Colicin E4-CT9 is Proteolytically Degraded after Discharge from Producing Cells in Liquid Cultures

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Colicin E4 was produced in very large amounts when Escherichia coli K12 strain W3110 pColE4-CT9 was grown in the presence of 0.5 μg mitomycin C ml⁻¹. The colicin was discharged from the producing cells when they lysed and was then degraded by a protease located on the surface of the producing cells. Colicins and proteolytic fragments derived from them could be concentrated from spent culture medium by filtration through Millipore membrane filters. Colicins are probably retained by these filters by hydrophobic interactions since binding was unaffected by changes in ionic conditions but was completely inhibited in the presence of ionic or non-ionic detergents.

INTRODUCTION

Escherichia coli K12 strains carrying small, high copy number colicin (Col) plasmids encoding colicins A, D, E1, E2, E3, E4, E5, E6, E7, K or N (Hughes et al., 1978; Pugsley, 1981; Mock & Pugsley, 1982) produce substantially increased amounts of colicin when grown under conditions which derepress the so-called 'SOS' system for DNA repair (Little & Mount, 1982; Pugsley, 1981). When treated with 0.5 μg mitomycin C ml⁻¹ (Little & Mount, 1982), these strains accumulate colicin to very high levels (4–8% of total cell protein, see examples in Mock & Pugsley, 1982) and then lyse (Pugsley & Rosenbusch, 1981). We have noted previously that colicin E4-CT9, which is closely related to the more extensively studied colicins E2 and E3, is discharged into the growth medium in much lower amounts than would be expected from the large amounts of the colicin E4 polypeptide which accumulate within the cells before they lyse (Mock & Pugsley, 1982). We considered four possible explanations for this observation: (1) the colicin was not discharged; (2) the colicin was discharged but became adsorbed to specific receptors (BtuB protein; Mock & Pugsley, 1982) on the surface of the producing cells; (3) the colicin E4 activity was inherently unstable or (4) the colicin was rapidly degraded after it was discharged. The results of experiments reported here indicate that the last interpretation was correct. As part of this work we used the ability of membrane filters to absorb colicin as a simple procedure for concentrating colicin from spent culture medium.

METHODS

Bacterial strains and media. Escherichia coli K12 Col⁺ strains listed in Pugsley (1981) and Mock & Pugsley (1982) were used throughout. These strains are derivatives of E. coli K12 W3110 and carry a single plasmid species introduced by transformation as described by Mock & Pugsley (1982). The strain from which the plasmid was originally derived is indicated by the plasmid and colicin designations (thus pColE4-CT9 is derived from Shigella sonnet strain CT9 of Horak (1975) and Males & Stocker (1982) and encodes colicin E4-CT9). All of the strains tested carried small, high copy number Col plasmids as defined previously (Mock & Pugsley, 1982) and lysed and discharged colicin within 3 h when grown in the presence of 0.5 μg mitomycin C ml⁻¹ (see Pugsley & Rosenbusch, 1981). The colicin indicator strain was BZB1019 (E. coli K12 hsdR met gal rpsL). Culture media were as used
previously (Pugsley & Rosenbusch, 1981) except that tryptone broth was supplemented with 0.5% (w/v) yeast extract (to make L broth) for some experiments. Mitomycin C was used at 0.5 μg ml⁻¹ and was added to cultures growing exponentially at $A_{560} = 0.1-0.2$.

**Procedures.** β-Galactosidase (Miller, 1972), alkaline phosphatase (Brockman & Heppel, 1968) and acid phosphatases (Dassa & Boquet, 1981) were assayed as described. Colicins were assayed by mixing dilutions of the preparation with $10^6$ indicator cells in a final volume of 1 ml tryptone broth or L broth and incubating for 20 min at 37 °C. The mixtures were then plated out for survivors. One unit of colicin was the reciprocal of the dilution required to kill 50% of the indicator cells. Lacunae (colicin-laden cells) were counted as previously described (Pugsley & Rosenbusch, 1981). PAGE in the presence of SDS was as described in Pugsley & Schnaitman (1979) and Pugsley & Rosenbusch (1981) and employed the Tris/glycine buffer system with 9% acrylamide in the separation gels. Molecular weight standards were phosphorylase B ($M_c 97 400$), bovine serum albumin (66 000), catalase (60 000), fumarase (49 000), ovalbumin (45 000), aldolase (40 000), acetylcholine esterase (33 500), bovine carbonic anhydrase (29 000), lysozyme (13 700) and cytochrome c (12 900). Gel filtration in Sepharose 6B (Pharmacia) was performed in columns with a height : diameter ratio of > 40 and with resin equilibrated with 20 mM-Tris/HCl, pH 7.2 containing 1 mM-Na$_2$O$_2$ as preservative. Samples (1% of total bed volume) were spent culture media from mitomycin-treated ColE2-P9$^+$ cultures in L broth. The medium was concentrated to 1/20 original volume by filtration through Amicon Diaflo PM10 filters and then dialysed against column buffer. Eluted fractions were assayed for colicin and were examined by SDS–PAGE.

**RESULTS**

Proteolysis of discharged colicin E4-CT9

The basic phenomenon examined in this report is that mitomycin-treated W3110 pColE4-CT9 cells synthesise large amounts of colicin which then apparently disappears from the culture as the cells lyse (Fig. 1). This behaviour contrasts sharply with that of W3110 pColE2-P9 cells.
which discharge active colicin when they lyse (Pugsley & Rosenbusch, 1981). The following set of observations led to the conclusion that colicin E4 was discharged by the lysing cells but that it was degraded by one or more proteases on the surface of the producing cells.

(1) Colicin polypeptides are easily identifiable as major bands when mitomycin-treated Col+ cells are examined by SDS-PAGE (Mock & Pugsley, 1982). This approach was used here to show that colicin E4 was synthesized in large amounts and then discharged by mitomycin-treated ColE4-CT9+ cells (Fig. 2). In addition, the number of lacuna-forming (colicin-laden) cells in mitomycin-treated ColE4-CT9+ cultures increased to nearly 100% of total cells after 40 min and then declined markedly (Fig. 1). We have previously suggested that this decline in lacuna count is due to the fact that the cells have discharged their colicin before being plated out.

(2) BtuB− mutants of W3110 pColE4-CT9 (selected as resistant to bacteriophage BF23 and shown to be resistant to all BtuB colicins; Kadner et al., 1979; Mock & Pugsley, 1982) lysed after mitomycin treatment, but colicin did not appear in the culture medium in increased amounts relative to BtuB+ cultures (data not shown). This indicates that the colicin was not discharged and then adsorbed on to specific cell surface receptors on the producing cells.

(3) The addition of the protease inhibitor p-aminobenzamidine (PAB) to ColE4-CT9+ cultures in tryptone broth + 0.5 μg mitomycin ml−1 in the period shortly after the onset of lysis resulted in a substantial increase in the amount of colicin E4 recovered from the culture medium (Fig. 1). Furthermore, colicin in the culture medium from cells not treated with PAB was
Fig. 3. Proteins in spent medium from mitomycin-treated cultures of various Col+ derivatives of W3110. Cells were grown in tryptone broth + mitomycin for 5 h before they were removed by centrifugation and the supernatant medium was filtered through Millipore type HA filters. Proteins were eluted from the filters and examined by SDS–PAGE. The positions to which authentic purified colicins migrated are indicated at the left of the gel. The colicin produced by the strain from which the sample was prepared is shown at the top. The positions of molecular weight standard proteins are indicated at the right of the gel. The plasmids carried by the producing strains were pColA-CA31, pColD-CA23, pColE1-K53, pColE2-P9, pColE3-CA38, pColE4-CT9, pColE5-O99, pColE6-CT14, pColE7-K317 and pColK-K235. Each sample represents 0.2 ml culture medium.

(4) Spent medium from mitomycin-treated Col+ cultures was examined for the presence of colicin polypeptide by filtering the clarified medium (centrifuged at 20000 g for 2 × 10 min) through Millipore type HA (cellulose ester) membrane filters (mean pore diameter 0.45 μm). As described in more detail below, these filters retain colicins and other proteins from the culture medium. The colicins could be eluted from the filter directly into SDS–PAGE sample buffer at 100 °C. Results in Fig. 3 indicate that colicins were the major proteins in the retentates when spent culture medium from ColA-CA31+, ColE1-K53+, ColE2-P9+, ColE3-CA38+, ColE5-O99+, ColE6-K317+ and ColK-K235+ cultures was filtered, but the filter retentates from the ColE4-CT9+ and ColE7-K317+ cultures were almost totally devoid of protein with the same Mr, as authentic colicins E4 or E7. The culture media contained, instead, large amounts of a number of smaller polypeptides which could have been derived from the colicin polypeptide by limited proteolysis. To show that this was the case, the ColE4-CT9+ cultures were treated with the protease inhibitors PAB, procaine, phenethyl alcohol or tosyl-L-lysine-chloromethyl ketone (Régnier, 1981) just after the onset of lysis (Fig. 4). Proteolysis of colicin E4 was substantially reduced in the presence of these inhibitors.

stabilized by removing the cells by centrifugation (Fig. 1). This suggests that a protease on the surface of the producing cells was degrading the discharged colicin.
Proteolysis of colicin E4 by producing cells

Fig. 4. Effects of protease inhibitors on the stability of colicin E4 released by mitomycin-treated W3110 pColE4-CT9 growing in tryptone broth. Inhibitors were added 10 min after the onset of lysis, and incubation was continued for a further 3 h before the cells were removed and the samples filtered as indicated in the legend to Fig. 3. The inhibitors used were: A, 5 mM-procaine; B, 30 mM-procaine; C, 30 mM-phenethyl alcohol; E, 5 mM-p-aminobenzamidine; F, 10 mM-p-aminobenzamidine; G, 30 mM-p-aminobenzamidine; H, 10 mM-tosyl-L-lysine-chloromethyl ketone (TLCK); I, 2 mM-TLCK; J, 5 mM-TLCK and K, 10 mM-TLCK. Sample D was not treated with an inhibitor. Unlettered pointers at the left indicate probable proteolytic fragments of colicin E4. Molecular weight markers and colicin E4 are indicated at the right of the gel.

Retention of colicins by membrane filters

The technique of concentrating colicin from spent culture media seemed to be so simple and potentially useful that more detailed investigation was justified. Further experiments indicated that the ability of Millipore HA filters to retain colicin could be saturated (Fig. 5). Experiments described here were all done under non-saturating conditions. Some colicins were only poorly retained by the filters. An example of this is shown in Fig. 3 in which the recovery of colicin D-CA23 seems to be unusually low when compared with the recovery of other proteins from the spent culture media. This is because >90% of the colicin D was always present in the filtrate.

Results in Fig. 6 show that not all of the proteins present in the spent culture medium from the lysed ColE2-P9+ culture were retained by the membrane filters. It was not possible to identify any of the other proteins which bound to the filters, but some proteins always seemed to be retained irrespective of the Col+ strain used (Fig. 3). Other proteins in the filter retenates could represent breakdown products of the colicin. Experiments with culture medium from a derivative of W3110 pColE2-P9 carrying a phoS mutation (which causes constitutive synthesis of alkaline phosphatase) and grown in tryptone broth + 1 mM-isopropyl-β-thiogalactopyranoside (to derepress β-galactosidase synthesis) + 500 μg mitomycin ml⁻¹ indicated that β-galactosidase and alkaline and acid phosphatases were present only in the filtrates (data not
Fig. 5. Saturation of Millipore type HA (cellulose ester) membrane filters with colicin from the supernatant medium of a culture of W3110 pColE2-P9 grown for 4 h in tryptone broth + 0.5 \( \mu \)g mitomycin ml\(^{-1}\). Various volumes of medium containing \( 2 \times 10^8 \) units colicin E2 ml\(^{-1}\) were filtered, and the final 1 ml of filtrate were collected and assayed (O). The filters were then immersed in SDS-PAGE sample buffer and heated to 100°C for 5 min. Samples of the retenate were loaded on SDS-polyacrylamide gels together with known amounts of bovine serum albumin. The gels were stained and photographed, and an integrated densitometry scan of the film negative was used to calculate the amount of colicin retained by the filters (●).

Fig. 6. Comparison by SDS-PAGE of proteins present in cells and supernatant medium from a culture of W3110 pColE2-P9 growing in tryptone broth + 0.5 \( \mu \)g mitomycin ml\(^{-1}\). Sample A represents whole cell proteins obtained as in Fig. 2. The cells were harvested 60 min after the addition of mitomycin and the sample is equivalent to 0.25 ml culture. Sample B represents proteins present in the supernatant medium 4 h after the addition of mitomycin. The proteins were concentrated by ethanol precipitation (Pugsley & Rosenbusch, 1981). The sample represents 0.2 ml culture. Sample C represents proteins retained by a Millipore type HA filter through which sample B had been filtered. The sample volume is equivalent to 0.2 ml culture. Molecular weight standard proteins are indicated at the right of the gel.
Proteolysis of colicin E4 by producing cells

Proteolysis of colicin E4 by producing cells shown. Colicin E2 was also retained by membrane filters when mitomycin-treated cells were broken in a French pressure cell and the soluble proteins (the supernatant fraction obtained after centrifugation at 50000 g for 2 h) were filtered.

Colicins were eluted from membrane filters in inactive form by immersing them in a solution of SDS at 100 °C. Active colicin E2 could be obtained by precipitating the eluate with cold acetone and then redissolving the precipitate in 20 mM-Tris/HCl buffer (pH 7.2). This procedure did not work for colicin E1, which was inactivated by heating in SDS. Active colicin could be quantitatively eluted from filters by immersing them in buffer containing the non-ionic detergents Triton X-100 or octylpolyoxyethylene. The detergents had to be present at greater than the critical micelle concentrations to be effective (approximately 0.02% for Triton X-100 and 0.2% for SDS and octylpolyoxyethylene). Colicins could not be eluted from the filters by mechanical means such as sonication.

By what mechanism are the colicins retained by the membrane filters? A priori it seemed unlikely that monomeric colicin was retained by virtue of its size, but the possibility remained that the colicins were present as large aggregates or in vesicles. Results from three experiments eliminated these possibilities: (1) colicin E2 migrated in the included volume on a Sepharose 6B column, and eluted together with bovine serum albumin (M_r 66000) which was added as a standard. Colicin E2 eluted from this column was retained by membrane filters, but the serum albumin was not. (2) Colicin E2 in spent culture medium was not sedimented by centrifugation at 100000 g for 4 h. (3) Colicin E2 was equally well retained by Millipore cellulose acetate and cellulose nitrate filters (0.45–0.5 μm mean pore diameter) but was not retained by Nuclepore polycarbonate membrane filters with pore diameters from 0.01 μm to 5 μm. The retention of colicin E2 by cellulose ester filters was unaffected by heating the filters at 100 °C in SDS solutions, by heating the sample to 100 °C for 5 min, by adjusting the pH of the sample to 2 or 12 (with HCl or NaOH, respectively) or by adding 8 M-urea or 1 M-NaCl to the sample. Filters did not retain colicin if the samples contained detergent at above the critical micelle concentration. These results suggest that the colicins are retained by hydrophobic interactions with the filters. This may also be the mechanism by which phage MS2 particles are retained by membrane filters (Farrah, 1982).

DISCUSSION

The results presented clearly establish that colicin E4-CT9 is discharged from mitomycin-treated ColE4+ cells when they lyse and is then degraded proteolytically. Other colicins may also be degraded when they are discharged but, with the exception of colicin E7-K3 17, the extent of degradation is much lower than with colicin E4. Proteolysis of discharged colicin E4 occurred only in the presence of the producing cells. Thus, although E. coli contains many soluble proteases which could be released when the cells lyse (Swamy & Goldberg, 1982), the protease(s) which degrade colicin E4 remain cell-associated. Outer membrane proteins do not appear in the culture medium when Col+ cells lyse after mitomycin treatment (Pugsley & Rosenbusch, 1981; A. P. Pugsley, unpublished data), and thin sections of lysed cells do not reveal extensive outer membrane damage when examined by electron microscopy (Mock, 1980). Thus at least one of the proteases responsible for colicin E4 degradation is apparently exposed on the surface of the cells.

Outer membrane proteases have been identified in several previous investigations (Régnier, 1981; Pacaud, 1982). Of particular interest is the recent report that colicins A and E1 are degraded by an outer membrane protease on the surface of indicator cells (Brey, 1982). Extensive degradation of these colicins may not have been observed in the present study because the colicin was present in far greater amounts than those used by Brey (1982). We must then assume that colicins E4 and E7 are exquisitely sensitive to the protease or that they are more accessible to it. In this context, we have noted that while colicins A, E1, E2, E3, E5 and E6 can be extracted with high yield by treating mitomycin-grown Col+ cells with Triton X-100 + 100 μM EDTA, extraction of ColE4+ or ColE7+ cells results in the complete disappearance of colicin activity within 10 min (M. Mock & A. P. Pugsley, unpublished results). These results...
suggest that these colicins are unusually sensitive to the protease. The results also suggest that the protease involved in colicin E4 degradation is not the same as the Triton-sensitive outer membrane protease identified by Régnier (1981), but this difference could again be explained by problems of substrate accessibility.

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