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Isolation, Characterization, and Mode of Action on *Escherichia coli* Strains of Microcin D93

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Microcin D93 is an antibiotic substance produced by *Escherichia coli* strains which harbor the 5.5-kilobase plasmid pMccD93. Its production is unaffected by the use of different carbon and ammonia sources, different phosphate concentrations, or mitomycin C. We developed a method for purifying this microcin based on gel permeation chromatography and reverse-phase high-pressure liquid chromatography. The antibiotic appears to be a small, hydrophilic, basic peptide, active on *E. coli* and *Proteus*, *Citrobacter*, and *Pseudomonas* species and much more active on *recA* strains than on their isogenic wild type. Diminution of the rate of DNA biosynthesis without any apparent effect on other macromolecules appears to be a primary effect in the action of microcin D93.

Microcins are dialyzable antibiotics produced by enterobacteria (3). All the microcins so far described are small peptides with molecular weights ranging from 500 to 5,000 (4), with the exception of microcin A15, which seems to be a derivative of the amino acid L-methionine (2). Both production and immunity to these antibacterial agents are coded by plasmid DNA, and their synthesis is not inducible by agents that activate the SOS repair system to DNA damage (7). Except for the plasmidic determination, these characteristics differ from those of colicins, which are antibiotic proteins ranging in size from 27,000 to 80,000 daltons whose synthesis is inducible (15). There is one exception: colicin V, which was the first antibiotic of this group to be described. It is a dialyzable antibacterial peptide, the synthesis of which is not inducible. For these reasons, it has been recently suggested that colicin V might be included in the microcin group (22).

Microcins have been genetically classified according to cross-immunity criteria into five groups (4). A somewhat large fraction of the microcinogenic strains so far isolated (ca. 30%) produces two different microcins. One of these strains is *Escherichia coli* LP93, which produces a microcin the activity of which is reversible by L-methionine (2) and also produces another, microcin D93, whose activity is nonreversible by this amino acid. By mobilization with an antibiotic resistance plasmid and subsequent curing of it, transconjugants on *E. coli* BM21 have been obtained which only harbor the nonconjugative 5.5-kilobase plasmid pMccD93, which codes for microcin D93 synthesis and immunity (4). By using one of these strains, we developed a procedure for obtaining this microcin. In this report, we also show a preliminary structural characterization of the antibiotic and provide experimental data on its mechanism of action on *E. coli* strains.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this work are listed in Table 1. Strains used to determine the antimicrobial spectrum of microcin D93 were clinical isolates from our laboratory collection.

Assay of microcin activity. The production of microcin under both aerobic and anaerobic conditions was tested as previously described (3). BBL Gas-Pak jars (Becton Dickinson) were used to achieve an anaerobic environment. Antibiotic activity was estimated by the critical dilution method under conditions previously described (11). Dilutions of tetracycline were used as standards. One activity unit (a.u.) was defined as described by García-Bustos et al. (11).

The antimicrobial spectrum of microcin D93 was studied on 46 strains belonging to 17 different species as described previously (2). Purified preparations with an activity of 1,000 a.u./ml were used as a microcin source.

Media and growth conditions. *E. coli* 93F was grown in minimal medium M63 (19) with 0.2% (wt/vol) of the appropriate carbon source. MOPS (morpholinepropanesulfonic acid) (20) and 121-salts (16) media were used to measure the influence of different phosphate concentrations on the production of microcin. Substitution of ammonium sulfate by 400 μg of equivalent nitrogen per ml as either L-proline or Casamino Acids (Difco Laboratories, Detroit, Mich.) in the composition of M63 medium was made to evaluate the influence of different nitrogen sources on the production of microcin D93. Lactose, glucose, raffinose, xylose, sucrose, mannitol, glycerol, and lactate were also used as alternative carbon sources to evaluate their effect on the production of the antibiotic.

Induction by mitomycin C was attempted by inoculating single colonies of the producing strain, *E. coli* 93F, in M63 agar plates which contained 0.1 to 1 μg of the inducer per ml. Plates were incubated for 2 days at 37°C and spray seeded with *E. coli* BM21. After 18 h of incubation, production of microcin was evaluated by measuring the diameter of the zone of inhibition. *E. coli* RYC513 and *E. coli* RYC9000 were used as positive and negative controls, respectively, of induction.

Microcin preparation. *E. coli* 93F was grown in minimal medium M63 with 0.2% glycerol at 37°C with vigorous shaking. At the end of the exponential phase of growth, cells

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TABLE 1. Bacterial strains used

Strain	Relevant genotype
E. coli BM21[a]	F⁻ *gyrA*(λ⁺)
E. coli 93F[b]	Like BM21(pMccD93)
E. coli pop3351[c]	F⁻ *araD139* Δ*lacU169* Δ*mal*B1 *rpsL relA thiA*
E. coli RYC819[c]	Like pop3351, *recA56*
E. coli RYC513[c]	Like pop3351(pColE2)
E. coli RYC9000[c]	Like pop3351(pColV)

[a] From our laboratory collection.
[b] From F. Baquero.
[c] From M. Herrero.

were removed by centrifugation, and supernatants were 500-fold concentrated as described previously (3).

The 500-fold concentrates were passed through octadecyl silica columns (Sep-Pak C-18; Waters Associates, Inc., Milford, Mass.). Active fractions were pooled, lyophilized, redissolved in water, and chromatographed on Sephadex G-15. Active fractions were pooled, lyophilized, and redissolved in water.

Reverse-phase high-pressure liquid chromatography (RP-HPLC) was performed with a model 204 liquid chromatograph fitted with a 480 wavelength detector (Waters). The column used was a LiChrosorb RP-18 column (0.4 by 2 cm) (E. Merck AG, Darmstadt, Federal Republic of Germany). All chromatographic runs were done at room temperature.

Thin-layer chromatography was developed on DC-Alufolien cellulose (Merck) with pyridine–*n*-butanol–acetic acid–water (189:122:38:151 [vol/vol/vol/vol]) as the mobile phase.

Protein concentration was determined by the Bradford method (5).

Characterization of microcin D93. The net charge of microcin at pH 7 was determined by observing the displacement of the inhibition zone after electrophoresis in situ (40 V for 5 h) of 32 a.u. of microcin dropped into a well cut out of M63 agar in petri dishes, followed by biological autography with *E. coli* RYC819. Other characterization tests were made as previously described (3). Purified preparations of microcin were used in all cases.

Action of microcin D93 on sensitive strains. Sensitive strains were grown in M63 medium with 0.2% glucose to the mid-exponential phase of growth. At this moment, cells were collected by centrifugation and resuspended in M63 with glucose. An appropriate number of cells were put into flasks containing M63 with glucose and different concentrations of microcin D93. Viability was estimated by plating dilutions of the cultures for the desired lengths of time.

Incorporation of labeled precursors into macromolecules. Exponentially growing cells (2×10^8/ml) of the sensitive strain obtained as described above were deposited in flasks containing M63 with 2 mg of glucose per ml, 15 μg of uridine per ml, 15 μg of leucine per ml, 5 μg of thymidine per ml, and 25 μg of 2′-deoxyadenosine per ml and different concentrations of microcin.

Flasks were incubated at 37°C in prewarmed tubes containing 0.1 μCi of the corresponding precursor, namely DL-[4,5-^{3}H]leucine (43 Ci/mmol) for proteins, [*methyl*-^{3}H]thymidine (42 Ci/mmol) for DNA, and [6-^{3}H]uridine (22.6 Ci/mmol) for RNA. Incorporations were stopped with 5 ml of 5% trichloroacetic acid. Samples with thymidine or uridine were maintained for 3 h at 4°C, and the samples with leucine were boiled for 15 min. Each sample was filtered through a Whatman GF/C filter and washed with cold 5% trichloroacetic acid. The radioactivity retained on the filters was estimated in a Beckman LS-100 scintillation counter.

RESULTS

Microcin D93 preparation. Microcin D93 was produced by *E. coli* 93F both aerobically and anaerobically and was active against *E. coli* BM21 under the same conditions of growth. Treatment with concentrations of mitomycin C ranging from 0.1 to 1 μg/ml did not result in an enhancement of microcin levels, and no significant effect was seen in the production of the antibacterial agent by using different nutrients. The best preparations of microcin D93 were obtained from supernatants of *E. coli* 93F grown with aeration in M63 with glycerol and harvested at the end of the exponential phase of growth. We used glycerol instead of glucose as the carbon source because it exerted a protective effect over the microcin during the 500-fold-concentration step (see below); by using this nutrient it was possible to recover 100% of the microcin activity contrasting with 10% when glucose was used as the carbon source.

Supernatants from cultures of *E. coli* 93F were subjected to 500-fold concentration and methanol precipitation as described elsewhere (3). These concentrates must be considered as a first purification step in which proteins and most of the salts are eliminated by the methanol precipitation (Table 2).

Concentrated preparations of microcin were passed through a Bondapak C-18 column. In this hydrophobic matrix, the antimicrobial agent was eluted with water while pigments and lipids were fully retained. Despite the poor purification obtained (Table 2), this step was necessary to diminish the viscosity of preparations and preserve the RP-HPLC column (see below) from irreversible binding of substances such as pigments or fatty acids, which in some cases could present unspecific inhibitory effects on sensitive strains.

Active preparations from the previous stage were subjected to gel filtration through Sephadex G-15 (Fig. 1A). Microcin activity was eluted near the void volume of the column partially mixed with a basic compound that eluted slightly after the antibacterial agent.

Separation of microcin from the rest of pigments was facilitated by interaction of these with the gel, which made them elute long after the total volume of the column.

After neutralization, active fractions, pooled, lyophilized and redissolved in water, were subjected to RP-HPLC. Despite our having passed the preparations through a Bondapak C-18 column, the column was saturated when more than 800 a.u. were injected, so 700 a.u. were processed each time. In the first attempts, no recovery of the activity was obtained after chromatography. This loss did not agree with the behavior of microcin in Bondapak C-18 (see above) and appeared to be a phenomenon unrelated to the chromatographic procedure. The absorption of biological com-

TABLE 2. Purification of microcin D93

Step	Total activity (10^3 a.u.)	Sp act (10^2 a.u./mg of protein)	Yield (%)	Purification (fold)
Supernatant	162	18	100	1
500-fold concn	153	230	95	13
Bondapak C-18	149	280	92	16
Sephadex G-15	64	740	39	41
RP-HPLC	32	1,780	20	99

pounds to glass is not unusual. Because we suspected a similar behavior for microcin, we used polypropylene tubes for collecting fractions. No loss of activity was seen with this method.

After two consecutive separations by this system, the second of which is shown in Fig. 1B, reinjection of microcin D93 gave only one peak (Fig. 1C) with behavior corresponding to a hydrophilic molecule. Thin-layer chromatography of this fraction gave only one spot with ninhydrin and none with iodine. After going through this process, microcin D93 was purified 100-fold with a yield of 20%.

Structural characterization. Microcin D93 was active after incubation (4 h) at pH 12 but not at pH 2. It lost 90% of its activity after 1 h of incubation at 70°C but remained active for at least 1 year at −20°C. The antibiotic presented a positive net charge at neutral pH and was not sensitive to pronase, subtilisin, or chymotrypsin, but incubation for 1 h with trypsin or proteinase K caused an inactivation of 10%. When incubation with these last two proteases continued for 6 h, the inactivation was total. Gel filtration chromatography of the microcin on Sephadex G-10 and G-15 and Bio-Gel P-2 gave an approximate molecular weight lower than 1,000.

According to these data and to the behavior of microcin when subjected to RP-HPLC, we can conclude that microcin D93 seems to be a small, basic, hydrophilic peptide.

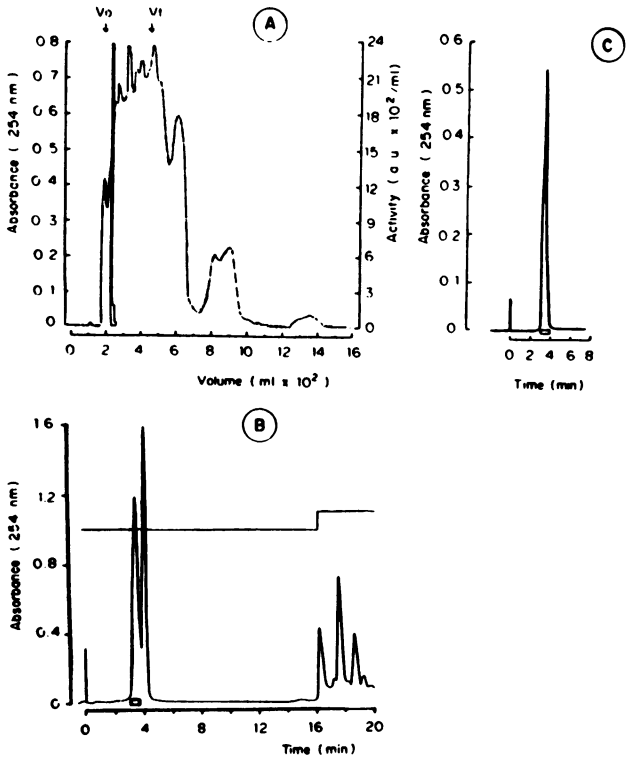

FIG. 1. (A) Gel filtration chromatography on Sephadex G-15 of preparations of microcin D93 obtained after 500-fold concentration and C-18 chromatography steps. The size of the column was 95 by 2.6 cm. Elution was made with water at a flow rate of 50 ml/h. Fractions of 8 ml were taken, and antibiotic activity was evaluated as described in the text. (B) Second RP-HPLC step in the purification of microcin D93. The first eluent was water, and the second eluent was methanol. The flow rate was 0.8 ml/min, and 0.4-ml fractions were taken. (C) Analytic RP-HPLC of microcin D93. Conditions were as described in panel B. In all cases, □ shows microcin.

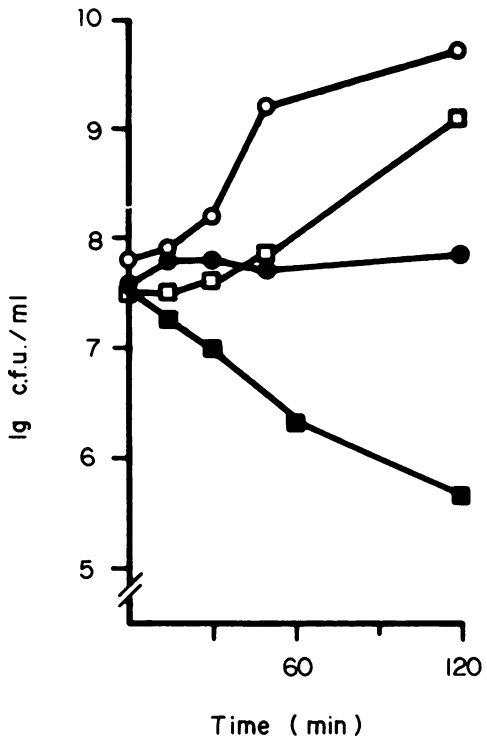

FIG. 2. Activity of microcin D93 on RecA$^-$ and RecA$^+$ strains. Viability of 5 × 10^7 cells of *E. coli* pop3351 and *E. coli* RYC819 was measured during growth without microcin or with 50 a.u. of the antibiotic per ml. Symbols: ●, strain pop3351 with microcin; ○, pop3351 without microcin; ■, RYC819 with microcin; □, RYC819 without microcin.

Antimicrobial specificity of microcin D93. Microcin D93 showed a narrow antimicrobial spectrum which includes *E. coli*, *Citrobacter freundii*, *Proteus mirabilis*, *Pseudomonas maltophilia*, and *Pseudomonas aeruginosa*. The strains of *Candida*, *Enterobacter*, *Klebsiella*, *Rhodotorula*, *Salmonella*, *Staphylococcus*, and *Bacteroides* tested were not susceptible to the concentrations of antibacterial agent used for this study. By measuring the inhibition zone diameter, it appeared that *Pseudomonas* was the most sensitive genus, but these data must be considered as only qualitative since sufficient quantities of purified preparations of the antibiotic are not yet available for the determination of the MICs of microcin D93 for these microorganisms.

Effect of microcin D93 on viability of sensitive strains. There are several methods for screening substances which exert an action on DNA (13). Some of them, based on the differential viability of strains which are defective in DNA-repairing systems with respect to the wild type, have recently been described (14, 18, 21). We used *E. coli* RYC819, which is defective with respect to the SOS repair system to DNA damage. In a first preliminary screening with microcinogenic strains, we saw that *E. coli* 93F was more active on *E. coli* RYC819 than on the *recA*$^+$ *E. coli* pop3351 (data not shown). The same effect was seen when purified antibiotic was used. Microcin concentrations that were bacteriostatic to *E. coli* pop3351 appeared as clearly bactericidal for *E. coli* RYC819 (Fig. 2). The effect of the antibacterial agent on this strain was strongly affected both by its concentration and the size of the bacterial inoculum (Fig. 3). As with conventional

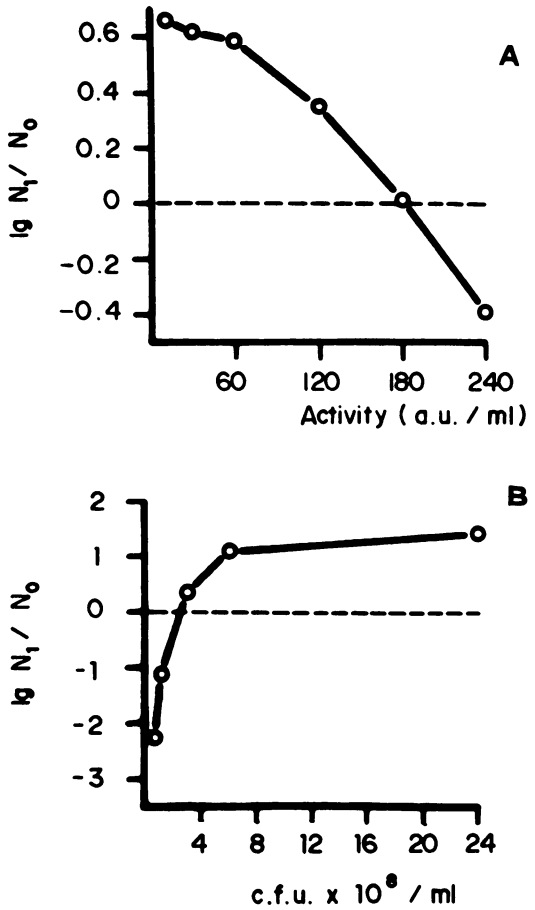

FIG. 3. Influence of microcin concentration and inoculum size on the viability of sensitive cells. (A) Viability of *E. coli* RYC819 with an inoculum size of 2×10^8 CFU/ml was measured against different concentrations of microcin D93. (B) Viability of different concentrations of *E. coli* RYC819 cells in M63 medium with microcin D93 (180 a.u./ml). In both cases, viability was estimated as the fraction N_1/N_0, where N_1 is the number of CFU per milliliter 1 h after inoculation and N_0 is the same at time zero. Microcin was added at the beginning of the experiment.

antibiotics, we defined the minimal inhibitory dose (MID) as the dose of microcin that exerted a bacteriostatic effect on a bacterial inoculum with a fixed size. This value corresponded to 180 a.u./ml when an inoculum of 2×10^8 cells of *E. coli* RYC819 was used as the sensitive marker strain.

Effect of microcin D93 on rate of incorporation of labeled precursors into macromolecules. The rate of biosynthesis of DNA, RNA, and proteins was measured when different quantities of the antibiotic were added to active growing cells of *E. coli* RYC819. Doses of antibiotic 10-fold lower than the MID were sufficient to diminish the rate of DNA biosynthesis by 20% with respect to the control without affecting either RNA or protein biosynthesis (Fig. 4). It was necessary to treat the cells with 20-fold-higher doses to reduce RNA biosynthesis to the same level, while no appreciable reduction in the rate of biosynthesis of protein was observed. Using the MID of microcin, the rate of incorporation of labeled thymidine diminished by 75% with respect to the control, in contrast to 13% for labeled uracil. Incorporation of labeled leucine into proteins was unaffected. The rate of incorporation of diaminopimelic acid into the cell wall was also unaffected during the first hour after an MID of microcin D93 was added to the culture (data not shown).

When the wild-type strain *E. coli* pop3351 was treated with 120 a.u. of microcin per ml, the rate of DNA biosynthesis diminished by 30% with respect to the control without affecting either RNA, protein, or cell wall biosynthesis.

These data are consistent with the enhanced susceptibility of SOS system-deficient cells and point to a primary effect of microcin D93 on the metabolism of bacterial DNA.

DISCUSSION

Since the discovery of microcins in 1976 (3), several works related to their characterization and mode of action have been published (1, 2, 8, 9, 12), but few results related to the purification of these antibiotics have been reported. This situation can be attributed to the low amount of microcin excreted by the producing strains (0.5 μg/ml for microcin C7 [11], 5 μg/ml for microcin A15 [2], and 30 ng/ml for microcin D93 [unpublished data]) and to the difficulties of obtaining good yields in the purification of certain microcins (6; A. Duro, Ph.D. thesis, Universidad Complutense de Madrid, Madrid, 1980). This situation does not occur with colicins, the production of which is amplifiable by mitomycin C and which respond to classical methods for purifying proteins, with the exception of colicin V which, since its discovery in 1925, has still not been purified (22).

Two different methods were used to concentrate microcins from culture supernatants: 500-fold concentration (3) and retention on hydrophobic matrices (6, 11). Although the second method has shown better results in some cases (11), microcin D93 was not retained by Bondapak C-18, and so 500-fold concentrates were utilized to recover the antibi-

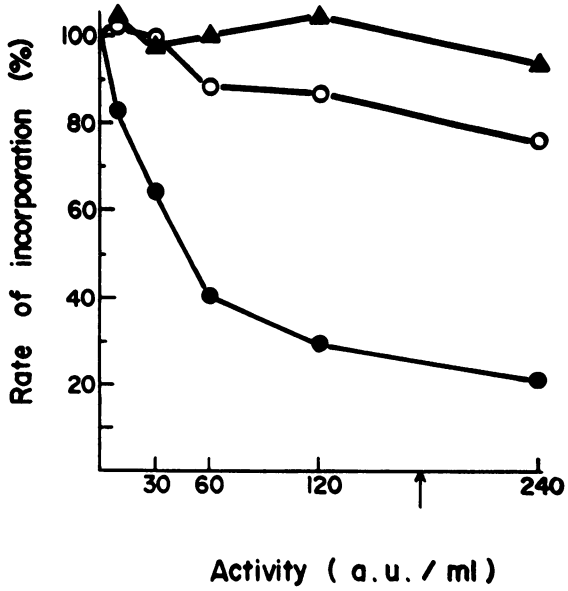

FIG. 4. Influence of different concentrations of microcin D93 on the rate of incorporation of labeled precursors into macromolecules was studied with *E. coli* RYC819. The 100% rate of incorporation was estimated to be the incorporation rate when no microcin was added to the medium. Symbols: ○, rate of incorporation of labeled uridine into RNA; ▲, rate of incorporation of labeled leucine into proteins; ●, rate of incorporation of labeled thymidine into DNA. The arrow indicates the MID of microcin D93 for *E. coli* RYC819 under our assay conditions.

otic. Loss of activity associated with concentration was avoided by the use of glycerol instead of glucose as the carbon source. In this way, we improved 10-fold the recovery efficiency of the antibacterial agent in the first purification and concentration step. As a result of the poor yield obtained when microcins are purified by methods including strongly ionic-exchange resins, we used gentle purification procedures (namely gel filtration chromatography and RP-HPLC) to purify microcin D93. These methods allow purification of the antibiotic 100-fold with an efficiency of 20% (Table 2).

Microcin purified by this method appears to be a hydrophilic, small, basic peptide resistant to the action of several proteases. These characteristics and the fact that the antibacterial agent is both produced and active on minimal medium in anaerobic conditions make sense with the proposed ecological action of microcins in the intestinal tract (3, 4, 7). The narrow antibacterial spectrum that, with the exception of *Pseudomonas* species, includes species related to the producing strain which could directly compete with *E. coli* for the same niches in the intestinal ecosystem is also in line with this hypothesis.

Studies on the mode of action of microcin D93 on sensitive *E. coli* strains led us to conclude that the antibacterial agent exerts a primary action on DNA bacterial metabolism (Fig. 2 and 4). This action is dose dependent and seems to be highly influenced by the inoculum size (Fig. 3). Although this effect could be due to the excretion of low amounts of an antibiotic inhibitor by sensitive strains, as described for other antibiotics (10), supernatants of *E. coli* RYC819 do not show any measurable antimicrocin activity. The fact that mutants naturally resistant to microcin D93 are easily detected (data not shown) could point to the presence of surface receptors to the antimicrobial agent that, as occurs in the case of colicins (17), might retain part of the antibiotic, preventing its action at high cellular concentrations. These mutants and topics related to resistance to microcin D93 are being studied in our laboratory.

According to their structural properties and to the cross-resistance showed by their producing strains, it has been proposed that microcin D93 and D15 are the same or a very similar type of molecule and are closely related to microcin D140 (4). Although the studies on the mechanisms of action have been made with different strains, differences reported for the mode of action of the three antibiotics have caused us to reevaluate the supposition of a structural identity among them. Further biochemical and genetic studies will be necessary to establish the relationship among microcins belonging to group D. Despite the advances shown in this paper, quantities of purified microcin D93 are unfortunately too low to complete the necessary studies on the structure and mechanism of action of this molecule, and the problem with microcins D15 and D140 is similar.

Genetic approaches are under way in our laboratory to improve the production and to establish the relationship among the plasmids responsible for synthesis of D-type microcins.

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