

Microcin E492, a Low-Molecular-Weight Peptide Antibiotic Which Causes Depolarization of the *Escherichia coli* Cytoplasmic Membrane

VICTOR DE LORENZO^{1,2†} AND ANTHONY P. PUGSLEY^{2*}

Instituto de Investigaciones Biomedicas, Facultad de Medicina de la Universidad Autónoma de Madrid, Madrid 28029, Spain,¹ and Unité de Génétique Moléculaire, Institut Pasteur, Paris 75724, France²

Received 1 October 1984/Accepted 10 January 1985

Microcin E492 is a 5,000- to 7,000-molecular-weight peptide antibiotic which depolarizes the cytoplasmic membrane of sensitive *Escherichia coli* K-12 cells. The microcin has many features in common with colicin V but is distinguished from it by the absence of microcin E472 immunity in ColV⁺ strains and by the fact that colicin V-insensitive Cir mutants of *E. coli* K-12 are microcin sensitive. Neither of the two plasmids detected in the producing strain of *Klebsiella pneumoniae* appear to carry the determinants for microcin E492 production or immunity, which is in contrast to the situation found hitherto with other microcins and colicins.

Microcin is the name chosen to describe a novel group of peptide antibiotics produced by many types of bacteria including the family *Enterobacteriaceae* (2, 9). These antibiotics are strikingly different from most of the more extensively characterized colicins and related bacteriocins in that they are much smaller (apparent molecular weights, <7,000) and are produced and released from the cells by a process which does not depend on the activation of the SOS regulatory circuit and which neither is lethal nor involves lysis (2, 18). Early studies indicated that microcins, like colicins, were probably plasmid encoded (1), but this has not been rigorously demonstrated in every case. Studies on the modes of action of some microcins have revealed similarities with certain colicins, but other microcins appear to have unique activities (2, 9, 18).

The microcin which is the subject of the present report, E492, is produced by a clinical isolate of *Klebsiella pneumoniae* and has been partially purified from the cell-free medium of cultures of the producing strain by high-pressure liquid chromatography (8, 10). These preliminary studies revealed that microcin E492 is a highly hydrophobic and basic molecule with an apparent molecular weight of 5,000 when examined by gel filtration (8). Microcin activity was lost after treatment with the proteases pronase or chymotrypsin, indicating that this microcin is probably a polypeptide containing 30 to 50 amino acids. As with other microcins, however, microcin E492 could not be visualized directly in stained polyacrylamide gels after electrophoresis (8), probably because only very small amounts of material were applied to the gels and because the gel system used was incapable of resolving small, hydrophobic polypeptides such as microcin. We therefore adapted the electrophoresis system used previously to detect small polypeptides (21). Microcin E492 remained active in the presence of sodium dodecyl sulfate (SDS), and we were therefore able to localize microcin after electrophoresis by placing slices cut from the gel onto seeded soft L broth agar (17) and then incubating the plates to detect zones of growth inhibition. When strain BZB1011, BZB1019, or BM21 was used (Table 1), a

zone of activity was detected at a position corresponding to a molecular weight of 5,000 to 7,000. No activity was detected when strain BZB1192 (*tonB*; microcin E492 insensitive) was used as the indicator. When gels were stained with Coomassie blue or with silver (25), a poorly defined band was detected in the same position as the active microcin (Fig. 1). Two other, slower-migrating bands were also detected by both staining methods, and an additional, faster-migrating, diffuse band was detected by silver staining (Fig. 1). Similar results were obtained when samples of ¹⁴C-amino acid- or [³⁵S]methionine-labeled microcin extracts were tested. In these cases, however, the material comigrating with active microcin was resolved into two bands (Fig. 1; data not shown). Ideally, one should confirm that one or both of these bands correspond to microcin by testing extracts of medium from nonproducing strains, but we have thus far failed to obtain Mcc⁻ mutants by nitrosoguanidine or Mucts Ap Tn9 mutagenesis of strain RYC492; the only mutants detected by patch tests (21) appeared to produce increased amounts of a microcin inhibitor, possibly enterochelin (10, 19; unpublished data). The molecular weight of microcin E492 determined by this method is in good agreement with that determined by gel filtration (8), but the present estimate may be incorrect due to the abnormal migration of hydrophobic molecules such as microcin E492 in SDS-polyacrylamide gels (4).

A microcin E492 plasmid? The microcin E492-producing strain RYC492 is resistant to 30 µg of kanamycin per ml. DNA extracts prepared from this strain by the methods of Holmes and Quigley (12), Kado and Liu (13), or Portnoy and White (quoted in reference 6) contained two plasmids (pVC1, ca. 60 kilobases, and pVC2, ca. 4.3 kilobases) when examined by agarose gel electrophoresis in 0.5 to 1.2% agarose gels with Tris acetate buffers (15). Plasmid pVC1 could be conjugally transferred (17) to *Escherichia coli* K-12 BZB1019 or to *K. pneumoniae* UNF (Table 1) with selection for kanamycin resistance. The recipients were shown to carry pVC1 but not pVC2, and all of them failed to produce microcin and were susceptible to it. No transconjugants were obtained when selection for microcin immunity, either alone or in conjunction with kanamycin resistance, was used. These results make it unlikely that pVC1 carries the determinants for microcin production or immunity.

* Corresponding author.

† Present address: Department of Biochemistry, University of California, Berkeley, CA 94720.

TABLE 1. Bacterial strains

Strain	Plasmids, genotype, and phenotype	Source or reference
<i>K. pneumoniae</i>		
RYC492	pVC1 pVC2 Km ^r Mcc ⁺ Mcc ^{imm+}	10
UNF5023	<i>hisD2 hsdR rpsL</i> Mcc ⁻ Mcc ^{imm-}	C. Emerlich
<i>E. coli</i> K-12		
BM21	λ ⁺ <i>gyrA</i>	F. Moreno
BZB1019	<i>met gal rpsL hsdR</i>	21
BZB1011	<i>gyrA</i>	21
RK5	BM21 <i>tonB</i>	This study
BZB1022	<i>cir</i>	This study
BZB1192	<i>gyrA tonB</i>	This study

Plasmid pVC2 was extracted from the microcin-producing strain and used to transform (15) *E. coli* K-12 BZB1019 with selection for microcin immunity. No transformants were obtained. The plasmid, which was shown to be amplifiable by chloramphenicol treatment, was then purified and digested with various restriction endonucleases to construct a physical map. Samples of the plasmid then were digested with *SalI* (one site), *BamHI* (two sites; 2,000- and 2,300-base-pair fragments), *BglII* (two sites; 1,900- and 2,400-base-pair fragments), or *HindIII* (2 sites; 1,800- and 2,500-base-pair fragments), all of which cut at disperse sites on the plasmid (data not shown), and the fragments were ligated (15) into pBR322 (15) cut at the same site (or at *BamHI* to receive the pVC2 *BglII* fragments). The ligation mixtures were used to transform strain BZB1019 (15) with selection for ampicillin resistance. Plasmids carrying all possible fragments of pVC2 were thus obtained, but none of the transformants produced microcin or were immune to it. Plasmids extracted from representatives of each class of transformants were used to transform *K. pneumoniae* UNF5023 with selection for ampicillin resistance (15). Once again, none of the transformants produced microcin. Since it is unlikely that all of the restriction endonuclease cleavage sites would be in genes essential for microcin production or immunity, or both, we conclude that these determinants are probably not located on pVC2. This raises the interesting possibility that microcin E492 determinants, unlike those for colicins and other microcins (2, 9, 18), are located either in the chromosome or in a thus far undetected and nonmobilizable large plasmid.

Mode of action of microcin E492. Preliminary experiments indicated that the addition of microcin to growing *E. coli* K-12 cultures led to a rapid loss of ability to incorporate precursors into RNA, DNA, proteins, and cell wall components (data not shown). The culture turbidity did not decline, although growth was almost immediately arrested, β-galactosidase was not released, and there was no change in the rate of extracellular *o*-nitrophenyl-β-D-galactoside hydrolysis by preinduced cytoplasmic β-galactosidase (not shown). These results rule out the possibility that microcin E492 caused cell lysis or large-scale increases in membrane permeability, and we therefore investigated the possibility that this microcin, like colicins A, E1, I, K, and V (14), affected the energy-transducing processes of the cytoplasmic membrane.

Microcin E492 effected a rapid arrest of leucine, serine, phenylalanine, and diaminopimelic acid accumulation by susceptible *E. coli* K-12 cells (Fig. 2). Since these substrates include representatives of those compounds accumulated by both phosphate bond energy-dependent and membrane potential-dependent transport systems (3), this result indicates

that the energetic state of the cells was severely affected by microcin treatment. There was no effect on leucine uptake when the microcin was replaced by 90% methanol (the solvent used to dissolve microcin) or by an inactive preparation obtained from a "low-producing" mutant strain (see above) or when *TonB* (microcin-insensitive) strains were used. These results indicate that the observed effects are indeed due to microcin.

Further evidence for the detrimental effect of microcin E492 on the energetic state of the cells was provided by the observation that microcin potentiated the lytic effects of the detergents Triton X-100 (Fig. 3) and SDS (not shown) (5, 24). Once again, strains carrying the *tonB* mutation were unaffected by microcin plus detergent.

To ascertain whether microcin E492 affected the transmembrane electrical potential directly, we used the lipophilic ion tetraphenylphosphonium (TPP⁺), which is passively accumulated as a function of the electrical potential (22). Cells which were preloaded with TPP⁺ were unable to retain the ion in the presence of microcin E492 (Fig. 4). Since there was no evidence for nonspecific increases in membrane permeability provoked by microcin (see above),

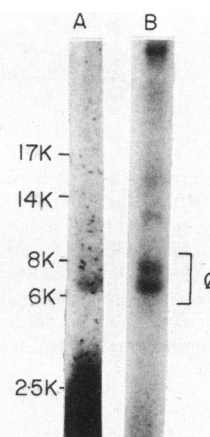


FIG. 1. SDS-polyacrylamide gel electrophoresis of partially purified microcin E492 from strain RYC492. Sample A was stained with silver (25) after electrophoresis, and sample B was prepared from the cell-free medium of a culture grown in the presence of ¹⁴C-amino acids (0.2 μCi/ml, 1.8 mM). Microcin was extracted from cell-free medium from overnight cultures in citrate minimal medium (8, 23) by passing it directly through Sep-Pak C18 silica columns (Waters Associates, Inc.) as previously described (8). The cartridges were washed with 60% methanol, and the active microcin was eluted with 90% methanol. The extract was centrifuged at 50,000 × g for 1 h to remove particulate matter. The extracts were stored at -20°C in 90% methanol until they were used. Further purification was not applied because of the extensive loss of activity incurred (8). Samples for electrophoresis were dried under vacuum in a SpeedVac centrifuge (Savant Instruments, Inc.) and resuspended in SDS sample buffer without heating. The electrophoresis system was essentially the same as that used previously (21), except that the gradient gel was replaced by a uniform gel containing 11.5% acrylamide, 0.59% bisacrylamide, and 8 M urea. After electrophoresis, the gels were fixed in 20% trichloroacetic acid in 50% ethanol and then either stained with silver (A) or treated with En³Hance (New England Nuclear Corp.) and exposed to Kodak X-Omat film for 1 month (B). Molecular weight standards were cyanogen bromide fragments of myoglobin (British Drug Houses). The region marked ∅ corresponds to the position to which active microcin migrated, as revealed by slicing an unfixed gel and placing the slices on agar plate seeded with an indicator strain before overnight incubation.

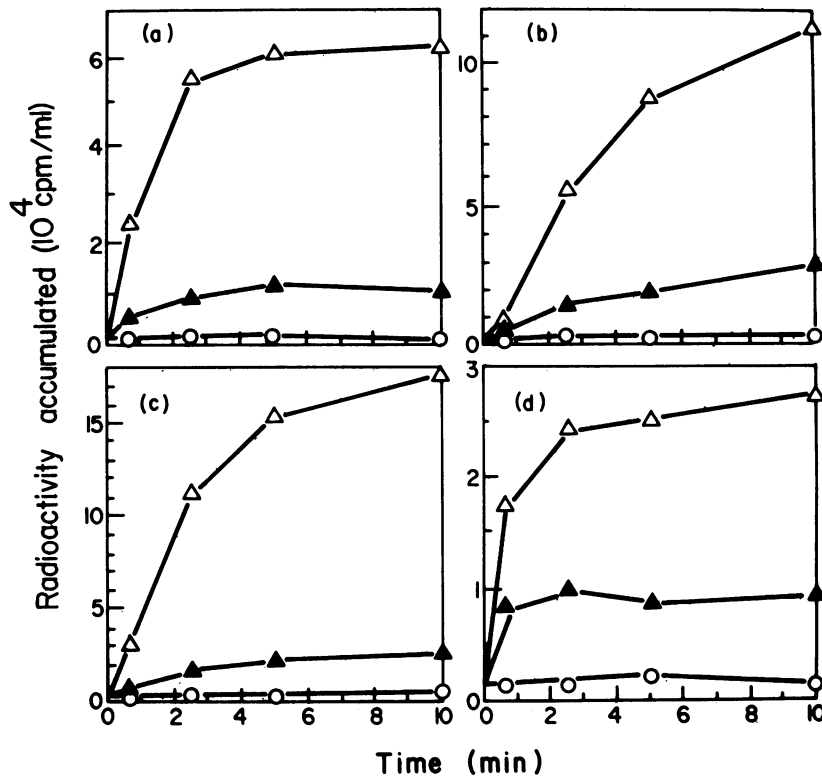
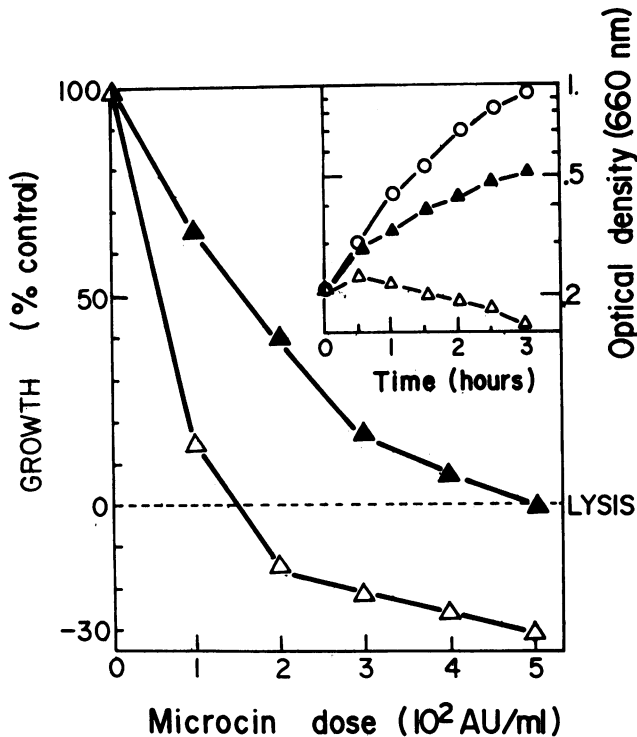


FIG. 2. Effects of microcin E492 on amino acid transport in *E. coli* K-12. Transport assays were performed essentially as described by Berger and Heppel (3). Strain BM21 (Table 1) was grown in M9 medium (17) containing 0.4% glucose to an optical density at 660 nm of 0.35. Chloramphenicol (80 μ g/ml) was then added, and the cells were held briefly on ice until use. In the transport assay, the cells were warmed to 37°C, and the microcin (sufficient to prevent colony formation by 95% of the cells) was added together with one of the following: [14 C]leucine (25 μ M, 25 Ci/mol), [3 H]diaminopimelic acid (10 μ M, 60 Ci/mol), [14 C]serine (20 μ M, 25 Ci/mol), or [14 C]phenylalanine (50 μ M, 30 Ci/mol) (all from Amersham International). Samples were removed periodically and filtered through nitrocellulose filters which were then dried and counted (20). Symbols: Δ , control cell, no microcin; \blacktriangle , microcin-treated cells; \circ , toluenized (fully permeabilized) cells. (a) [14 C]leucine; (b) [3 H]diaminopimelic acid; (c) [14 C]serine; (d) [14 C]phenylalanine.



this result indicates that microcin E492 depolarizes the cytoplasmic membrane.

Microcin E492 is not colicin V. Several properties of microcin E492 are similar to those of colicin V. These include their similar, small size (11), hydrophobicity (8, 26), and modes of action (26; this paper). In addition, mutations in *tonB* (as in strain BZB1192 or RK5) made cells insensitive to both agents (7; unpublished data). The two agents were differentiated, however, by the fact that a series of 10 *E. coli* K-12 strains carrying different natural ColV plasmids (sup-

FIG. 3. Effects of 1% Triton X-100 on the growth of *E. coli* K-12 BM21 in the presence of microcin E492. The combined effects of microcin and detergent were determined as described previously (24) with minor modifications. Cells were grown to an optical density at 660 nm of 0.2. The culture was then divided into several 5-ml lots in sidarm flasks with or without 1% Triton X-100 to which various dilutions of a partially purified microcin preparation were then added. The values shown are increases in culture turbidities after an additional 3 h of incubation relative to the maximum (100%) obtained without detergent or microcin. Triton X-100 alone did not affect growth. Symbols: \blacktriangle , cultures containing microcin alone; Δ , cultures containing microcin plus Triton X-100. The inset shows the effects of Triton X-100 on the growth of strain BM21 treated with a subinhibitory dose of microcin. Symbols: \circ , growth of control culture containing only Triton X-100; Δ , culture containing only microcin (200 arbitrary units [AU] per ml [8]); \triangle , culture containing Triton plus microcin.

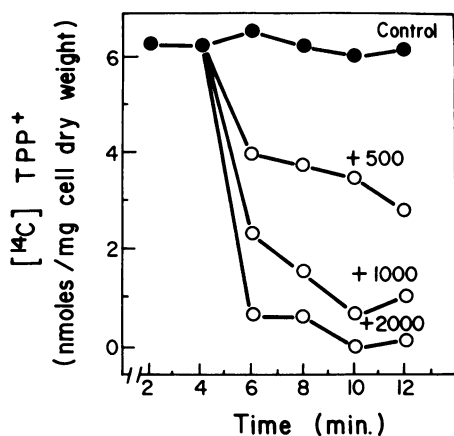


FIG. 4. Effects of microcin E492 on the accumulation of TPP⁺ by *E. coli* K-12 cells. Cells from an exponentially growing culture of strain BM21 (Table 1) in M9 medium (17) plus 0.4% glucose were permeabilized with EDTA (22) and then resuspended to an optical density at 660 nm of 3.0 (ca. 1 mg of cell [dry weight] per ml) in buffer containing 50 mM K₂HPO₄ (pH 6.6), 10 mM MgSO₄, and 0.8% glucose. The cells were then mixed with 3 μM tetraphenyl borate for 90 s at 37°C. ¹⁴C-TPP⁺ (30 μM, 6.3 Ci/mol; Amersham) was then added, and the cells were allowed to equilibrate for 2 min. Of this cell suspension, 0.9 ml was then mixed with 0.1 ml of an appropriate dilution of microcin, and 0.1-ml samples were removed periodically, filtered through GFC filters (Whatman, Inc.), washed with 5 ml of 0.1 M LiCl, dried, and counted (20). Nonspecific retention of TPP⁺ was determined by using cells permeabilized with toluene. Symbols: ●, endogenous levels of ¹⁴C-TPP⁺ retained by the cells; ○, effects of adding various amounts of microcin (in arbitrary units per milliliter [8]) at 4 min.

plied by P. Fredericq) and *E. coli* K-12 strains carrying recombinant ColV plasmids described by Frick et al. (11, 26) all showed high-level immunity to colicin V but were fully susceptible to microcin E492 when tested by the endpoint dilution method (16) (data not shown). Furthermore, strains carrying a *cir* mutation (e.g., BZB1022) were not susceptible to colicin V (7) but fully susceptible to microcin E492 (data not shown). We conclude that colicin V and microcin E492 are different, although they may be closely related.

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