycin (which prevented growth of the producer but not of the indicator strain) per ml, substantial activity was found (800 to 1,600 AU/ml). Later, we found that microcin could be eluted from the filters with either methanol or methanol-water (5:1, vol/vol).

A purification scheme based on the observation that the antibiotic could be efficiently adsorbed on activated charcoal and eluted in active form with 50% pyridine, 1% Triton X-100 (at 60°C), or 80% acetone was designed. We chose to use aqueous acetone for subsequent work. Charcoal also adsorbed pigments from the spent medium. We found that a substantial amount of this contaminating material could be removed by washing the charcoal with 40% acetone. Microcin remained adsorbed under these conditions and was then eluted with 80% acetone. Most of the remaining colored material was removed by precipitation at −20°C in *n*-propanol and preparative TLC. Although at this stage the antibiotic had been significantly purified, it was not pure enough for structural analysis. Final purification was achieved by HPLC (see Materials and Methods for experimental details). Recovery was approximately 50% on an antibiotic-units basis. The purified material was lyophilized, weighed, and dissolved in water to a known concentration. The activity was stable for at least 1 year during storage at −20°C.

General properties of Mcc25. The addition of excess isoleucine to M63 agar plates did not reverse the inhibitory effect of the antibiotic. This ruled out the possibility that Mcc25 was valine (several natural isolates have been found to hyperproduce and excrete this amino acid, which is toxic to *E. coli* K-12). Moreover, the action of Mcc25 was not antagonized by a mixture of the 20 amino acids.

The molecule contained charged sites, as evidenced by its binding to Dowex 50 W X2 at pH 3 and subsequent elution with 0.1 M sodium acetate buffer, pH 5. After TLC or paper chromatography, no reaction was obtained at microcin spots (detected by bioautography) when developed chromatograms were treated with ninhydrin or fluorescamine. However, acid or alkaline hydrolysis followed by chromatography and treatment with ninhydrin revealed the presence of several amino acids. These experiments suggested that Mcc25 was a peptide with a blocked amino terminus.

Concerning its physical properties, Mcc25 was resistant to extremes of pH (ranging from pH 2 to 12) and was fully active after exposure to 100°C for 30 min and even after autoclaving (120°C, 15 min). It was soluble in water, methanol-water (50:50), and methanol at 800 μg/ml, which was the highest concentration tested.

Mcc25 seemed to exist predominantly in an extracellular form. Very little activity (at best, 5% of the total activity of a culture) could be recovered from the cell mass after sonic disruption, osmotic lysis (30), lysis with lysozyme-sodium dodecyl sulfate (4), extraction with cold 1 M acetic acid (36), or boiling in 100 mM acetic acid–1 mM EDTA (12). Control experiments proved that the chemicals used did not inhibit the activity of microcin under the extraction conditions used.

Effect of medium composition on production of microcin. The production of Mcc25 by strain AY25 was strongly influenced by the composition of the growth medium. It was optimal in minimal medium and became hardly detectable in LB medium. Accordingly, no zone of inhibition was produced when strain AY25 was stabbed onto an LB plate which was incubated for 24 h and subsequently seeded with a lawn of sensitive cells. We wondered which component of the rich medium was inhibiting microcin production. To address this question, AY25 was grown in three different media, each one lacking a distinct component of LB (tryptone, yeast extract, or NaCl). It was found that growth in yeast extract repressed the synthesis of microcin, whereas in media consisting of tryptone or tryptone plus NaCl, production almost paralleled that in minimal medium. This suppressing effect of yeast extract on microcin production is currently under investigation.

When lactose or glycerol was substituted for glucose as

TABLE 2. Composition analysis of microcin25[a]

Amino acid	No. of residues
Glu	1
Ser	1
Gly	5
His	1
Thr	1
Ala	1
Pro	2
Tyr	2
Val	2
Ile	2
Phe	2

[a] Two independently purified samples gave identical results.

the carbon source, microcin levels in stationary-phase cultures of strain AY25 increased twofold (from 800 to 1,600 AU/ml). This effect, although weak, suggested that microcin synthesis may be subject to carbon catabolite repression.

Mcc25 was produced under both aerobic and anaerobic conditions.

Amino acid analysis. The amino acid composition of Mcc25 is shown in Table 2. The molecule has a relatively high content of glycine (25%) and hydrophobic residues. The presence of aromatic amino acids could be predicted from the absorption spectrum, which showed a maximum at 276 nm. No cysteine residues were found.

On the other hand, fluorescence measurements showed no emission band at 340 nm when the molecule was excited at 290 nm. The absence of indole fluorescence can be considered good evidence that the peptide lacks tryptophan.

Molecular weight. The natural Mcc25-producing isolate, *E. coli* AY25, was selected in our laboratory by a screening procedure which detected only those inhibitors able to diffuse through dialysis membranes (molecular weight cutoff, 10,000) onto a susceptible strain growing in minimal medium. In addition, Mcc25 could not pass through benzoylated membranes (molecular weight cutoff, 2,000). Based on these data, the inhibitor appeared to have a molecular weight in the range of 2,000 to 10,000. On the other hand, gel

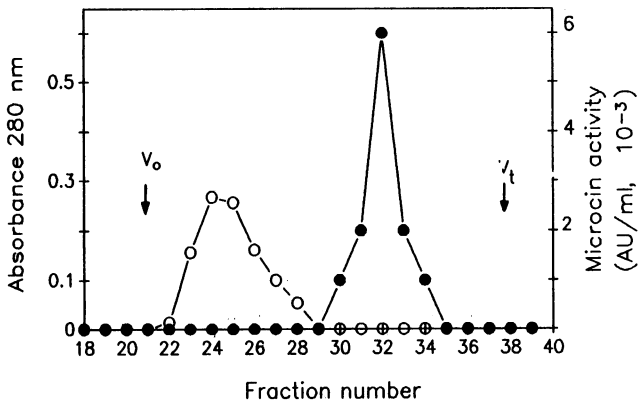

FIG. 1. Sephadex G25 chromatography of Mcc25. Experimental conditions are given in Materials and Methods. Arrows mark the emergence of blue dextran (V_0, void volume) and of cobalt chloride (V_t, total volume). Elution of glucagon was monitored by A_{280} (○). Mcc25 was detected by its antibiotic activity (●, in kilounits per milliliter).

TABLE 3. Antimicrobial activity of Mcc25[a]

Species	No. of sensitive strains/no. tested
Escherichia coli	19/25
Salmonella typhimurium	0/7
Salmonella newport	1/1
Salmonella enteritidis	2/5
Salmonella derby	0/1
Salmonella C_2	0/1
Shigella flexneri	6/7
Shigella sonnei	0/3
Klebsiella pneumoniae	0/1
Klebsiella sp.	0/1
Proteus sp.	0/1
Pseudomonas mendocina	0/1
Bacillus subtilis	0/2
Lactobacillus acidophilus	0/1
Saccharomyces cerevisiae	0/1

[a] Strains to be tested were grown as a lawn on LB plates, onto which a sample of purified microcin (1.5 μg) was spotted and dried. After overnight incubation at 37°C, the plates were examined for growth inhibition zones.

filtration of pure microcin showed that it was small enough to be retained on Sephadex G25. The elution profile of this column, shown in Fig. 1, indicated that Mcc25 was smaller than glucagon, a 3,460-Da peptide.

The amino acid composition and the data from the gel filtration experiment suggested that the molecule was a peptide made of 20 amino acids, with a molecular weight of about 2,100.

Antimicrobial activity of Mcc25. A survey of the sensitivity of several bacterial genera to Mcc25 showed that the antibiotic was active against *E. coli*, *Salmonella*, and *Shigella* strains of clinical origin (Table 3). We have estimated the MICs of microcin in LB, M63-glucose, and M63-glucose enriched with casein enzymatic hydrolysate for *E. coli* strains AB1133 and BM21 and for a sensitive strain of *Salmonella newport* (Table 4). For the *E. coli* strains, enrichment of the culture medium led to an increase in sensitivity to Mcc25. *E. coli* AB1133 was more sensitive than BM21 in all media tested. At present, we have no explanation for this differential susceptibility. The possibility that the *gyrA* mutation in strain BM21 reduces sensitivity to Mcc25 is currently being investigated.

The MIC for AY29 (plasmid-cured derivative of AY25) in LB medium was comparable to that for other sensitive *E. coli* strains (Table 4). Strain AY25, on the other hand, could grow in the presence of as much as 5 μg of the antibiotic per ml, indicating that microcinogenic cells are immune to

TABLE 4. MICs of Mcc25

Strain	MIC (μg/ml) in test medium[a]:		
	M63-G	M63-GH	LB
E. coli BM21	5.0	0.62	0.08
E. coli AB1133	2.5	0.08	0.02
E. coli RYC816	2.5	ND[b]	0.04
E. coli AY29	ND	ND	0.04
S. newport	0.01	0.01	0.01

[a] M63-G, M63-glucose; M63-GH, M63-glucose supplemented with 0.1% casein enzymatic hydrolysate.

[b] ND, not determined.

exogenously added microcin when growing in rich medium, a condition under which they did not produce the antibiotic.

Finally, note that the lowest MIC of microcin in the three media tested was for the *S. newport* strain and that the medium composition had no effect on sensitivity.

Kinetics of synthesis of Mcc25. The antibiotic activity of cultures of strain AY25 in M63-glucose minimal medium was measured during both exponential growth and the stationary phase. No assayable amounts of the inhibitor could be detected in supernatants from cells in the exponential phase of growth, even in 70-fold-concentrated samples. The activity appeared when cultures moved into the stationary phase, reached a maximum titer of 800 to 1,600 AU/ml at 20 to 30 h after the onset of antibiotic synthesis, and had not declined after 4 days of incubation. Strain SBG101 (MC4100 harboring the wild type Mcc25-producing plasmid) still showed this type of control of Mcc25 production (data not shown). Note that SBG101 is a relaxed mutant, which rules out a possible involvement of *relA*-dependent starvation effects on the expression of Mcc25 genetic elements.

Effects of microcin on cell growth and viability. When cultures of the sensitive strains AB1133, BM21, and RYC816 growing exponentially in LB medium were treated with microcin at 0.08 μg/ml, the bacterial mass continued to increase but at a slower rate than in control cultures. Thus, the mass doubling times for strains AB1133 and BM21 in the presence of Mcc25 were about 50 min, compared with 30 min for cultures without antibiotic. Also, the final plateau absorbances were below those of control cultures.

Viability studies were performed in LB medium for BM21 and the sensitive *S. newport* strain with concentrations of microcin two and eight times higher than the MIC for each strain. For BM21, treatment with microcin at 0.16 and 0.64 μg/ml for 7 h caused a drop in viable-cell counts to 47 and 12%, respectively, of the initial values. For *S. newport*, after the same time of exposure to microcin at 0.02 and 0.08 μg/ml, viable-cell counts had fallen to 70 and 37%, respectively, of the initial values. Given the relatively high frequency of mutation to Mcc25 resistance (mentioned above), it was possible that the prolonged incubation period allowed the overgrowth of resistant mutants, causing the number of viable cells to increase. This was in fact occurring, as demonstrated by the following experiment.

A culture of BM21 growing exponentially in LB medium was challenged with 0.64 μg of microcin per ml. Two samples of the culture were taken at various times. One of them was plated on LB, and the other was plated on LB containing microcin to assess the proportion of viable cells that were microcin resistant. Two hours after addition of the antibiotic, the viable-cell count had dropped to 25% of the initial value, and no mutants appeared on the selective plate. After 7 h of incubation, 12% of the cells remained viable, but practically all of them were mutants, since the number of colonies on both selective and nonselective plates was almost identical.

Mcc25 causes cell filamentation. *E. coli* strains BM21, AB1133, and RYC816 growing in LB medium showed filamentous growth when treated with Mcc25 at the MIC. Higher concentrations of the antibiotic (0.6 and 2.5 μg/ml) also induced filamentation. Microscopic examination showed that the cells gradually elongated over the first few hours of incubation in the presence of the antibiotic, until long aseptate filaments were produced (Fig. 2). Therefore, the mass increase seen in microcin-treated cultures represented predominantly an increase in cell length.

Sensitive *Salmonella* and *Shigella* strains also filamented. Microscopic observation of the halo of growth inhibition produced by a drop of microcin on lawns of the sensitive strains indicated in Table 4 revealed greatly elongated filaments dispersed all over the transparent zone. Some filaments developed large spherical bulges, which suggested that normal cell elongation might also be affected by the antibiotic. Resistant mutants and strains harboring the genetic determinants for immunity to Mcc25 did not filament. It should be noted that for strains BM21, RYC816, and AB1133, the block to division was relieved once microcin was removed from the medium. However, not all of the filaments recovered, and some of them eventually lysed.

It was interesting to examine microcin-resistant mutants for changes in penicillin-binding protein 3 (PBP3), which plays an essential role in septation. It is well known that the β-lactam antibiotics furazlocillin and piperacillin induce filament formation through their selective binding to PBP3. We examined a set of 23 spontaneous Mcc25-resistant mutants, derived from strain AB259, for decreased susceptibility to piperacillin. In a preliminary screening by the streak-plate method (23), some mutants showed a slight increase in resistance to the antibiotic. The MIC of piperacillin for two of these mutants, as determined by a serial dilution assay (23), was 3 μg/ml, whereas for the wild-type it was 2.5 μg/ml. At least for one of these strains, the mutation leading to microcin resistance has been mapped to about 8 min on the *E. coli* chromosome (results not shown), which is well separated from *pbpB* (*ftsI* or *sep*), the gene encoding PBP3, located at 2 min on the map.

Filamentation induced by Mcc25 does not appear to be mediated by the SOS system. A possible explanation for the filamentous phenotype was that the SOS system was being induced by Mcc25. This possibility seemed to be ruled out by the fact that strain RYC816 (carrying the *recA56* mutation, which eliminates *recA* protease activity) also grew into filaments when exposed to the antibiotic (Fig. 2D). Moreover, two other *recA* mutants, HB101 (*recA13*) and DH5α (*recA1*), showed the same behavior.

To further prove that the SOS pathway was not involved in filamentation, we examined the effect of microcin treatment on prophage induction with strain BM21(λ). As is well known, treatment of a lysogen with agents that induce the SOS response also causes massive induction of the resident lambda prophage. Exponentially growing cells of strain BM21 were treated with 0.16 μg of microcin per ml, which is sufficient to induce filamentation. After being diluted in fresh medium, the control and microcin-treated cultures yielded 1.5×10^3 PFU/ml. Following incubation for 2 h, phage titers had increased to 60×10^3 PFU/ml in the control and to 7×10^3 PFU/ml in the microcin-treated culture. The difference in phage production may be ascribed to the different cell densities of the cultures, since growth of cells which had been exposed to microcin was delayed relative to that of the control (0.15×10^6 versus 3.3×10^6 cells per ml in the control). Since activation of the SOS response would have resulted in much higher plaque counts, we concluded that no phage induction occurred in the presence of the antibiotic.

DISCUSSION

In this article, we describe a novel peptide antibiotic, Mcc25, which is released into the culture medium by a fecal strain of *E. coli*. The activity was easily detectable in supernatants from cultures grown in minimal medium. Growth in rich medium suppressed microcin production. Amino acid composition analysis of purified Mcc25 showed

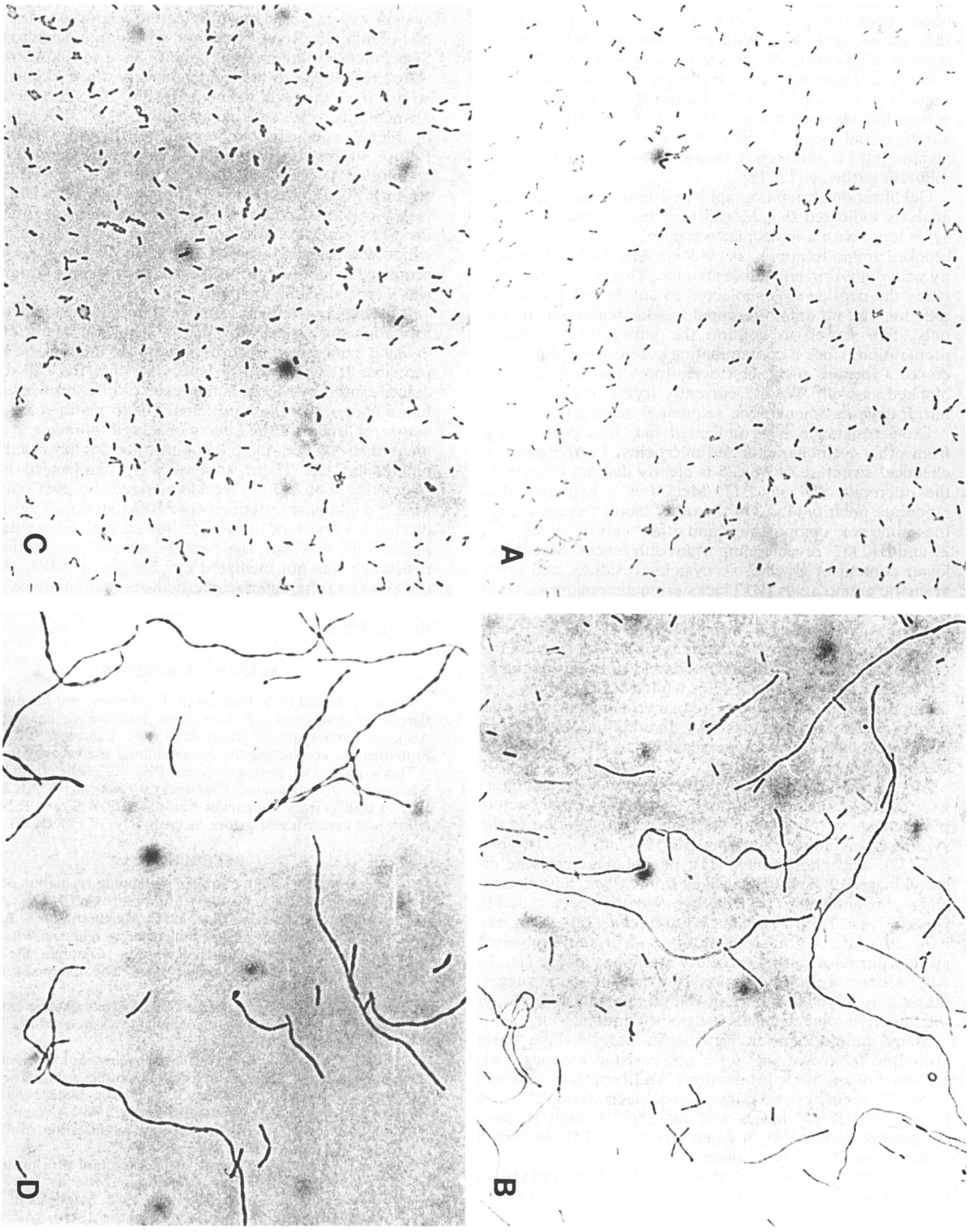

FIG. 2. Division arrest by Mcc25. Filaments were induced as indicated in the text. (A) BM21, control. (B) BM21 treated with Mcc25. (C) RYC816, control. (D) RYC816 treated with Mcc25. Magnification, ×200.

a predominance of uncharged and hydrophobic residues. No cysteine residues were found, which is in agreement with the observation that the majority of exoproteins appear to lack this amino acid (18). With regard to the relatively high content of glycine (25%), it is interesting that several colicins contain a large number of glycine residues, which are thought to contribute to the transport of the polypeptides across the inner membrane (33). Microcin B17, a 3.2-kDa antimicrobial peptide, also shows a high percentage of glycine (60%), although it seems to be excreted through a different pathway (12, 16).

Gel filtration chromatography and amino acid composition analysis indicated that Mcc25 may be a peptide 20 amino acids long, with a molecular weight of about 2,100. It has a blocked amino terminus, as evidenced by its lack of reactivity with ninhydrin and fluorescamine. This was corroborated when the peptide was subjected to automated Edman degradation and no amino-terminal residue was seen. Incidentally, this served to confirm the purity of the microcin preparation, since a contaminating peptide would have generated a residue at the first cycle (provided it was not end blocked as well). We are currently trying to cleave Mcc25 into fragments amenable to sequential degradation.

Cross-resistance tests indicated that Mcc25 is different from other well-characterized microcins. Furthermore, the chemical structure of Mcc25 is clearly distinct from that of the microcins C7 and B17. MccC7 is a heptapeptide of moderate polarity (15). On the other hand, a comparison of the amino acid compositions and other features of microcins 25 and B17 (12) revealed important differences. Mcc25 has a lower content of glycine, no cysteine residues, and several aromatic amino acids (B17 lacks aromatic residues). Unlike Mcc25, B17 is undetectable in unconcentrated supernatants but is easily extractable from cell pellets (12, 20). On the other hand, the bactericidal activity of B17 disappears from cultures after having reached maximal values during the first 2 h of the stationary phase (20), while Mcc25 activity, once accumulated in the medium, remained constant in the stationary phase for as long as 96 h. In addition, *recA* mutants have been shown to be hypersensitive to B17 (21), but this was not observed with Mcc25.

Mcc25 production was found to start as soon as cultures entered the stationary phase of growth. Many other bacterial products show this growth stage-specific regulation of their synthesis, including microcins B17 (20) and C7 (31), glycogen (32), acid phosphatase (11), phosphoenolpyruvate carboxykinase (19), and PBP6 (8), all from *E. coli*; a protease (7) and a phospholipase (17) from two *Serratia* species; and the pectate lyase from *Erwinia chrysanthemi* (22). Also, most antibiotics are synthesized at the end of the exponential growth phase or during the early stationary phase (1). The wide occurrence of this stationary-phase induction suggests that it may have an important role in the cell life cycle, yet the underlying mechanisms are poorly understood.

Many antibiotic-producing species are sensitive during growth to their own antibiotic, and resistance appears with the onset of antibiotic production (26). In contrast, immunity to Mcc25 seemed to be constitutive, since strain AY25, the natural Mcc25 producer, was resistant to high levels of exogenous microcin even when growing in LB medium, in which no antibiotic is produced.

Concerning the mode of action of Mcc25, it appears that the antibiotic disrupts cell division. Sensitive cells exposed to it continue to elongate but fail to septate and thus form filaments. Cell filamentation can arise as a consequence of the blocking of DNA replication and subsequent SOS induction, interruption in chromosome segregation, or inhibition of septation (25). The possible involvement of the SOS system was explored by studying the response to the antibiotic of different RecA$^-$ mutants, which are unable to invoke SOS functions under stress conditions. The RecA$^-$ mutants tested responded to the treatment very much like wild-type strains (i.e., they still filamented) (Fig. 2D). Furthermore, if filamentation resulted from an inhibition of DNA replication by Mcc25 and subsequent induction of the SOS system, RecA$^-$ strains should exhibit an increased sensitivity to the antibiotic. As shown in Table 4, the MIC of microcin for strain RYC816 (*recA56*) was similar to that for BM21, the isogenic RecA$^+$ strain. These observations suggested that the SOS pathway was not involved in the filamentation, which was further substantiated by the fact that no lambda prophage induction resulted when the lysogenic strain BM21 was exposed to the antibiotic.

It is well known that a number of proteins mediate septum formation during cell division (13, 14, 27). Many of these division components are peripheral or integral membrane proteins. It is tempting to speculate that the cell division defect caused by Mcc25 is the result of interference with the function of one of these proteins. The formation of filaments was reminiscent of the effects observed following the treatment of *E. coli* with the β-lactam antibiotics furazlocillin and piperacillin (5). These antibiotics are known to interact selectively with PBP3. We have tested the possibility that Mcc25 might be interfering with PBP3 function. The analysis of mutants resistant to Mcc25 showed that susceptibility to piperacillin was not significantly altered, suggesting that resistance was not mediated by changes in PBP3. Work is under way to characterize microcin-resistant mutants, which should eventually help to clarify the mechanism of action of this antibiotic.

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