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# Comparison of Colicins B-K260 and D-CA23: Purification and Characterization of the Colicins and Examination of Colicin Immunity in the Producing Strains

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Colicins B-K260 and D-CA23 were purified by ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography and were compared with respect to a number of physical and chemical properties. Both colicins were shown to be proteins and were found to have similar molecular weights, isoelectric points and amino acid compositions. The two colicins also have substantial antigenic similarities but are distinguished by the presence of non-cross-reacting antigens and by differences in stability and in sensitivity to heat and reducing conditions. In addition, strains of *Escherichia coli* K-12 producing colicins B-K260 and D-CA23 are not cross-immune. The similarities noted between the two colicins are compatible with their use of a common cell surface receptor while having different modes of action.

Bacteriocins are a diverse group of antibiotic-like proteins produced by many species of bacteria. Colicins are bacteriocins produced by *Escherichia coli* and by related species of bacteria. The production of colicins is controlled by plasmids (col factors; 34), and their activity is limited to *E. coli* and to related organisms (for reviews, see references 13, 16, 34).

Colicins range in size from 18,000 (colicin M-K260; 2) to 92,000 (colicin D-CA23; daltons 42). Several colicins have been purified and characterized, and comparisons of their physical and chemical properties demonstrate the structural diversity of this group of bacteriocins (13, 16, 19). This diversity also extends to the mode of action of colicins on sensitive cells (13). Various studies have shown that colicins with similar modes of action do not necessarily have similar structures, and vice versa (19, 42).

The original classification of the colicins into the various types was based on activity spectra against bacterial isolates and against colicin-resistant mutants of *E. coli* (6, 7). Further differentiation of closely related colicins was based on cross-immunity between the producing strains; bacteria producing a colicin are generally immune (resistant) to that colicin but not to unrelated colicins (9). Recent evidence from our laboratory has indicated that the original identification of colicins B and D as different colicin types may have been in error. We have been unable to isolate mutants of *E. coli* K-12 that are resistant to only one of the two colicin types (3), and both colicins use the same cell surface receptor and are strongly inhibited by the siderophore (iron chelator) enterochelin (31, 32). The colicin B- and D-producing strains originally used by Fredericq in the classification of these colicins (7) have subsequently been shown to produce other colicins in addition to B or D (10, 43). However, strains of *E. coli* that produce only colicins B or D are now available (3, 42), and it is thus possible to compare the two colicins in greater detail. In this paper we compare the physical and chemical properties of purified colicin B-K260 with those of purified colicin D-CA23 and with those determined for the latter colicin by Timmis (42). We find that colicins B-K260 and D-CA23 are indeed very similar but are distinguished from each other by differences in stability and antigenicity. In addition, strains producing colicins B-K260 and D-CA23 are not cross-immune.

## MATERIALS AND METHODS

**Bacterial strains.** Strains of *E. coli* K-12 and colicinogenic bacteria carrying col B or col D factors are listed in Table 1. Other colicinogenic bacteria were as described previously (4).

**Culture media.** Nutrient broth, nutrient agar, soft nutrient agar, and minimal salts media were as described previously (33).

**Transfer of col factors.** Transfers of col factors to strain P1684 were performed in nutrient broth buffered to pH 7.8 with $Na_2HPO_4$ and containing 5 mg of trypsin per ml. Donor and recipient cells in the logarithmic phase of growth were mixed in the ratio 1:5 (final concentration, approximately $10^8$ cells/ml) and incubated for 1 to 3 h at 37°C. The

TABLE 1. *Strains of E. coli K-12 and colicinogenic bacteria used in this study*

| Strain | Relevant genetic markers | Colicin resistance | Original source of plasmid | Colicins produced[a] | Source/reference |
|---|---|---|---|---|---|
| AB1133 | *thr leu pro his argE str supE* | –[b] | – | – | A. L. Taylor |
| P1552 | as AB1133, but *aroE spc* | – | – | – | (33) |
| P1687 | as AB1133, but *bfe cir* | A, E1, E2, E3, 1a, S1, V | – | – | This laboratory |
| P1693 | as AB1133, but *tonA cir* | 1a, 1b, M, S1, V | – | – | This laboratory |
| P1684 | *trp spc* | – | – | – | Derivatives of JC6256 |
| JC6256 (R64-11) | *trp* | – | – | – | N. Willets |
| JC6256 (R136 fi$^-$) | *trp* | | | | |
| | – | – | – | – | N. Willets |
| AG097 | – | – | K260 | B | B. Stocker |
| T20 | – | – | K260 | B | This paper |
| K89 | – | – | K89 | B, M | P. Fredericq |
| CA18 | – | – | CA18 | B, M | P. Fredericq |
| K252 | – | – | K252 | B, I, V | P. Fredericq |
| AG084 | – | – | K77 | B, M | B. Stocker |
| K44 | – | – | K44 | B, I, V | P. Fredericq |
| UB1082 | – | – | CA23 | D | K. Timmis (42) |
| II | – | – | II | D, E1, I, Q | J. Smarda (40) |

[a] According to records in this laboratory.
[b] –, No data available.

mixtures were then diluted in nutrient broth and plated onto nutrient agar containing 100 μg of spectinomycin per ml for counter-selection. In the case of self-transferring col factors, selection for col$^+$ recipients was by immunity to a crude colicin preparation (2 to 5 arbitrary units/ml of agar) obtained from the donor strain (see below). In the case of R136 fi$^-$ or R64-mediated transfer of col factors, donor cells were first mated with bacteria (JC6256) carrying the appropriate R factor (Table 1). Mating mixtures were diluted in glucose minimal salts medium and plated onto glucose minimal agar; counter-selection was for ability to grow in the absence of tryptophan and selection for R$^+$ recipients was with 20 μg of tetracycline per ml. Col and R factors were then cotransferred to strain P1684 as above, with selection for R$^+$ recipients with or without selection for colicin immunity. All recipient colonies were screened for colicin production, spectinomycin resistance, and tryptophan requirement, and recipients carrying representatives of each col plasmid type were selected for further study.

**Isolation of a strain producing increased amounts of colicin B-K260.** Strain AG097 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (24), plated directly onto nutrient agar, and incubated for 24 h at 37°C. Single colonies were transferred to two sets of fresh nutrient agar plates and incubated for 24 h at 37°C. Growth on one set of plates was then killed with chloroform vapor, overlayed with $10^6$ cells of strain P1552 in 3 ml of soft agar, and reincubated. Isolates showing substantially increased colicin production (increased size of killing zone) were streaked for purity, and single colonies were retested for colicin production. One isolate, named T20, was retained.

**Preparation of colicins.** The preparation and purification of colicins B-K260 and D-CA23 are described in the text. Partially purified colicins H-CA58, E3-CA38, Ib-P9, K-K235, M-K260, and S1-P1 were as used previously (31). Other colicins used in this study were prepared by growing the producing strains in well-aerated nutrient broth cultures to an optical density (at 660 nm) of 0.60. Mitomycin C (0.4 μg/ml, Sigma) was then added, and incubation was continued for a further 240 min or less if cell lysis occurred. Cells and cell debris were removed from the culture supernatant (crude colicin) by centrifugation (10 min at 4,600 × *g*) and membrane filtration (0.45-μm mean filter pore diameter).

**Assay of colicins and detection of colicin activity in polyacrylamide gels.** Colicins were assayed by the arbitrary units method as previously described (35), using doubling dilutions of the colicin preparation. Indicator strains P1687 and P1693 were used to detect only colicins B and D of those produced by multicolicinogenic bacteria (see Table 1). Colicins B-K260 and D-CA23 were assayed throughout using strain P1552 as indicator. Colicin activity in polyacrylamide gels (see below) was detected either by embedding the gel in soft nutrient agar containing $10^6$ indicator cells/ml or by slicing the gels and placing each section (0.3 mm) onto the surface of soft nutrient agar containing the indicator cells. Both test systems were incubated at 37°C for 24 h, and the diameters of the inhibition zones obtained by the latter procedure were used to compare the colicin activities recovered from the gels.

**Colicin resistance patterns and colicin activity spectra.** Plate tests for testing colicin resistance/sensitivity were as described previously (4).

**Ion-exchange chromatography and gel filtration.** Whatman diethylaminoethyl-cellulose (DE52) was used for all ion-exchange column chromatography. Ion-exchange and gel filtration columns were equilibrated with the appropriate buffer before use. Samples for each gel stage of filtration were concentrated to less than 10% of the column liquid volume (less than 1% for high-resolution gel filtration) by dialysis against Aquacide II (Calbiochem) or by Diaflo membrane filtration (Amicon), both at 4°C, or by freeze-drying and resuspending in a reduced volume of solvent (colicin D-CA23 only). All samples were extensively dialyzed against the appropriate buffer at 4°C before being loaded onto columns. All other procedures for gel filtration and column chromatography were as recommended by the manufacturers of the filtration or chromatography media. Fractions from the columns were collected and examined for colicin activity, for optical density at 280 nm, and, where appropriate, for phosphate molarity measured as conductivity. The phosphate buffer (PB) was $Na^+$ buffer at pH 7.1.

**PAGE.** Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed using the continuous Maizel gel system (23) with 7.5% acrylamide gels in tubes and with the samples solubilized in the phosphate buffer ($Na^+$, pH 7.3, 0.1 M) containing 1% SDS and 0.1% $\beta$-mercaptoethanol. PAGE in the absence of SDS was performed by the method of MacGreggor et al. (22). Gels were fixed, stained, and examined as described previously (31).

**Isoelectric focusing.** Isoelectric focusing was carried out in acrylamide disc gels containing ampholytes (LKB: wide range, pH 3 to 10). The gels contained 4.8% acrylamide (Eastman), 0.2% bisacrylamide (Eastman), 0.085% *N,N,N',N'*-tetramethylethyldiamine, 2% ampholytes, and 0.034% ammonium persulfate (added as a 2% solution) in distilled water. The mixture was degassed before the addition of the persulfate. Gels were run in the electrolyte bath normally used for PAGE, with 0.01 M phosphoric acid and 0.02 M NaOH as acidic and basic electrolyte solutions, respectively. Excess persulfate was discharged from the gels by prerunning for 30 min at 1 mA/gel. Protein (5 to 50 $\mu$g/gel in 10% sucrose) was then loaded, and the gels were run for 20 h at 4°C. An initial voltage of 50 V was applied (1 mA/gel), rising to 400 V for the final 4 h to complete the focusing. Gels were fixed, stained, and scanned in the normal way (31), with extensive dialysis against distilled water between the fixing and staining stages to remove the ampholytes. The pH gradient along the gels was determined by slicing unfixed gels, resuspending the sections in 1 ml of distilled deionized water for 1 h at 20°C, and measuring the pH.

**Preparation and assay of antisera.** Antisera were prepared in rabbits using multisite intramuscular vaccination with 1 mg of purified colicin B-K260 or D-CA23 in complete Freund adjuvant. The rabbits were vaccinated at 0 and 50 days and bled at 70 days. Colicin neutralization titers of the antisera were determined by mixing 0.1 ml of doubling dilutions of the antisera with an equal volume of nutrient broth containing 1 arbitrary unit of the appropriate colicin per ml. The mixture was incubated for 30 min at 37°C and assayed for residual colicin as above. Neutralization titers were determined from the last dilution of antiserum giving complete inactivation of the colicin.

**Immunochemical analyses.** Immunodiffusion and immunoelectrophoresis experiments were performed using 1% agarose (Calbiochem) in veronal buffer (pH 8.6; 41) unless otherwise stated. Immunodiffusion tests were performed as described by Ouchterlony and Nilsson (27). Rocket immunoelectrophoresis and cross-immunoelectrophoresis were as described by Weeke (46, 47). Agarose gels were then rinsed extensively in saline and then distilled water and fixed and stained with Coomassie blue as for acrylamide gels (31). PAGE and isoelectric focusing gels loaded with 5 to 50 $\mu$g of protein and run as described above were sometimes used in place of the normal agarose first dimension in cross-immunoelectrophoresis by slicing the acrylamide gels longitudinally, dialyzing against veronal buffer for 30 min, and embedding one-half of the acrylamide gel at the edge of the antibody-containing agarose gel. Antisera were used at final concentrations of 5 $\mu$l/cm$^2$ of agarose gel in each case.

An Ouchterlony-type immunodiffusion test was linked to a colicin activity test in a procedure similar to that described by Ozaki et al. (28). The assay was performed in soft nutrient agar containing 100 $\mu$g of streptomycin and $10^6$ cells of strain P1552 per ml. Antibody (100 $\mu$l) and antigens (100 $\mu$g) were incubated at 4°C until clear precipitin lines formed (usually 3 days) and then at 37°C for 8 h to permit growth of the indicator cells.

**Chemical analyses.** Protein was determined by the method of Schacterle and Pollack (38) using bovine serum albumin as standard. Hexoses were determined by the phenol sulfuric acid method (18) using glucose as standard, and 2-keto-3-deoxyoctonic acid was determined by the method of Warandehar and Shaw (45). Amino acid analyses were performed in a Beckman model 120C amino acid analyzer modified with Chromatronix type LC9MA columns and using Durrum DC1A resin and Durrum pico buffer system III. Samples were hydrolyzed under nitrogen at 110°C for 24 h in 1 ml of 6 N HCl containing 0.1% phenol.

## RESULTS

**Purification of colicin D-CA23.** The method used to purify colicin D-CA23 was essentially that of Timmis (42). Colicin D was extracted from sonicated cells of strain UB1082 as described previously (31; 10-liter nutrient broth culture induced with 0.4 $\mu$g of mitomycin C per ml) and purified by sequential steps of ammonium sulfate precipitation (31), gel filtration on Sephadex G200, exchange chromatography on Whatman DE52 cellulose, gel filtration on

Sephadex G200, exchange chromatography on Whatman DE52 cellulose, and high-resolution gel filtration on Sephadex G100. The final four stages of purification are illustrated in Fig. 1. The active material eluted from the Sephadex G100 column (stage 7) was free of detectable hexose and 2-keto-3-deoxyoctonic acid. This material was dialyzed extensively against distilled water at 4°C and lyophilized, and is referred to throughout as purified colicin D-CA23.

**Purification of colicin B-K260.** An attempt was made to purify colicin B-K260 from strain AG097 using the procedure described above for colicin D-CA23. This attempt failed for several reasons: strain AG097 produced considerably less colicin than strain UB1082, and yields were therefore very low; sedimentation of the crude colicin occurred on standing; and considerable loss of activity occurred during the final stages of purification and during slow freezing and thawing. The latter problems were overcome to some extent by using higher molarity PB to solubilize the colicin and by flash freezing the colicin in ethanol-dry ice mixtures at −20°C. To overcome the problem of low yields of colicin B-K260, we treated a suspension of strain AG097 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Materials and Methods) and isolated a derivative (T20) that produced increased amounts of colicin in plate tests. Strain T20 was shown to produce only colicin B by its activity spectrum against 18 different classes of colicin-resistant mutants (see references 3, 4).

Strains T20, AG097, and UB1082 were examined for colicin production in nutrient broth after induction with mitomycin C. Twenty-milliliter volumes of broth were inoculated with 2 ml of a standing 16-h broth culture (optical density, 1.0 to 1.2) of each of the three strains and incubated with good aeration at 37°C until the optical density (at 660 nm) reached 0.60. Mitomycin C was then added at final concentrations of 0.2 and 0.4 μg/ml, and incubation was continued for a further 150 min before the cells were harvested by centrifugation. Control cultures (no mitomycin C) were also harvested at this time. The cells were resuspended in 100 mM PB and sonicated for 10 min at 4°C, and cell debris was removed by centrifugation at 46,000 × *g* for 60 min at 4°C. The culture supernatants and cell sonicates were then assayed for colicin activity using strain P1552 on nutrient agar containing 100 μg of streptomycin per ml (Table 2). The production of both colicins B and D was shown to be strongly induced by mitomycin C, as reported previously (12, 42), and strain T20 clearly produced more colicin activity than strain AG097.

For the large-scale production of colicin B-K260, 6 liters of a well-aerated 16-h nutrient broth culture (optical density, 1.1) of strain T20 was used to inoculate 60 liters of nutrient broth in a fermentor. The broth was incubated with good aeration until the optical density (at 660 nm) reached 0.60, at which stage mitomycin C (0.2 μg/ml) was added. Incubation was continued for a further 150 min, and the cells were harvested by centrifugation. The cells (86 g, wet weight) were resuspended in 1 liter of PB (100 mM) at 4°C and disrupted in a French

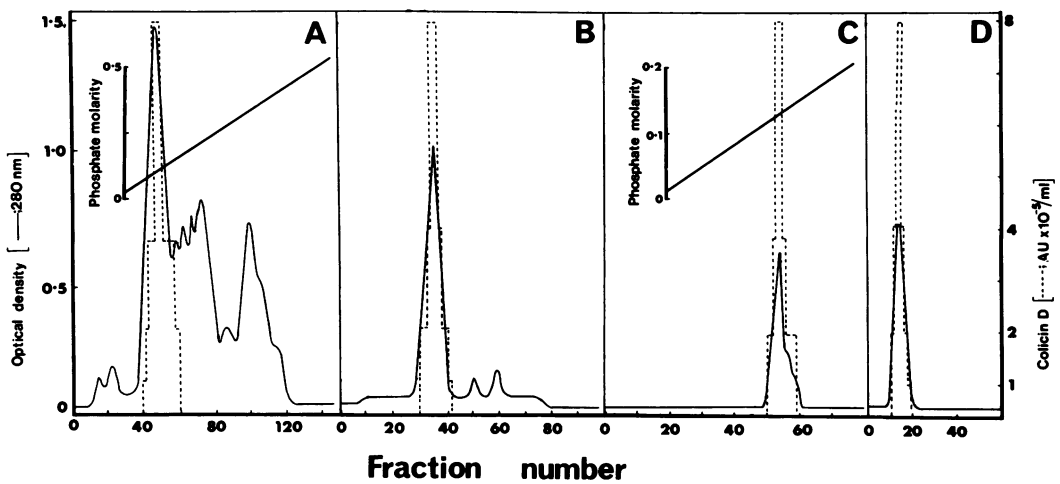


FIG. 1. *Purification of colicin D-CA23 by ion-exchange chromatography and gel filtration. (A-D) represent the final four stages of purification detailed in the text. (A) Stage 4, anion-exchange chromatography on DE52 cellulose; (B) stage 5, gel filtration on Sephadex G200; (C) stage 6, anion-exchange chromatography on DE52 cellulose; (D) stage 7, gel filtration on Sephadex G100.*

pressure cell (Aminco). Cell debris was removed by centrifugation at 46,000 × g for 90 min at 4°C. The colicin B-K260 in the supernatant was further purified as detailed in Table 3. Considerable amounts of high- and low-molecular-weight material were removed by gel filtration on Sephadex G200 (stage 3). The final four stages of purification are illustrated in Fig. 2. Samples of the colicin at various stages of purification were assayed for colicin activity and for protein, hexose, and 2-keto-3-deoxyoctonic acid content (Table 4). The colicin eluted from the Sephadex G100 column (stage 7), which was free of detectable hexose and 2-keto-3-deoxyoctonic acid, and was divided into two parts. One part was divided into small aliquots and frozen at −20°C, and the other was dialyzed against distilled water and lyophilized. Both samples are referred to throughout as purified colicin B-K260. Unless otherwise stated, the purified colicin B-K260 frozen in PB, which retained colicin activity, was used in the experiments described in this paper. The final yield of purified colicin B-K260 obtained by this method from strain 720 was 36-fold greater than that obtained from cells of strain AG097 under identical conditions. This indicates that the increased colicin activity obtained from strain T20 was due to increased colicin production and not to altered immunity factor.

**Immunochemical analysis of purified colicins B-K260 and D-CA23.** Antisera prepared against purified colicins B-K260 and D-CA23 strongly inhibited killing by all colicins of types B and D tested. Each of these colicins was produced by apparently independently isolated strains of bacteria (Table 1), and identical amounts of each colicin (as killing units) were inactivated by the same titers of the antisera (1:1,056 of anticolicin B-K260 and 1:2,122 of anticolicin D-CA23). Neither of the two antisera had any effect on killing by colicins H-CA58, K-K235, M-K260, Ib-P9, S1-P1, and E3-CA38, and normal rabbit serum had no effect on killing by any of the colicins tested.

Purified colicins B-K260 and D-CA23 were examined by the Ouchterlony immunodiffusion technique using homologous and heterologous antisera. Both colicins produced strong single precipitin lines against homologous and heter-

TABLE 2. *Titration of colicins produced by strains AG097, T20, and UB1082 after induction with mitomycin C*

| Strain | Colicin produced | Mitomycin C (μg/ml) | Colicin in culture supernatant (AU/ml)[a] | Colicin in cell sonicate (AU/mg of protein) |
|---|---|---|---|---|
| AG097 | B-K260 | | <1 | <1 |
| | | 0.2 | 1 | $1.15 \times 10^2$ |
| | | 0.4 | 1 | $1.35 \times 10^2$ |
| T20 | B-K260 | | 4 | 23.5 |
| | | 0.2 | $1.28 \times 10^3$ | $1.62 \times 10^4$ |
| | | 0.4 | $1.28 \times 10^3$ | $2.05 \times 10^4$ |
| UB1082 | D-CA23 | | 4 | 25.6 |
| | | 0.2 | $2 \times 10^3$ | $4.63 \times 10^3$ |
| | | 0.4 | $1.6 \times 10^4$ | $2.21 \times 10^4$ |

[a] AU, Arbitrary unit.

TABLE 3. *Purification scheme for colicin B-K260*

| Stage of purification | Purification procedure |
|---|---|
| 1 | Precipitation of cell extract with 30 to 50% saturated $(NH_4)_2SO_4$ at 4°C; precipitate redissolved in 60 ml of 50 mM PB |
| 2 | Low-resolution gel filtration on Bio-Gel P100 (50 to 100 mesh, 70 by 2.5 cm) in 50 mM PB at 4°C |
| 3 | Divided into two lots for low-resolution gel filtration on Sephadex G200 (70 by 2.5 cm) in 50 mM PB at 4°C |
| 4 (Fig. 2A) | Anion-exchange chromatography on Whatman DE52 cellulose (55 by 2.5 cm); colicin eluted with steps of 50 mM PB (600 ml), 150 mM PB (600 ml), and 500 mM PB (600 ml) at 20°C; active material eluted from this column was concentrated before proceeding to stage 5 |
| 5 (Fig. 2B) | Anion-exchange chromatography on Whatman DE52 cellulose (50 by 2.5 cm); colicin eluted with 300 ml of 50 mM PB followed by 1,000-ml linear gradient of 50 to 200 mM PB at 20°C |
| 6 (Fig. 2C) | High-resolution gel filtration on Sephadex G200 (80 by 2.5 cm) in 50 mM PB at 4°C |
| 7 (Fig. 2D) | High-resolution gel filtration on Sephadex G100 (100 by 2.5 cm) in 50 mM PB at 4°C |

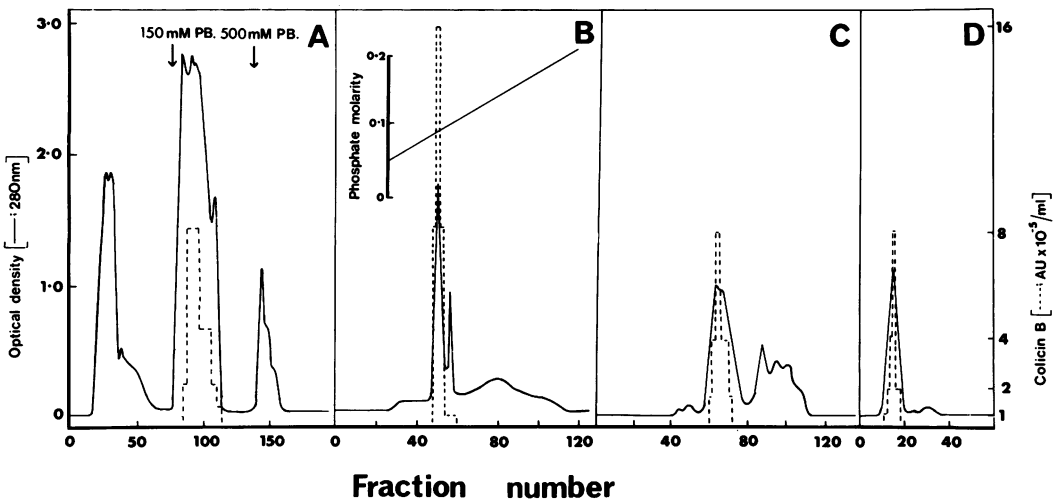


FIG. 2. *Purification of colicin B-K260 by ion-exchange chromatography and gel filtration. (A-D) represent the final four stages of purification detailed in Table 3. (A) Stage 4, anion-exchange chromatography on DE52 cellulose; (B) stage 5, anion-exchange chromatography on DE52 cellulose; (C) stage 6, gel filtration on Sephadex G200; (D) stage 7, gel filtration on Sephadex G100.*

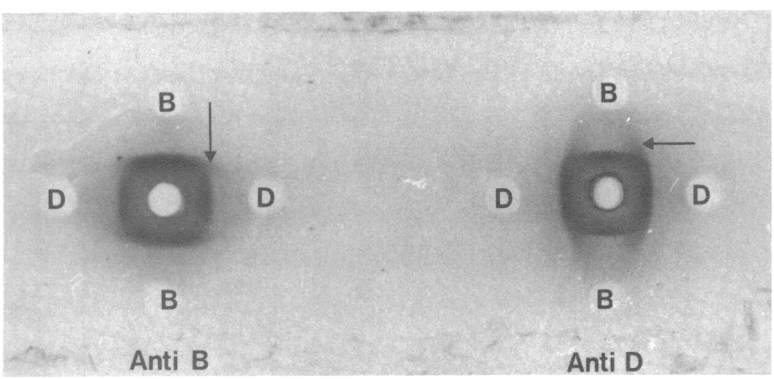


FIG. 3. *Examination of purified colicins B-K260 (B) and D-CA23 (D) by the Ouchterlony immunodiffusion test against homologous and heterologous antisera (anti-B and anti-D). The antibody (10 μl) and antigens (10 μg) were allowed to diffuse for 24 h at 20°C before fixing and staining. Note particularly the spurring reactions present only in reactions with homologous antisera (indicated by arrows).*

TABLE 4. *Colicin activity and chemical analysis of colicin B-K260 at different stages of purification*

| | Stage of purification | % Recovery of colicin activity | Sp act (AU/mg of protein) | Protein (mg) | Hexose (μg) | KDO (μg)[a] |
|---|---|---|---|---|---|---|
| | Crude colicin | 100 | $2.8 \times 10^4$ | 5,856 | $4.9 \times 10^4$ | $2.5 \times 10^3$ |
| 1 | $(NH_4)_2SO_4$ precipitate | 78 | $3.6 \times 10^4$ | 3,325 | $4.7 \times 10^3$ | 603 |
| 2 | Bio-Gel P100 | 56 | $5.1 \times 10^4$ | 2,916 | 583 | 599 |
| 3 | Sephadex G200 | 48 | $8.4 \times 10^4$ | 874 | 37 | 0 |
| 4 | DE52 cellulose | 36 | $2.1 \times 10^5$ | 262 | 0 | 0 |
| 5 | DE52 cellulose | 23 | $5.9 \times 10^5$ | 61 | 0 | 0 |
| 6 | Sephadex G200 | 17 | $7.2 \times 10^5$ | 36 | 0 | 0 |
| 7 | Sephadex G100 | 7.5 | $7.8 \times 10^5$ | 12 | 0[b] | 0 |

[a] KDO, 2-Keto-3-deoxyoctonic acid.
[b] No detectable hexose in 1 mg of material.

ologous antisera, with reactions of antigenic identity between the two colicins indicated by merging of the precipitin lines (Fig. 3). Spurring of the precipitin lines (Fig. 3) indicates the presence on both colicin molecules of non-cross-reacting determinants. In addition, we occasionally noted the appearance of a second antigenic species, detected as a very faint precipitin line outside the major line, in samples of the purified colicin B-K260 that had been lyophilized. This second precipitin line was formed only in reactions with homologous antiserum and was only detected in unstained preparations. We suggest that this antigen is a non-cross-reacting component, which is split from the colicin B-K260 molecule in distilled water or by lyophilization (colicin B-K260 is inactivated by dialysis against distilled water).

To confirm that the major precipitin lines formed in the Ouchterlony test resulted from the inactivation of colicin by antiserum and were not due to the presence of contaminating material, we coupled the Ouchterlony test to a colicin activity test (Materials and Methods). The precipitin lines marked the boundary of colicin activity in each case (Fig. 4A). Purified colicin B-K260, which had been partially inactivated by dialysis against distilled water, was used to demonstrate that the spurring of the colicin D-CA23 precipitin line noted above was due to precipitation (and inactivation) of the colicin and not to the presence of contaminating antigens (Fig. 4B). The distortion of the colicin D-CA23 killing zone shown in Fig. 4B is due to competition between colicins B-K260 and D-CA23 for common receptor sites on the indicator cells (31).

Further examination of the purified colicins B-K260 and D-CA23 by rocket immunoelectrophoresis confirmed the presence of strong cross-reactions between the colicins and again indicated the presence of a colicin-specific antigen in the lyophilized colicin B-K260, which was identified on a second precipitin line in Fig. 5 (indicated by arrow). Similar reactions were also indicated by cross-immunoelectrophoresis, in which agarose gels were used for the first electrophoretic dimension (data not shown). Further resolution of the antigenic components of the purified colicins was obtained by using PAGE or isoelectric focusing for the first electrophoretic dimension in the cross-immunoelectrophoreses (Fig. 6 and 7). Figure 8 shows a typical result obtained by these procedures. Both colicins were resolved into three components by these procedures, but only one of these components in each case had colicin activity (Fig. 6 and 7). In the case of purified colicin D-

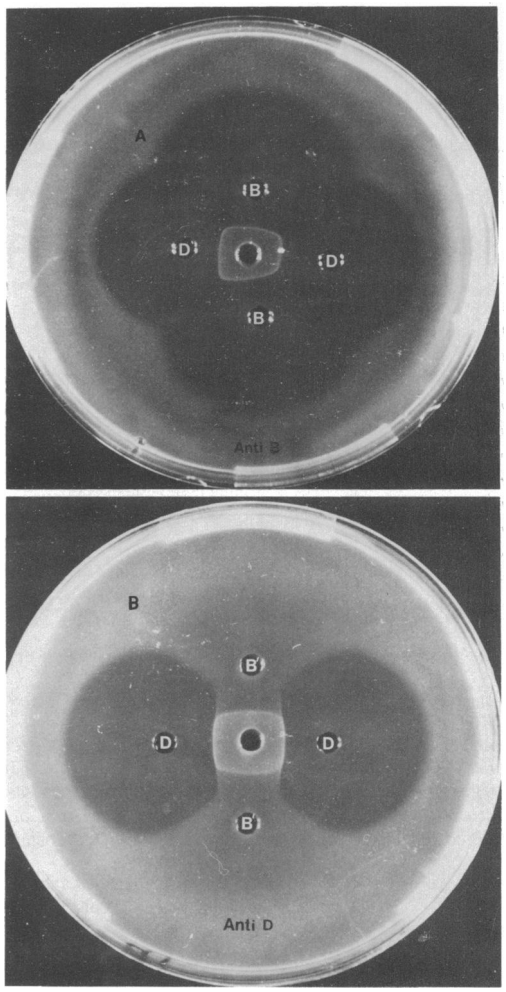


FIG. 4. *Examination of purified colicins B-K260 and D-CA23 by a coupled colicin activity-Ouchterlony immunodiffusion test. (A) shows the reactions between the two colicins (B and D) and antiserum prepared against purified colicin B-K260 (anti-B). An identical pattern was obtained with antiserum against the purified colicin D-CA23. (B) shows a similar reaction between active colicin D-CA23 (D), lyophilized (partially inactivated) colicin B-K260 (B'), and antiserum against purified colicin D-CA23 (anti-D, 50 μl). The clear zone in each case indicates the area of colicin killing of the indicator strain incorporated into the agar.*

CA23, one or both of the remaining components may be inactive colicin with electrophoretic mobilities (Fig. 6) and isoelectric points (Fig. 7) different from those of the active colicin. In the case of the purified colicin B-K260, however, one or both of the inactive components may be breakdown products derived from the active colicin, since less than 10% of the colicin B-

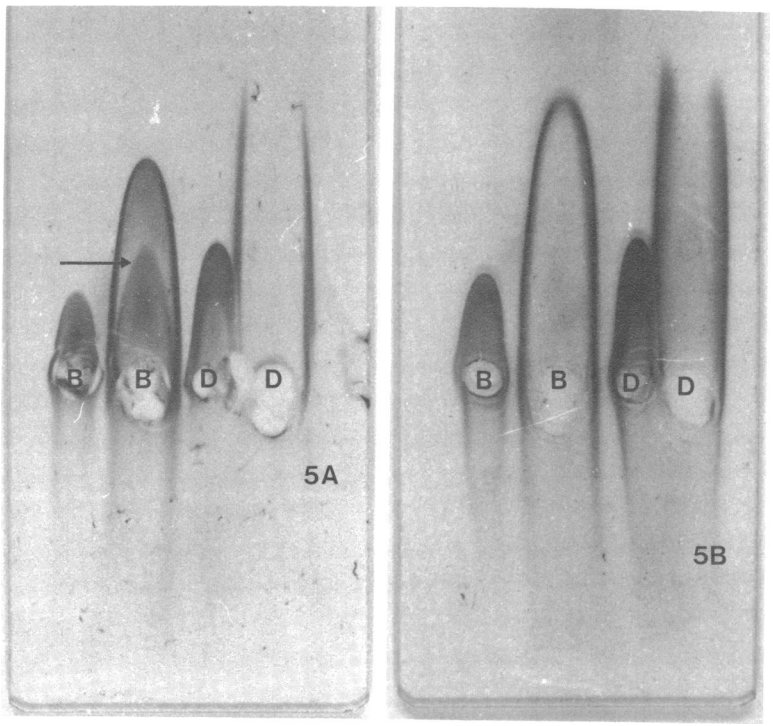


FIG. 5. *Examinaton of purified lyophilized colicins B-K260 (B) and D-CA23 (D) by rocket immunoelectrophoresis against homologous and heterologous antisera. The antigen wells contained 2.5 μg (left) and 15 μg (right) of the appropriate colicins, and the gels were run for 20 h at 80 V/gel slab. (A) shows the reactions between the two colicins and antiserum against purified colicin B-K260. (B) shows the reaction between the two colicins and antiserum against purified colicin D-CA23.*

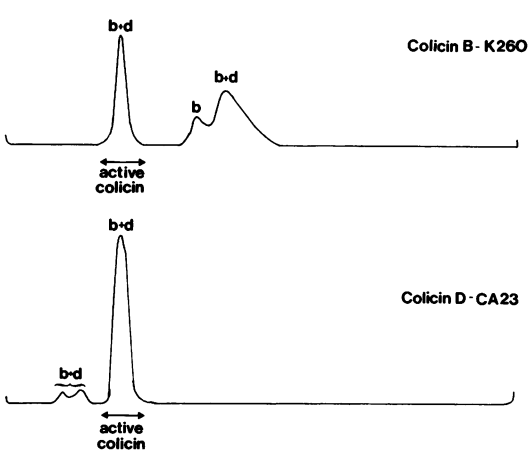


FIG. 6. *Densitometry scans of purified colicins B-K260 and D-CA23 examined by PAGE in the absence of detergent. The figure also shows the location of colicin activity in the gels and identifies the protein peaks that gave precipitin lines with antisera prepared against the purified colicin B-K260 (b) or D-CA23 (d) when the gels were examined by the cross-immunoelectrophoretic technique. The direction of electrophoresis was from left to right.*

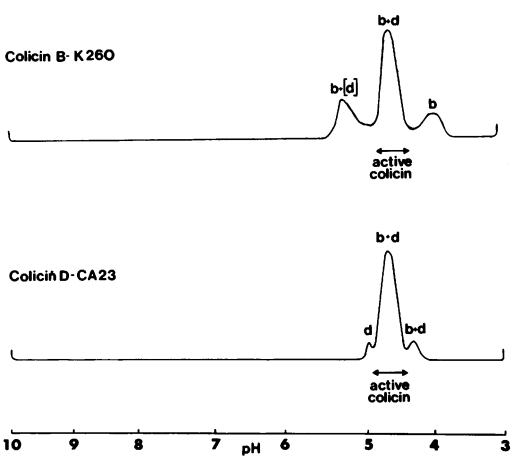


FIG. 7. *Densitometry scans of purified colicins B-K260 and D-CA23 examined by isoelectric focusing in polyacrylamide-ampholyte gels. The figure also shows the location of colicin activity in the gels and identifies the protein peaks that gave precipitin lines with antisera prepared against the purified colicin B-K260 (b) or D-CA23 (d). The scale shows the pH gradient along the length of the gel.*

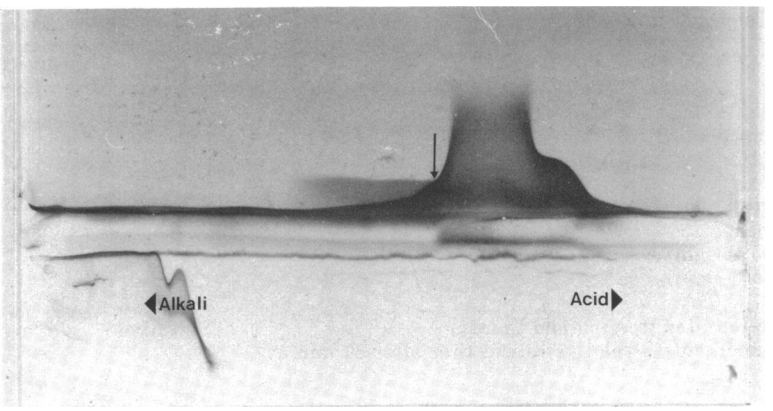


FIG. 8. *Examination of purified colicin D-CA23 by a coupled isoelectric focusing-cross-immunoelectrophoresis technique using homologous antiserum. The acrylamide-ampholyte gel was loaded with 50 μg of protein and subjected to electrophoresis as described in Materials and Methods (first dimension). The second dimension of electrophoresis was at 80 V for 18 h at 20°C. The alkaline end of the acrylamide-ampholyte gel was removed for convenience.*

K260 activity was recovered from the gels compared with the recovery of colicin D-CA23 under the same conditions. Isoelectric focusing revealed the presence of a serologically non-cross-reacting component in both purified colicins (Fig. 7). That present in the colicin B-K260 may be the same component identified in the Ouchterlony test as the additional precipitin line obtained with lyophilized colicin, but the colicin D-CA23 component may be a contaminating protein not detected previously (Fig. 8).

**Physical and chemical analysis of purified colicins B-K260 and D-CA23.** Purified colicins B-K260 and D-CA23 were compared with respect to a number of physical and chemical properties (Table 5). Although the two colicins had several features in common, there were some notable differences relating to the stability of colicin activity. We have already noted that colicin B-K260 was rapidly inactivated by dialysis against distilled water and by repeated slow freezing and thawing. The results in Table 5 also show that this colicin is unstable at room temperature and is more sensitive than colicin D-CA23 to heat and to the detergent SDS, particularly in the presence of the reducing agent β-mercaptoethanol. The instability of purified colicin B-K260 at room temperature can be reduced to a certain extent by the addition of bovine serum albumin (protein ratio, 1:50), as has been reported previously for colicin E2 (25).

Examination of the purified colicin B-K260 by PAGE in the presence of SDS revealed the presence of two components (labeled I and II in Fig. 8 and 9) whose combined molecular weights were equal to the molecular weight of active colicin B-K260, as determined by gel filtration on Sephadex G200 (Fig. 10). This suggests that treatment with SDS and β-mercaptoethanol results in the breakdown of colicin B-K260 into two components, with a resulting loss of colicin activity. It may be that these two components are identical to the inactive proteins found by PAGE in the absence of SDS and by isoelectric focusing (Fig. 6 and 7). We do not consider that the difference in molecular weights of colicins B-K260 and D-CA23 as demonstrated by gel filtration (Fig. 10, 11) is necessarily significant, as it is probably within experimental error.

**Amino acid analyses of purified colicins B-K260 and D-CA23.** Table 6 shows the results of amino acid analyses of purified colicins B-K260 and D-CA23. The amino acid composition determined for colicin D-CA23 is very similar to that determined for colicin B-K260 and also to that obtained by Timmis (42) for his preparation of purified colicin D-CA23. However, there is one notable difference between our results and those obtained by Timmis, namely, the absence of cysteine in either of our purified colicins. Control checks with added cysteine on the sensitivity of our amino acid analysis procedure demonstrated that the levels of half-cysteine found by Timmis in his preparation of purified colicin D-CA23 would have been detected by our technique.

**Activity spectra of colicins B-K260 and D-CA23.** Strains T20 and UB1082 were used to screen a large number of different gram-negative bacterial isolates for sensitivity to the two colicins B-K260 and D-CA23. The two colicinogenic strains produced similar inhibition zones in the agar plate test. In no case did we detect

TABLE 5. *Comparison of chemical and physical properties of purified colicins B-K260 and D-CA23*

| Property | Determination for: | |
|---|---|---|
| | B-K260 | D-CA23 |
| Nonprotein components[a] | Nil | Nil |
| Sensitivity to proteases[b]—half-life (min) at 37°C in: | | |
| 100 μg of Pronase per ml | <1 | <1 |
| 100 μg of trypsin per ml | 5 | 10 |
| Sensitivity to heat | | |
| Half-life at 56°C (min) | 3 | 35 |
| Half-life at 80°C (min) | <1 | 3 |
| Half-life at 37°C (days) | 8 | Stable |
| Sensitivity to repeated freezing and thawing | Sensitive | Stable |
| Sensitivity to detergents[c]—relative colicin titer after 30 min at 37°C in: | | |
| 0.5% SDS | 0.25 | 1 |
| 0.5% Sarkosyl | 0.25 | 0.25 |
| 0.5% Nonidet | 1 | 1 |
| 0.5% Triton X-100 | 1 | 1 |
| 0.5% cholic acid | 1 | 1 |
| 1% SDS + 0.1% β-mercaptoethanol | 0.125 | 1 |
| Molecular weight | | |
| SDS PAGE[d] | 58,000 + 34,500 | 92,000 |
| Sephadex G200[e] | 92,000 | 96,500 |
| Isoelectric point[f] | 4.6 | 4.7 |
| Maximum adsorption wavelength (nm) | 279 | 279 |

[a] See Table.
[b] Sensitivity to proteases, heat, and detergents was measured at a final concentration of 100 μg of colicin per ml in 50 mM PB.
[c] Sensitivity to detergents compared with control incubated in 50 mM PB.
[d] PAGE in the presence of SDS; see Fig. 8 and 9.
[e] Gel filtration on Sephadex G200; see Fig. 10.
[f] Isoelectric point of the protein band having colicin activity; see Fig. 7.

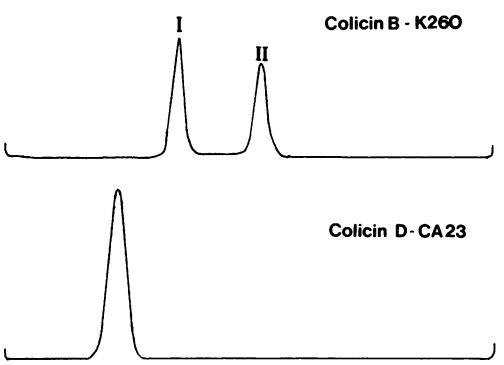


FIG. 9. *Densitometry scans of purified colicins B-K260 and D-CA23 examined by PAGE in the presence of SDS. The direction of electrophoresis was from left to right.*

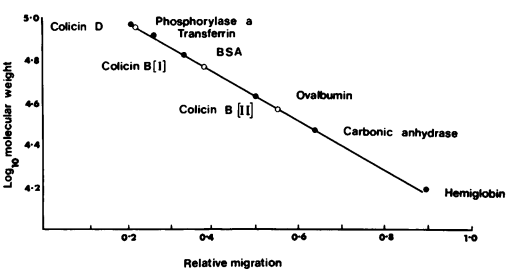


FIG. 10. *Determination of molecular weights of purified colicins B-K260 and D-CA23 by PAGE in the presence of SDS. Gels were loaded with mixtures of 10 μg each of the standard proteins (●) or the two colicins (○) in various combinations, and the migration of each protein relative to the electrophoresis front was determined after electrophoresis. The two components of purified colicin B-K260 revealed by this test are labeled I and II (see Fig. 9). BSA, Bovine serum albumin.*

any difference in the activity of the two colicins. Only *E. coli* (11 of 90 O antigen types tested) and *Shigella sonnei* (6 of 7 strains tested) were found to be sensitive to the two colicins. Strains of *Salmonella* (22, including several deep rough strains), *Serratia* (34), *Enterobacter* (11), *Klebsiella* (9), *Pseudomonas*, (2), *Aeromonas* (13), *Vibrio* (19), *Proteus* (2), *Yersinia* (2), and *Shigella boydii* (4) were resistant to both colicins. Fredericq (7, 8) and Papavasillous (29) have previously reported that both colicins have wider activity spectra against gram-negative

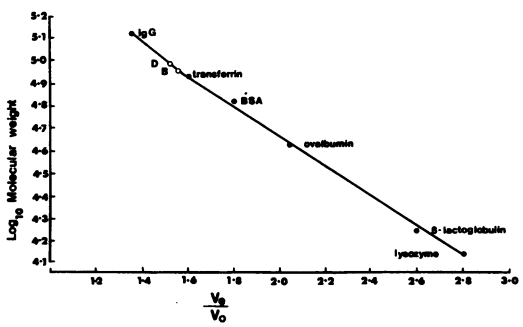


FIG. 11. *Determination of the molecular weights of purified colicins B-K260 and D-CA23 by gel filtration on Sephadex G200. The column (80 by 2.5 cm, equilibrated with 50 mM PB) was loaded with mixtures of 7 to 15 mg each of the various standard proteins (●) and 2 mg of active purified colicin B-K260 or D-CA23 (○) in various combinations. Fractions (1.2 ml) were eluted from the column with 50 mM PB and examined for colicin activity, for protein content (optical density at 280 nm), and, where appropriate, for colicin activity. Blue dextran was included in each run to permit determination of the column void volume ($V_o$). $V_e$ is the volume at which each component was eluted from the column. BSA, Bovine serum albumin.*

TABLE 6. *Amino acid compositions of purified colicins B-K260 and D-CA23*

| Amino acid | % of total amino acid content (M) | |
|---|---|---|
| | B-K260 | D-CA23 |
| Aspartic acid | 12.40 | 11.10 |
| Threonine | 4.04 | 4.54 |
| Serine | 8.62 | 7.68 |
| Glutamic acid | 12.01 | 10.57 |
| Proline | 6.03 | 6.55 |
| Glycine | 9.02 | 9.37 |
| Alanine | 8.48 | 8.53 |
| Valine | 9.15 | 9.19 |
| Methionine | 1.25 | 2.04 |
| Isoleucine | 5.08 | 5.09 |
| Leucine | 8.12 | 8.47 |
| Tyrosine | 2.59 | 2.20 |
| Phenylalanine | 2.24 | 2.38 |
| Tryptophan[a] | 1.82 | 1.63 |
| Lysine | 4.18 | 5.05 |
| Histidine | 0.60 | 0.96 |
| Arginine | 4.23 | 4.63 |
| Cysteic acid | 0 | 0 |
| Half-cystine | 0 | 0 |

[a] Derived from tyrosine value and alkaline adsorption spectra (5).

bacteria than found here. This may be due to the production of other colicins by the colicinogenic bacteria in use at that time. In particular, the extremely wide activity spectrum reported for colicin D (7, 8) is probably due to the production by *E. coli* strain CA23 of colicin X, which belongs to a different group of colicins than D-CA23 (3, 42).

Previously, we were unable to isolate mutants of *E. coli* K-12 resistant to only one of the colicins B and D (3). We have now extended this observation with over 300 independently isolated colicin B-K260- or D-CA23-resistant mutants of strains AB1133 and P1552. Mutants were isolated under various conditions of iron deprivation or supplementation and in the presence or absence of enterochelin synthesis to exclude the possibility that interactions between iron uptake systems and colicin killing were preventing the isolation of some mutant classes (31). All mutant classes were of the previously described types (*cbt, cbr, exbC, exbB,* and *tonB;* see references 3, 31), except two representatives of a new phenotypic class of colicin B- and D-resistant mutants (*exbD;* excretion of inhibitor of colicin B, phenotype D). These mutants were isolated as colicin D-CA23-resistant derivatives of strain AB1133 (able to produce enterochelin) and were resistant (tolerant in the triple-layer plate test) to colicins B-K260, D-CA23, G-CA46, H-CA58, Ia-CA53, Ib-P9, S1-P1, and V-CA7 only. The mutants also hypersecreted enterochelin (see reference 33) and became fully sensitive to all colicins upon insertion of the *aroE* marker, which blocks enterochelin biosynthesis (31). We conclude that hypersecretion of enterochelin in this strain blocks killing by colicins B and D in a manner similar to that reported previously for colicin G, H, Ia, Ib, S1, and V action on *exbC* and *exbB* mutants (31).

**Cross-immunity between colicin B- and D-producing strains.** To study the colicin immunity of *E. coli* carrying col B or col D factors, we transferred col factors of each type to strain P1684. The factors K252, K89, K77, K260, and II were self-transferred at frequencies ranging from 0.01 to 0.1% of potential recipients. Col factor CA18 was not self-transferred at detectable frequencies, but transfer of this col factor could be mediated by R factors R64 and R136 $fi^-$ at rates of 0.6 to 0.9% of potential recipients (70 to 80% cotransfer of col factor and R factors). Col factor CA23 was transferred only in conjunction with the I-like R factor R64 (0.01% of potential recipients, 15% cotransfer of R64 and CA23). Col factor K44 could not be mobilized.

Derivatives of strain P1684 carrying each of the above col factors were tested for colicin production and for immunity to colicins produced by each other and by other strains producing colicins in groups A and B (Davies and Reeves classification; see references 3, 4). Only strains P1684 (K260) and P1684 (CA23) pro-

duced single colicins (Table 7); P1684 carrying other col B or col D factors produced colicins of types similar to those produced by the donor strains (Table 1). The tests used to detect colicin production by the P1684 derivatives would, however, only detect colicins secreted in relatively high concentrations. It is evident that colicins D-CA23 and B-K260 do not show cross-immunity and that colicin D-CA23 differs from all of the colicins of type B tested. Thus, although by the usual criterion of resistance colicin D-CA23 would be classed as a colicin of type B, it is nonetheless distinctive in its immunity. This result was also confirmed by titrating the activity of purified colicins B-K260 and D-CA23 against the $col^+$ derivatives of strain P1684. It is also evident that all the col B factors confer immunity to at least two other colicins in addition to B (Table 7). Indeed, plasmid CA18 conferred immunity to all colicins in group B except D (colicin Q not tested). In addition, we note that P1684 (II) was immune to colicins of type B but not to colicin D-CA23. We conclude that this plasmid codes for the production of colicin B and not colicin D (40). R factors R64 and R136 $fi^-$ did not confer any colicin immunity.

## DISCUSSION

The results presented in this paper indicate that considerable purification of colicins B-K260 and D-CA23 was achieved by the techniques used. Although some of the data are compatible with the possible presence of contaminating proteins in our preparations, we believe that a more likely explanation is that some inactive colicin was present in both samples, and in particular in the lyophilized colicin B-K260. In most cases in which cross-immunoelectrophoresis experiments revealed the presence of two antigenic components, of which only one had colicin activity (Fig. 6 and 7), we noted that merging of the precipitin lines occurred (for example, see Fig. 8), indicating antigenic homology between the active colicin and the inactive component. Only in the case of a minor protein present in purified colicin D-CA23 was this merging not demonstrated (Fig. 8), and we concede that this protein could be a contaminant.

The finding that colicins B-K260 and D-CA23 are very similar in many respects is of considerable importance to our understanding of several reported findings with colicins of types B and D. The substantial similarity between the amino acid compositions of the two colicins may account for their similar antigenic determinants; these antigenic determinants presumably have very similar amino acid sequences and tertiary structures. A similar situation may also apply to the receptor-recognition regions of the two colicins, since both use the same cell surface receptor in *E. coli* K-12 (32).

Colicins of types B and D have been reported to have quite distinct modes of action. Colicin D-CA23 specifically inhibits protein synthesis without affecting deoxyribonucleic acid or ribonucleic acid synthesis (44), whereas colicin B-K77 inhibits protein, ribonucleic acid, and deoxyribonucleic acid synthesis (11) and may

TABLE 7. *Colicin production and colicin immunity of strain P1684 carrying various col B and col D factors as determined by the cross-streak plate test*

| Plasmid carried | Colicins produced[a] | Immunity/sensitivity to colicins produced by col B and col D factors[b] | | | | | | | | Immunity to other colicins[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | K260 | CA23 | CA18 | K77 | K89 | K 25 2 | II | K44 | |
| K260 | B | I | S | S | S | S | S | S | S | 1b-P9, 1a-CA53 (S1-P1) |
| CA23 | D | S | I | S | S | S | S | S | S | |
| CA18 | B, M | I | S | I | I | I | I | S | S | (G-CA46), H-CA58, 1a-CA53, 1b-P9, M-K260, S1-P1, V-CA7 |
| K77 | B, M | I | S | I | I | I | I | S | S | 1b-P9, M-K260 |
| K89 | B, M | I | S | I | I | I | I | I | S | 1b-P9, M-K260 |
| K252 | B, M | I | S | I | I | I | I | I | S | M-K260, V-CA7 |
| II | B,[d] E1, I-S1, V-Q[e] | I | S | I | I | I | I | I | I | E1-K53, 1a-CA53, 1b-P9, M-K260, (S1-P1), V-CA7 |

[a] Determined using mutants of known colicin resistance (3, 4; Table 1). Resistance to one of the colicins produced by multicolicinogenic strains was indicated by the appearance of a narrower inhibition zone.
[b] All col factors in strain P1684 except K44. I, Immune (scores as resistant); S, sensitive.
[c] Parentheses indicate partial tolerance (hazy or narrow inhibition zone).
[d] Originally called colicin D.
[e] We are unable to distinguish between colicins 1a, 1b, and S1 and V and Q in these tests.

therefore be similar to colicin K, which affects energy metabolism (21). Although colicin B-K77 may not be identical to the colicin B-K260 studied here, there is substantial antigenic similarity between the two colicins, and *E. coli* producing colicin B-K77 is immune to colicin B-K260. Furthermore, recent results in our laboratory indicate that colicin B-K260 inhibits amino acid uptake, a characteristic of colicins that affect energy metabolism, whereas colicin D-CA23 does not (unpublished data). We therefore suggest that the differences in antigenic structure, and in sensitivity to heat and to reducing agents, and minor differences in amino acid composition are consistent with the differences in modes of action of the two colicins B and D. Colicins E2 and E3 also use a common receptor site on the cell surface of *E. coli* K-12 (37, 39), but have quite distinct modes of action (1, 36) and are only partially cross-immune (17). These colicins also have very similar amino acid compositions, but colicin E2 is sensitive to reducing conditions whereas colicin E3 is not (15), and colicins E2 and E3 each have non-cross-reacting as well as common antigenic specifities (19). This raises the possibility that the common antigenic determinants in colicins B and D and in colicins E2 and E3 are part of the receptor-recognition regions of these colicins, whereas the non-cross-reacting antigens are parts of the "active" regions of the colicin molecules. Colicins Ia and Ib, which are distinguished chiefly by the lack of cross-immunity between the producing strains (20), may also have non-cross-reacting antigens (19). An alternative hypothesis may therefore be that the non-cross-reacting antigens of colicins B and D, E2 and E3, and Ia and Ib are part of the immunity-recognition regions of these colicins. It should be noted, however, that in no case has a range of independently isolated colicins of the two types been compared, and some of the antigenic differences could even be unimportant.

Colicins are generally regarded as secreted proteins, although large amounts of the colicin often remain cell bound (Table 2) even in the absence of the homologous colicin receptors on the producing cells (42). The mechanisms by which colicins are secreted by colicinogenic bacteria remain largely unknown. However, most of the colicins studied in detail have been shown to contain very little or no cysteine (19) and thus conform to the generally accepted view that proteins secreted by bacteria contain very little of this amino acid (30). The only report of a colicin containing appreciable amounts of cysteine is that of Timmis (42) for colicin D-CA23. However, we find that our preparations of colicin D-CA23 and the closely related colicin B-K260 contain no detectable cysteine.

The finding that colicins B and D are very similar also raises some questions relating to the apparent differences between plasmids coding for the production of these colicins. We have previously shown that both colicins are part of a larger subdivision of colicins, which we call group B. Mutants of *E. coli* K-12 resistant to colicins in group B (B, D, G, H, Ia, Ib, M, Q, S1, and V) show no cross-resistance to colicins in group A (A, E1, E2, E3, K, L, N, S4, and X) and vice versa (3, 4). This division into two major groups in general parallels a similar division of col factors into self-transferring and non-self-transferring types (13, 14). Colicins in group B in our classification are generally produced by large self-transferring col factors. The only exception to this observation is colicin D-CA23, which we classify as a group B colicin but which is produced by a small, non-self-transferring col factor. The col factor CA23 is clearly a simple plasmid, coding only for the production of one colicin, and is therefore different from col B factors, which are self-transferring (with the possible exception of K44) and code for the production of at least one additional colicin and produce immunity to at least two other colicins (Table 7). The plasmid K260 used here apparently carries that part of a col I factor coding for immunity, although not producing colicin I. The col factor CA23 is considerably smaller than plasmids coding for production of and immunity to the B colicins (13, 43), and it is possible that this plasmid has been formed by the fragmentation of a larger plasmid with resulting loss of sex factor activity. On the other hand, we note that the complex col B factors also carry col I or col V factor activity (Table 7), and col I and col V factors themselves are known to have sex factor activity (26).

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