

The Nature of the Colicin K of *Escherichia coli* K235

(*Proteus mirabilis*/bacteriocin/mitomycin C)

WALTHER F. GOEBEL

The Rockefeller University, New York, N.Y. 10021

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ABSTRACT When *E. coli* K235 is grown in the presence of mitomycin C it elaborates part of its colicin as a protein, which has been purified by gel filtration and electrofocusing to yield a product that is serologically homogeneous. The colicin K of *E. coli* K235 is chemically and serologically identical with that of *Proteus mirabilis*. Like the colicin of the latter, the *E. coli* bacteriocin occurs in multiple forms that exhibit slight differences in mobility upon electrophoresis in a polyacrylamide gel.

The exact chemical nature of the colicins has been established only in the past several years, an achievement made possible by the addition of inducing agents to the culture media in which the colicinogenic microorganisms are grown (1). When we began our investigations of these potent bacteriocidal agents in 1953 not much was known of them. Our early work (2) revealed that both colicins K (3) and V (4), obtained from the culture medium of the respective microorganisms, were invariably associated with the O antigen of the bacterium in question—an observation that was subsequently corroborated by others (5). We showed, furthermore, that when type K colicinogeny was transferred from a strain of *Escherichia coli* to an unrelated dysentery bacillus by mating, the bacteriocin and O antigen of the latter were inseparable (6). Despite this, we have always been alert to the possibility that the two might well be separate entities. Indeed, we showed that when the O antigen-bacteriocin complex was dissociated into its components, the lipopolysaccharide and protein, it was the protein that bore the bacteriocin (3). Thus, the protein nature of these agents was at that time firmly established.

The first investigator to obtain a bacteriocin from cultures of a colicinogenic microorganism by induction with ultraviolet light was Reeves (7), who isolated colicin F (now colicin E₂). He showed it to be a protein, albeit his substance was still contaminated with carbohydrate. Colicin K from a strain of *Proteus mirabilis* was the first of the bacteriocins to be obtained in a state of purity and to be unequivocally characterized as a protein (8). Its molecular weight, amino-acid composition, and immunological properties were all thoroughly described (9). Shortly thereafter, this same bacteriocin was obtained from a strain of *E. coli* K12 (10), and several years later a brief description of its isolation from *E. coli* K235 appeared (11). More recently, the isolation of colicins A, E, Q, and K, presumably in states of purity, was described (12). Some months after the characterization of colicin K from *P. mirabilis* (8) was made, an account of colicins E₂ and E₃ appeared (13). This was followed several years later by a description of colicins E₁, (14), I (15), and D (16). In all instances the bacteriocins, obtained from mito-

mycin-induced bacterial cells or from their culture medium, proved to be protein in nature.

In this communication we shall describe the isolation of colicin K from a culture of *E. coli* K235 induced with mitomycin C, and we shall show that the bacteriocin apparently exists in two forms—one a protein identical in all respects with the colicin K from *P. mirabilis*, the other the bacteriocin associated with the O antigen of the bacillus (and will be described in a later communication).

EXPERIMENTAL

Preparation of Colicin K. The colicinogenic bacillus *E. coli* K235, which elaborates colicin K and which was used in earlier studies from this laboratory, was grown in a casamino-acid medium at constant pH as described (3). When the bacteria had reached a concentration of 5×10^8 cells per ml mitomycin C (0.5 μ g/ml) was added. After 4 hr at 37°, the bacteria were killed with chloroform and the culture medium was allowed to stand overnight at 4°. The microorganisms were then harvested. The moist cells (400 g) were extracted 15 times in all with 800-ml portions of 1 M NaCl at pH 7.0 and 23° (9, 13). Each time the bacteria were centrifuged and the crude colicin was recovered from the cell-free supernate by precipitation with ammonium sulfate at 75% saturation. Extracts 3-15 yielded a total of 5.9 g of crude colicin K containing 5×10^6 units of bacteriocin per mg. [A unit of colicin and its method of determination have been described (3).] The bacteriocin was partially purified by precipitating a 2% solution of the crude colicin with ammonium sulfate and recovering the fraction that precipitated at 33-75% saturation. 2.8 g of partially purified colicin was thus obtained.

The protein was further purified by chromatography on Sephadex G-150, superfine grade (Fig. 1). It can be seen from the figure that upon elution two biologically active fractions emerged from the column in two distinct peaks. The effluents underlying each were combined, and the bacteriocidal material was recovered by precipitation with ammonium sulfate at 75% saturation. This was followed by centrifugation, dialysis, and lyophilization. Fraction 1 (72 mg), containing 8.0% nitrogen (17) and 48% carbohydrate (18), was composed primarily of the O antigen of the bacillus associated with the bacteriocin. The properties of this complex and its identification will be described in a later communication. The activity of fraction 1 was low; it contained 8×10^4 units of bacteriocin per mg. Fraction 2 (91 mg), on the other hand, was predominantly protein (12% N). Its biological activity was very high, for it contained 2.5×10^6 units of bacteriocin

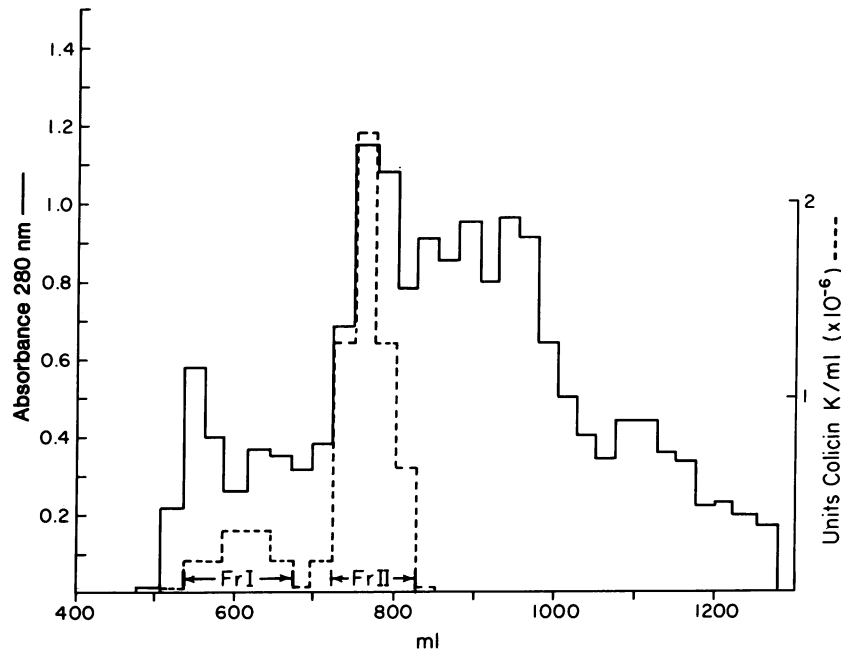


FIG. 1. Gel filtration of colicin K. Elution profile of bacteriocin from aqueous extracts of *E. coli* K235 on a G-150 Sephadex (superfine grade) column. Bed dimension: 5×87 cm; rate: 21.8 ml/hr; buffer: 0.025 M Tris-HCl-0.3 M NaCl at pH 8.0; sample: 0.58 g of colicin K in 30 ml of buffer. Absorbance at 280 nm, —; bactericidal activity (units of colicin K per ml $\times 10^{-6}$), - - -.

per mg. The latter was not pure, however, for when tested against an antiserum to crude colicin K that contains a multiplicity of antibodies to the intracellular components of *E. coli* K235 (19), it not only formed a strong colicin line, which merged with that of pure *P. mirabilis* colicin K (Fig. 2A), but in addition it showed several other serologically active components.

Electrofocusing. Fraction 2 obtained from the Sephadex G-150 column was further purified by electrofocusing. Thus, 100 mg of bacteriocin was subjected to isoelectric fractionation in a sucrose-ampholine gradient (pH 4.0-6.0), a column of 400 ml capacity (20). The experiment is shown graphically in Fig. 3, where it is seen that the bacteriocin is sharply localized at 290-330 ml between pH 5.2 and 5.8. This material (fraction 3, 35.2 mg) was recovered, after short dialysis to remove sucrose and ampholine, by precipitation with ammonium sulfate (75%). Fraction 3 contained 5×10^6 units of colicin K per mg. Fractions 1 and 2 had but 4×10^6 units each and were discarded. Fraction 3, the colicin, still gave a feeble serological reaction when tested against an antiserum elicited by a fraction obtained from a mitomycin-induced culture of the noncolicinogenic variant of *E. coli* K235 (19). In its mode of isolation this bacterial fraction corresponded in every respect to the crude colicin K fraction obtained from the colicinogenic variant of *E. coli* K235, save that it was devoid of colicin activity. Although the serological test is not illustrated here, it indicated that the colicin K was still contaminated with a very small amount of a bacterial substance other than the bacteriocin itself.

This impurity was readily eliminated by filtration of 50 mg of fraction 3, obtained from two preparations of electrofocused material, once more through Sephadex G-150, superfine grade (Fig. 4) to yield the bacteriocin (fraction 2, 28.7 mg) serologically homogeneous. This substance proved

to be serologically identical with the colicin K of *P. mirabilis* (Fig. 2B).

Properties of Colicin K from *E. coli* K235. The colicin of *E. coli* K235 is a protein. Its nitrogen content is 16.22%, carbon 50.36%, and hydrogen 6.98%. These values are in excellent agreement with those reported by Jesaitis (9) for *P. mirabilis* colicin K. The ultraviolet absorption spectrum of the *E. coli* colicin, though not illustrated, was typical of proteins. The material gave no qualitative test for carbohydrate.

The colicin K of *E. coli* K235 is a potent antigen. When mixed with Freund's adjuvant and injected intradermally (1 mg) in rabbits, the substance evokes excellent colicin-

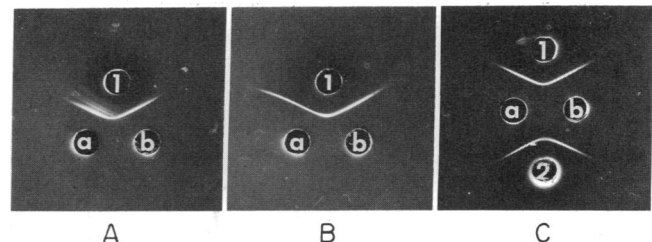


FIG. 2. Gel precipitation reactions of *E. coli* K235 colicin K. (A) Colicin K, obtained by chromatography on Sephadex G-150. (a) Colicin K (*E. coli* K235) fraction 2; (b) colicin K (*P. mirabilis*); (1) antiserum to crude colicin K from *E. coli* K235. (B) Colicin K purified by isoelectric fractionation and refractionation on Sephadex G-150. (a) Purified colicin K (*E. coli* K235); (b) colicin K (*P. mirabilis*); (1) antiserum to crude colicin K (*E. coli* K235). (C) Purified colicin K (*E. coli* K235) and colicin K (*P. mirabilis*) in homologous and heterologous antisera. (a) Purified colicin K (*E. coli* K235); (b) colicin K (*P. mirabilis*); (1) antiserum to purified colicin K (*E. coli* K235); (2) antiserum to purified colicin K (*P. mirabilis*). Concentration of antigen solutions, 0.5 mg/ml.

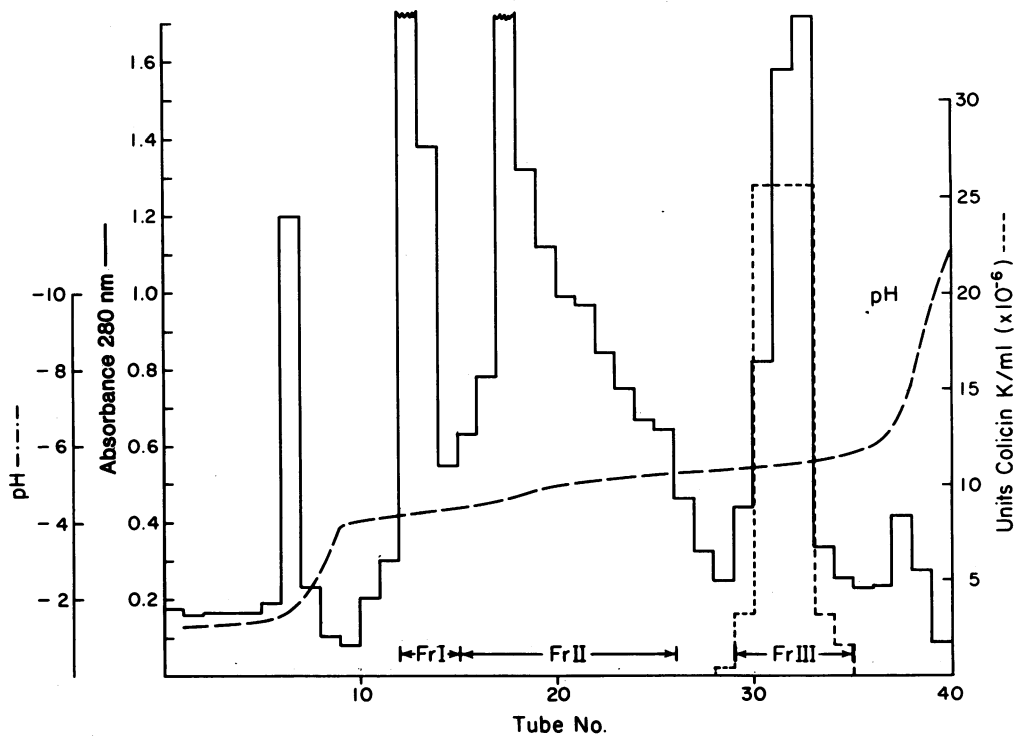


FIG. 3. Isoelectric fractionation of chromatographically purified colicin K from *E. coli* K235 in ampholine-sucrose gradient. The figure shows the absorbance, pH, and bactericidal activity of the effluents from the gradient column; 0.17-g sample of colicin K (Sephadex G-150, fraction 2) was electrofocused in a 400-ml column containing 0.8% ampholytes (pH 4.0-6.0) for 48 hr at 400 V and 4°. Contents of column were distributed in 40 tubes. Absorbance at 280 nm, —; bactericidal activity (units of colicin K per ml $\times 10^{-6}$), - - -.

precipitating and neutralizing antibodies. When such an antiserum is tested against the homologous purified colicin

and the colicin K of *P. mirabilis*, the two lines converge, thus indicating the identity of the two bacteriocins. Conversely, the two substances when tested against *Proteus* colicin antiserum also exhibit identity (Fig. 4C).

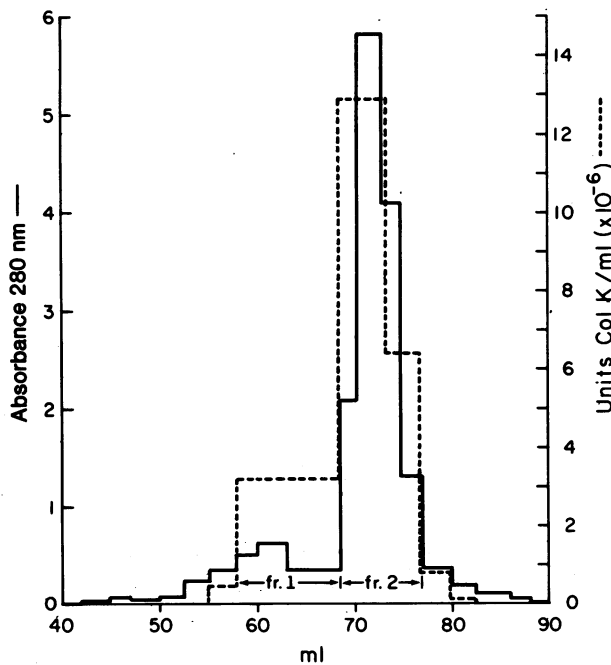


FIG. 4. Gel filtration of colicin K. Elution profile of bacteriocin purified by isoelectric fractionation on a Sephadex G-150 (superfine grade) column. Bed dimension: 1.38 \times 83 cm; rate: 1.6 ml/hr; buffer: 0.025 M Tris-HCl-0.3 M NaCl at pH 8.0; sample: 50 mg of colicin K in 4 ml of buffer. Absorbance at 280 nm, —; bactericidal activity (units of colicin K per ml $\times 10^{-6}$), - - -.

Gel Electrophoresis. The highly purified colicin K of *E. coli* K235, obtained by electrofocusing and refractionation through Sephadex G-150, superfine grade, was subjected to electrophoresis in polyacrylamide gel at pH 8.0 in a vertical cell (EC 470, E-C Apparatus Corp., Philadelphia). As can be seen in Fig. 5, the colicin K derived from *E. coli* K235, like that of *P. mirabilis*, occurs in multiple forms having different electrical charges. The two colicins form three bands that migrate anodically at identical relative rates. One can conclude from this and from the values of the amino-acid analysis given below that the two colicins, each from a different microorganism, are identical. In this regard it should be mentioned that colicins E₂ (13) and E₃ (21) also appear in multiple forms. The fact that the two bacteriocins migrate in the gel at identical rates indicates that the molecular weight of *E. coli* K235 colicin is the same as that of *P. mirabilis* (45,000). This assumption is further supported by the observation that when K235 colicin K is placed upon a G-200 Sephadex column, it emerges at the same elution volume as does *Proteus* colicin K.

APPENDIX

Dr. Tony Hugli*, The Rockefeller University, New York, N.Y. 10021.

* Present address: Scripps Clinic and Research Foundation, La Jolla, Calif.

Amino-Acid Analysis of Colicin K from *E. coli* K235. A sample of lyophilized colicin K from *E. coli* K235 weighing 3.03 mg was dissolved in 0.35 ml of water. Two 50- μ l aliquots of the protein solution were hydrolyzed for 24 and 48 hr, respectively, in 6 M HCl at 110° under reduced pressure. Tryptophan was determined on two independent 100- μ l aliquots of the solution that had been hydrolyzed for 24 and 60 hr with 4.2 M NaOH in the presence of 25 mg of potato starch according to the method of Hugli and Moore (22). The remaining 50 μ l of solution was treated with performic acid according to the method of Hirs (23) for determination of cysteine acid.

The analyses were performed by the automatic procedure of Spackman, Stein, and Moore (24), by the accelerated system (25) with Beckman-M 82 (0.9 \times 60 cm) and PA-35 (0.9 \times 10.5 cm) resins. Analytical measurements, performed on separate samples of this material, indicated 4.4% of moisture and 1.9% of ash, after drying under reduced pressure at 100° for 4 hr. Both of these corrections were made before the protein recovery values were calculated. The estimated total recovery of amino acids in the purified *E. coli* K colicin accounted for 85–87% of the corrected weight of the material, as based on a mean residue weight of 109.

The results of the analyses are recorded in Table 1, where they are compared with those of *Proteus* colicin K. The table shows that the two substances, each from different microorganisms, have nearly identical amino-acid compositions. In view of this, one must conclude that the two colicins are indeed identical and hence that the genetic mechanisms that

TABLE 1. Amino-acid composition of colicin K from *E. coli* K235 and *Proteus mirabilis* colicin K

Amino acid	Average or extrapolated values expressed as mol/100 mol of amino acid	
	<i>E. coli</i> colicin K	<i>P. mirabilis</i> colicin K*
Lysine	10.7	10.6
Histidine	1.1	1.2
Arginine	2.2	2.1
Aspartic acid	13.5	13.2
Threonine	4.6†	4.3
Serine	9.1†	9.3
Glutamic acid	14.0	14.5
Proline	1.7	1.7
Glycine	8.1	7.7
Alanine	8.7	8.7
Cysteine	0.0‡	0.2
Valine	7.9	7.4
Methionine	1.5	1.6
Isoleucine	4.2§	4.0
Leucine	6.9	6.8
Tyrosine	2.5†	2.5
Phenylalanine	2.8	2.7
Tryptophan	0.9¶	1.2
Total	100.4	99.7

* Data reported by M. A. Jesaitis (8).

† Value obtained by linear extrapolation to zero time.

‡ Uncorrected value obtained for cysteine acid after performic acid oxidation.

§ Value from 48-hr hydrolysis.

¶ Value obtained with the procedure of Hugli and Moore (18).

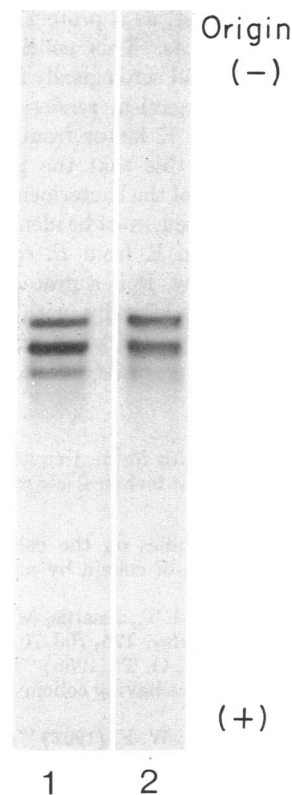


FIG. 5. Electrophoresis of colicin K from *E. coli* K235 in polyacrylamide gel. (1) Control: *P. mirabilis* colicin K; (2) purified *E. coli* K235 colicin K. 10- μ l Samples of solutions of both colicins containing 100 μ g were deposited into slots of a vertical electrophoresis cell containing 5% polyacrylamide gel in 0.077 M glycine–0.01 M Tris·HCl at pH 8.0. After electrophoresis for 4 hr at 400 V the gel was stained with 0.25% amido black, then destained.

control their synthesis are identical. We have made no effort to study other physical properties of the bacteriocin of *E. coli* K because those of the *P. mirabilis* bacteriocin have already been described (9).

DISCUSSION

The cultivation of colicinogenic bacilli in the absence of inducing agents is invariably accompanied by the release of a certain amount of bacteriocin into the culture medium. When the colicin is recovered it has, in those instances thus far studied, proved to be associated with the lipopolysaccharide antigen of the bacillus in question (2–6). The use of the inducing agent mitomycin C, on the other hand, enhances the elaboration of the bacteriocin to such an extent that the latter can now actually be isolated as a free protein from the culture medium (9) or extracted from the bacterial cells themselves (9, 13).

Our early work on colicin K clearly demonstrated that the colicin of *E. coli* K235 is part of the bacterial O antigen. Only by dissociating this complex by means of organic solvents was it possible to obtain the bacteriocin as a constituent of the protein moiety (3)—a fact that indicated that colicin K was not covalently bound to the lipopolysaccharide portion of the antigen. In the account presented here we corroborated this hypothesis. We have shown that in the presence of mitomycin C, *E. coli* K235 also elaborates its

bacteriocin, in part at least, as a protein unassociated with other bacterial constituents. This colicin K protein has proved to be chemically and serologically identical with that of *P. mirabilis*, a microorganism rendered colicinogenic by the transfer of the colicin K factor from *E. coli* K235 (9). One must conclude from this that the genetic mechanism that controls the synthesis of the bacteriocin in the two bacilli, which are otherwise unrelated, must be identical.

In summary: (i) Colicin K from *E. coli* K235 has been isolated in a state of purity. It is a protein that is identical both analytically and serologically with the colicin K of *P. mirabilis*. (ii) The colicin K of *E. coli* K235 exists in multiple forms that show different mobilities upon gel electrophoresis.

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