

## Characterization of Group B Colicin-Resistant Mutants of *Escherichia coli* K-12: Colicin Resistance and the Role of Enterochelin

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Nine classes of group B colicin-resistant mutants were examined to study the role of enterochelin in colicin resistance. Four of the mutants studied (*cbt*, *exbC*, *exbB*, and *tonB*) hypersecreted enterochelin. Enterochelin hypersecretion was apparently responsible for resistance of the *exbC* mutant to colicins G and H and for resistance of the *exbB* mutant to colicins G, H, Ia, Ib, S1, and V. All four mutants scored as colicin B tolerant, even in the absence of enterochelin synthesis. The mutants produced substantially increased amounts of two high-molecular-weight outer membrane polypeptides when grown under limiting iron conditions. The presence of these polypeptides was correlated with increased colicin B-neutralizing activity in the outer membrane preparations.

Colicins are protein antibiotics produced by some members of the *Enterobacteriaceae*. They act only on *Escherichia coli* and closely related species (24). The first stage of colicin action is adsorption to a specific surface receptor on the sensitive cell. Loss of a functional receptor will render the cell resistant to the colicin. Mutant cells that bind the colicin normally but are, nonetheless, resistant to the colicin are referred to as colicin tolerant (24).

The colicins have been divided into two groups, A and B, on the basis of cross-resistance patterns of a large number of colicin-resistant mutants of *E. coli* K-12 (3, 4). Guterman (8, 9), Guterman and Dann (10), and Davies and Reeves (3) have noted that some colicin B-resistant mutants of *E. coli* (*tonB* and *exbB*) secrete an inhibitor of colicin B action. This inhibitor was identified by Guterman (8, 9) as the siderochrome enterochelin (enterobactin), a cyclic trimer of 2,3-dihydroxybenzoyl serine (25). Davies and Reeves (3) found that enterochelin also strongly inhibits killing by colicin D. These workers also isolated a third class of colicin B-resistant mutants (*exbC*), which secreted an inhibitor of only colicin B when grown on nutrient agar.

In this paper we reexamine nine classes of mutants of *E. coli* K-12 that are resistant to the group B colicins to determine the role of enterochelin in colicin resistance. Four classes of mutants (*cbt*, *exbC*, *exbB*, and *tonB*) were found to hypersecrete enterochelin. All four classes of mutants were resistant to colicins B and D, but in no case was enterochelin secretion shown to

contribute to the colicin B and D resistance of these mutants. Enterochelin secretion did, however, contribute markedly to resistance to colicins G, H, Ia, Ib, S1, and V in two of the mutants. We also demonstrate that hypersecretion of enterochelin is associated with the appearance of substantially increased amounts of two polypeptides in the outer membranes of the mutants.

### MATERIALS AND METHODS

**Bacterial strains.** Strains of *E. coli* K-12 and colicinogenic bacteria used in this study are listed in Table 1. The colicin-resistant mutants used in this study have all been described previously (3). The *aroE* marker was inserted into these strains by cotransduction with spectinomycin resistance (*spc*) from strain AN366.

**Culture media and glassware.** Nutrient broth, nutrient agar, and soft nutrient agar were as described previously (3, 4). Minimal salts medium was a basic salts solution containing (in grams per liter):  $K_2HPO_4$ , 10.6;  $NaH_2PO_4 \cdot 2H_2O$ , 6.1;  $MgCl_2 \cdot 6H_2O$ , 0.17;  $(NH_4)_2SO_4$ , 2; and  $Ca(NO_3)_2$ , 0.01; adjusted to pH 7.2. Trace salt solutions were added as recommended by Neidhardt et al. (15). Analar-grade reagents were used throughout. The medium was supplemented where necessary with amino acids (0.1 g/liter), thiamine (0.001 g/liter), and 4-aminobenzoic acid and 4-hydroxybenzoic acid (0.007 g/liter). The *aroE* strains were grown in the absence of shikimic acid or 2,3-dihydroxybenzoic acid, unless otherwise stated, to suppress enterochelin synthesis. Glucose (5 g/liter) was used as a carbon source. Minimal media were solidified where required by the addition of 1.6% Difco agar.

Iron-depleted media were prepared by extracting  $10\times$  concentrated salts solutions with 100  $\mu$ g of 8-

TABLE 1. *Bacterial strains*

Strain no.	Colicin resist- ance genotype	Growth factor requirements and antibiotic resistances	Immediate source/reference
AB1133	S <sup>a</sup>	F <sup>-</sup> /thi, argE, his, proA, thr, leu, str	A. L. Taylor
P295	cbt	as AB1133	Mutants of AB1133 (3)
P535	exbC	as AB1133	
P575	exbB	as AB1133 but met	
P1245	exbB	as AB1133	
P585	tonB	as AB1133	
P625	cir	as AB1133	
P645	ivt	as AB1133	
P1235	cvt	as AB1133	
P1205	tonA	as AB1133	
P1209	cmt	as AB1133	
P1552	S	as AB1133 but aroE spc	Derivative of AB1133
P1553	cbt	as P1552	Derivative of P295
P1554	exbC	as P1552	Derivative of P585
P1555	exbB	as P1552 but met	Derivative of P575
P1556	tonB	as P1552	Derivative of P585
AN366	S	aroE, pabA, his, arg, ilv, purE, str, spc	G. Woodrowe
QD5003		P1, cml, ts, lysogen	B. Rolfe
AB1515	S	proC, purE, leu, trp, thi, str	G. Woodrowe
PL8-31	S	thr, leu, thi, proA, hisC, metG, metK, serA, glc, str	B. Bachmann
P1561	tsx	argG, metC, thiA, his, str	B. Stillman
H lip22	S	lip22	I. Young
χ478	tsx tonA	thi, metE, lysA, trpE, purE, proC, leu	B. Bachmann
AN156	S	proA, argE, pheA, tyrA, trp401, aroB, entA403	I. Young
AT2427	S	thi, cysC	B. Bachmann
PA-2-18	S	nadA, pro, argA, thi	B. Bachmann
AB2874	tsx	thi, ilvC, argE, his, proA, aroF363	B. Bachmann
AG097	colB <sup>+</sup> b		B. Stocker
K-12, CA23	colD <sup>+</sup>		K. Timmis
K-12, CA38	colE3 <sup>+</sup>		D. Helinski
CA46	colG <sup>+</sup>		P. Fredericq
CA58	colH <sup>+</sup>		P. Fredericq
CA56	colIa <sup>+</sup>		P. Fredericq
K-12, P9	colIb <sup>+</sup>		D. McCorquodale
K235	colK <sup>+</sup>		P. Fredericq
M32, T19	colM <sup>+</sup>		V. Braun
P1	colSI <sup>+</sup>		P. Fredericq
CA7	colV <sup>+</sup> c		N. Atkinson

<sup>a</sup> S, Colicin sensitive.<sup>b</sup> Colicinogenic.<sup>c</sup> May produce more than one colicin.

hydroxyquinoline (BDH) per ml. Iron-hydroxyquinoline and excess hydroxyquinoline were removed by repeated back-extraction with chloroform until hydroxyquinoline could not be detected in the chloroform phase by spectrophotometry (244 nm). Deionized distilled water was used in the preparation of all iron-free media.

Glassware for iron-depleted media was rinsed with 0.1 N HCl followed by copious quantities of deionized distilled water before being autoclaved.

**Iron assays.** The concentration of iron in media was determined by the Merckotest system (Merck). Assays were performed on 10× concentrated salts solutions and on 100× concentrated amino acid solutions. The test system was found to give reproducible results for iron concentrations over 3 μM. Media

were considered to be iron depleted when the final calculated iron concentration was less than 0.5 μM.

**Plate test for inhibitor secretion and colicin resistance.** Triple-layer plate tests for secretion of colicin inhibitor were performed as described by Davies and Reeves (3, 4), except that liquid colicin preparations of known activity were used in place of colicinogenic bacteria. Plate tests for colicin resistance were as described previously (3, 4).

**Ethyl acetate extraction of enterochelin and related compounds and analysis by thin-layer chromatography.** Enterochelin and related compounds were extracted from culture supernatants and from DE52 cellulose-fractionated culture supernatants with ethyl acetate (nanograde), concentrated by rotary evaporation, and examined by two-dimensional

thin-layer chromatography as described by O'Brien et al. (18). Cellulose F-coated plates (20 by 20 cm; Merck) were used for thin-layer chromatography, and plates were developed by spraying with a 1%  $\text{FeSO}_4$  solution (20).

**Fractionation of enterochelin and related compounds in culture supernatants by DE52 cellulose chromatography.** Cultures were grown at 37°C for 24 h in 1-liter volumes of iron-depleted minimal medium before harvesting by centrifugation at  $6,000 \times g$  for 20 min at 4°C.  $\text{FeSO}_4$  (400  $\mu\text{M}$ ) was added to the supernatants to stabilize the enterochelin and its hydrolysis products (25). The supernatant was then loaded directly onto a DE52 cellulose (Whatman) column (40 by 3 cm; equilibrated with 10 mM phosphate buffer, pH 7.1, at 4°C) at a flow rate of 1 liter/h. The column was washed with 1 liter of the phosphate buffer, and fractions were eluted with three linear gradients of  $\text{NH}_4\text{Cl}$  (pH 7.1): 0.01 to 0.1 M (1.5 liters), 0.1 to 2 M (2 liters), and 2 to 5 M (1 liter), all at 250 ml/h. Fractions of 10 ml were collected and examined for 2,3-dihydroxybenzoic acid-containing compounds by scanning spectrophotometry (300 to 350 nm; Perkin-Elmer model 124 spectrophotometer).

**Preparation of enterochelin.** Enterochelin was purified from culture supernatants of strain P585. Minimal medium containing 100  $\mu\text{M}$  2,2'-dipyridyl (BDH) and 80  $\mu\text{M}$   $\text{FeSO}_4$  was inoculated with washed cells and incubated with good aeration for 36 h at 37°C. Further  $\text{FeSO}_4$  was added during incubation to maintain the concentration of  $\text{FeSO}_4$  at the same molarity as the enterochelin in the medium. Cells were removed by centrifugation at  $6,000 \times g$  for 20 min, and the supernatant was loaded directly onto a DE52 cellulose column as described above. The column was washed with 250 ml of the phosphate buffer followed by 500 ml of 1 M  $\text{NH}_4\text{Cl}$  (pH 7.1). Enterochelin was eluted with 2 M  $\text{NH}_4\text{Cl}$  (pH 7.1). Fractions containing the red ferrienterochelin were extracted with ethyl acetate and crystallized by rotary evaporation. The dried enterochelin was dissolved in fresh ethyl acetate, washed successively with equal volumes of phosphate buffer (100 mM, pH 7.1) and distilled water, and concentrated to a final volume of 20 ml of rotary evaporation. Benzene (analar grade) was added to the ethyl acetate-enterochelin until faint turbidity persisted, and the enterochelin was crystallized by further rotary evaporation. The enterochelin was further dried over phosphorous pentoxide and stored at -20°C. Purity of the enterochelin was confirmed by dissolving in fresh ethyl acetate and analyzing by thin-layer chromatography. Ferrienterochelin was prepared by dissolving purified enterochelin in ethyl acetate, followed by rotary evaporation in the presence of an equimolar aqueous solution of  $\text{FeSO}_4$  until only the aqueous phase remained. Ferrienterochelin was filtered through cellulose acetate membrane filters before use. Enterochelin concentrations were determined from the extinction coefficient (19).

**Preparation and titration of colicins.** Colicins were prepared from cells grown to mid-log phase (optical density at 600 nm = 0.9) in well-aerated nutrient broth cultures at 37°C. Colicin production

was induced by the addition of mitomycin C at the concentrations shown in Table 2, and incubation continued for a further 3 h. Cells were harvested by centrifugation ( $6,000 \times g$ , 20 min, 4°C) and were washed once in 0.1 volume of phosphate buffer (100 mM, pH 7.1). Cells were finally resuspended in fresh phosphate buffer (50 ml/liter of original culture) and disrupted by sonication at 4°C for 30 min. Cell debris was removed by centrifugation ( $25,000 \times g$  for 60 min at 4°C), and the supernatants were stored at 4°C. Colicins in the supernatants were partially purified by  $(\text{NH}_4)_2\text{SO}_4$  precipitation. The  $(\text{NH}_4)_2\text{SO}_4$  cuts shown in Table 2 are those with highest colicin activity. Colicin titers were initially determined as described by Reeves (23) and are shown in Table 2 as arbitrary units per milliliter. We were consistently unable to obtain high yields of colicin H (or colicin G) by this or any other method. The activity spectrum of a colicin prepared from strain CA7 by this method did not correspond to that obtained with the colicinogenic bacteria in the triple-layer plate test. This preparation was therefore not used in the experiments described here.

Colicins were assayed against indicator strains under various conditions by mixing dilutions of the colicins (0.025 ml) with an equal volume of  $10^3$  washed viable cells suspended in growth medium. The mixtures were incubated at 37°C for 30 min and plated for survivors. Titters were recorded as dilutions giving 50% killing of the indicator cells.

**Preparation of outer membranes.** Triton-insoluble walls (outer membranes) were prepared using the method of Schnaitman (27) with minor modifications. Cells were grown to mid-log phase in well-aerated cultures, harvested by centrifugation ( $6,000 \times g$  for 20 min at 4°C), and washed once in 10 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4). The packed cells were flash frozen (-20°C) and cracked in an LKB X-press. HEPES buffer (10 mM) was then added to the cracked cells (4 ml of buffer per ml of cells) and mixed together with 5  $\mu\text{g}$  each of deoxyribonuclease and ribonuclease per ml (Sigma) for 30 s in an MSE blender. Cell debris was removed by centrifugation ( $5,000 \times g$ , 5 min). The crude cell envelope was then pelleted by centrifugation of the supernatant ( $40,000 \times g$ , 60 min), resuspended in 10 mM HEPES buffer

TABLE 2. Preparation of crude colicins

Colicin	Mitomycin C induction ( $\mu\text{g/ml}$ )	Cell sonicate colicin yield/ml of culture (AU <sup>a</sup> )	$(\text{NH}_4)_2\text{SO}_4$ cut (% saturation)	% Recovery in $(\text{NH}_4)_2\text{SO}_4$ cut
B-AG097	0.4	$5.6 \times 10^2$	30-50	91
D-CA23	0.4	$10^4$	30-50	83
E3-CA38	0.2	$1.6 \times 10^3$	20-40	78
H-CA58	0.2	4	20-40	56
Ib-P9	0.4	$10^4$	30-50	59
K-K235	0.5	$4.4 \times 10^3$	30-50	78
M-M32, T19	0.4	82	20-40	68
S1-P1	0.4	$1.6 \times 10^5$	30-50	78

<sup>a</sup> AU, Arbitrary units.

containing 2% Triton X-100 (BDH), and allowed to stand at room temperature for 10 min. The Triton-insoluble material was then pelleted by centrifugation ( $40,000 \times g$ , 60 min), the supernatant was discarded, and Triton extraction was repeated. The final pellet was resuspended in distilled water. Protein concentrations were determined by the method of Schacterle and Pollack (26).

**Polyacrylamide gel electrophoresis.** Triton-insoluble walls were solubilized for polyacrylamide gel electrophoresis by method II of Schnaitman (28). The samples were run on 7.5% polyacrylamide disc gels using the alkaline Bragg-Hou system (1). Gels were stained with Coomassie brilliant blue (17, 19) and, after destaining, were scanned in a Quick Scan densitometer (Helena Labs, Beaumont, Tex.).

**Assay of preparations for colicin-neutralizing activity.** Three systems were used for determinations of colicin-neutralizing activity. Method 1 was used to measure the colicin inhibitory activity of fractionated and unfractionated culture supernatants. Serial dilutions in distilled water were mixed with equal volumes of crude colicin and spotted onto nutrient agar plates previously spread with approximately  $10^6$  viable cells of the indicator strain, AB1133. Colicin concentrations were adjusted to the end point dilution just giving clear inhibition zones in the assay controls. Anticolicin activity was recorded as the last dilution at which inhibition of the colicin zone could be detected.

Method 2 was used to measure the anticolicin activities of purified enterochelin and ferrienterochelin. Dilutions of the enterochelin in iron-free minimal medium (0.025 ml) were mixed with an equal volume of approximately  $10^3$  viable indicator cells (strain P1552) resuspended in iron-free minimal medium, and the mixture was incubated for 30 min at 37°C. Colicin (0.025 ml diluted in iron-free minimal medium to a concentration giving 95% kill in the absence of inhibitor, equivalent to 3 to 5 lethal units adsorbed/cell) was then added, incubation was continued for a further 30 min, and aliquots were plated for survivors. Colicin inhibitory titers were recorded as the concentration giving 50% neutralization of colicin killing.

Outer membranes were assayed for colicin-neutralizing activity by method 3. Colicins and outer membrane diluted in phosphate buffer (100 mM, pH 7.1) were mixed and incubated as in method 2. A total of  $10^3$  viable cells of strain AB1133 were then added, and incubation was continued as before. Colicin neutralization titers were recorded as the amounts giving 50% inhibition of colicin killing.

**Transduction mapping.** All transduction experiments were performed using phage lysates prepared from cultures infected with the P1 *cml* temperature-sensitive bacteriophage from strain QD5003. The method of Pittard (21) was used throughout, and co-transduction frequencies were used to determine the distance between markers using the method of Wu (34).

## RESULTS

**Secretion of enterochelin.** *tonB*, *exbB*, and *exbC* mutants are known to secrete an inhibitor

of colicin B in the triple-layer plate test (3, 4). In the present study each of the nine group B colicin-resistant mutants was tested for production of inhibitor against colicins B, D, H, Ib, S1, and M in the triple-layer plate test. Only strains P535 (*exbC*), P575 (*exbB*), and P585 (*tonB*) produced inhibitor. Under our conditions, inhibition of colicins B and D was identical in all three mutants, with the *exbC* mutant secreting less inhibitor than the other two mutants. This conflicts with the results of earlier experiments, which indicated that the *exbC* mutant secreted inhibitor of colicin B only. In the present study, colicins B and D were used at the same concentration, whereas the use of agar seeded with colicinogenic bacteria, as used previously, resulted in the presence of low concentrations of colicin B and high concentrations of colicin D in the agar (unpublished data). Hence, inhibitor secreted by the *exbC* strain would not have been detected on plates seeded with the colicin D-producing strain.

The *tonB*, *exbB*, and *exbC* mutants also secreted inhibitor of colicin H but did not produce zones of inhibition against the other colicins tested. Inhibitor secretion by all strains was suppressed in agar containing an additional 8  $\mu\text{M}$   $\text{FeCl}_3$  [nutrient agar as prepared normally contained approximately 10  $\mu\text{M}$   $\text{Fe(III)}$ ], by growth under anaerobic conditions, or by using *aroE* derivatives of the mutants. Synthesis of shikimic acid, a precursor of enterochelin, is blocked in strains carrying the *aroE* marker. These strains, therefore, produce no enterochelin in the absence of precursors (see Fig. 1). These results confirm Guterman's suggestion (8, 9) that the colicin inhibitor is involved in some way in iron transport and is probably enterochelin.

**Activity spectrum of the inhibitor produced by each mutant in minimal medium.** Type strains of each class of mutant and the parent strain were grown for 24 h with shaking in minimal medium in the presence or absence of 20  $\mu\text{M}$  iron, and the culture supernatants were examined for inhibitors by assaying against colicins (method 1, see Materials and Methods). Strains P295 (*cbt*), P535 (*exbC*), P575 (*exbB*), and P585 (*tonB*) all secreted inhibitor of colicins B, D, H, Ib, and S1 when grown under iron-free conditions. Inhibitor secretion was suppressed in the presence of iron and was enhanced in the presence of 100  $\mu\text{M}$  2,2'-dipyridyl or desferrioxamine B (Desferal, Ciba-Geigy). These compounds bind iron strongly and, therefore, further deplete the level of available iron in the culture medium. None of the other mutants tested secreted inhibitor of the group B colicins.

The culture supernatants of all four strains

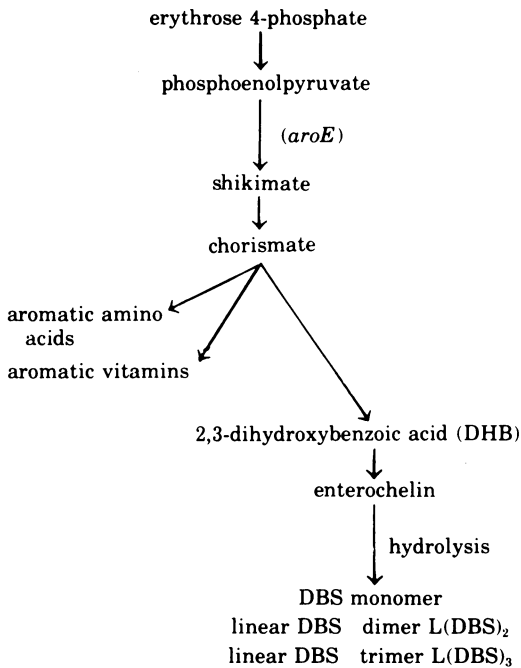


FIG. 1. Simplified biosynthetic pathway for enterochelin (according to Gibson and Pittard [7] and Rosenberg and Young [25]).

producing colicin inhibitor were found to contain enterochelin and its hydrolysis products (the monomer, dimer, and linear trimer of 2,3-dihydroxybenzoyl serine; see Fig. 1 and reference 20) as determined by thin-layer chromatography of ethyl acetate extracts. Supernatants of cultures shown to contain no colicin inhibitor contained only 2,3-dihydroxybenzoate (DHB), the secreted precursor of enterochelin (see Fig. 1).

One-liter cultures of the *tonB*, *exbC*, *exbB*, and *cbt* mutants were therefore grown, and the supernatants were fractionated by DE52 cellulose column chromatography as described in Materials and Methods. Fractions were assayed for colicin inhibitory activity (method 1) and also analyzed by thin-layer chromatography for enterochelin and related compounds. The results of a typical experiment are shown in Fig. 2. The figure shows the peaks of DHB- and 2,3-dihydroxybenzoyl serine-containing compounds, including enterochelin. High levels of anticolicin B activity were detected in all fractions containing dehydroxybenzoyl serine or enterochelin (Fig. 2). Colicins D and H were also strongly inhibited by these fractions, although the anticolicin H titer was up to 10-fold lower than the anticolicin B and D titers. Colicins Ib and S1 were inhibited only by fractions

containing high concentrations of enterochelin. No anticolicin E3, K, or M activity could be demonstrated in any of the fractions.

**Colicin-neutralizing activity of purified enterochelin.** Purified enterochelin and ferrienterochelin were assayed for anticolicin activity as described in Materials and Methods. The results (Table 3) indicate that ferrienterochelin is a far more effective inhibitor of colicin killing and confirm that only colicins B, D, H, Ib, and S1 are inhibited.

**Effect of iron chelators on colicin titers.** When colicins B, D, H, Ib, and S1 were diluted in 5  $\mu$ M solutions of enterochelin and assayed against strain AB1133, colicin titers were considerably reduced when compared with the titers of the colicins diluted in distilled water (Table 4). In contrast, desferrioxamine B, ethylenediaminetetraacetic acid (EDTA), and 2,2'-dipyridyl enhanced killing of strain AB1133 by these colicins. The enhanced killing could be eliminated by diluting the colicins in a mixture of equimolar concentrations of the chelator and  $\text{FeCl}_3$ . The weak iron chelator sodium citrate had no effect on colicin titers, even at high concentrations, but  $\text{FeCl}_3$  gave some protection against colicins B, D, and H and enhanced the protective effect of enterochelin (Table 3). It was interesting to note that only enterochelin, of all the compounds tested, had any effect on the titers of colicins B, D, H, Ib, and S1 on strain P1552 (*aroE*). However, this strain was already more sensitive to these colicins than the parent strain AB1133 (see below).

**Role of enterochelin in resistance to the group B colicins.** The role of enterochelin in colicin resistance of the four colicin B-resistant mutants was tested by comparing their colicin resistance patterns with those of their *aroE* derivatives. In every case, colicin resistance scored as tolerance and not as loss of receptor. This was confirmed by assaying the colicin-neutralizing activity of washed whole cells by method 3 (see Materials and Methods). *aroE* derivatives of the four mutants studied neutralized as much colicin B and D as cells of the colicin-sensitive strain P1552 (*aroE*) after growth in iron-supplemented minimal medium. The results in Table 5 indicate that hypersecretion of enterochelin may account for resistance to some or all of the colicins G, H, Ia, Ib, S1, and V in the *exb* mutants. None of the other group B colicin-resistant mutants showed any indication of alteration in the colicin resistance pattern associated with blocking enterochelin biosynthesis. The *aroE* mutants are also defective in the synthesis of aromatic amino acids, aromatic vitamins, and DHB; however,

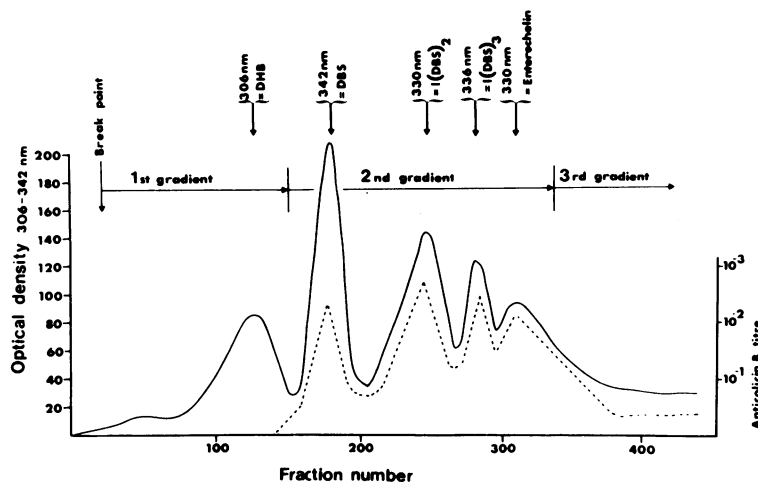


FIG. 2. DE52 cellulose column chromatography of culture supernatant of strain P585 (*tonB*). Fractions were assayed for the presence of DHB derivatives by scanning spectrophotometry (solid line) and thin-layer chromatography and for anticolicin B activity (broken line). DHB, 2,3-dihydroxybenzoic acid; DBS, 2,3-dihydroxybenzoyl serine.

TABLE 3. Titration of anticolicin activity of enterochelin and ferrienterochelin<sup>a</sup>

Inhibitor	Inhibitory concn ( $\mu\text{M}$ ) <sup>b</sup>				
	B <sup>c</sup>	D	H	Ib	S1
Enterochelin	20	20	200	— <sup>d</sup>	—
Ferrienterochelin	0.01	0.01	0.06	30	30

<sup>a</sup> The table shows the concentration of each compound giving 50% neutralization of colicin activity on strain P1552.

<sup>b</sup> No inhibition of colicins M, E3, or K with 200  $\mu\text{M}$  ferrienterochelin or enterochelin.

<sup>c</sup> Colicin.

<sup>d</sup> No inhibition with 200  $\mu\text{M}$  enterochelin.

none of these compounds had any effect on colicin when studied under the same conditions as enterochelin in Tables 3 and 4.

The role of enterochelin in colicin resistance of the *exb* mutants was confirmed by titrating the colicins against the *aroE* derivatives grown in minimal medium (10  $\mu\text{M}$  iron) in the presence or absence of shikimic acid or DHB as enterochelin precursors. The results in Table 6 show that growth in the presence of shikimic acid increased resistance of strains P1554 (*exbC*, *aroE*) and P1555 (*exbB*, *aroE*) to colicins H, Ib, and S1. Similar results were obtained with the same strains grown in the presence of DHB (200  $\mu\text{M}$ ). *aro*<sup>+</sup> strains (in the absence of shikimic acid or DHB) gave the same results as the *aroE* strains in the presence of the enterochelin precursors.

It is interesting to note that *aroE* strain P1552 was more sensitive to colicins H, Ib, and S1 and, particularly, to colicins B and D when

grown in the absence of enterochelin precursors (Table 6).

Outer membrane polypeptides of the group B colicin-resistant mutants. Davies and Reeves (3) have shown that *exbB* and *tonB* mutants have large amounts of two high-molecular-weight outer membrane polypeptides that are not present in outer membranes prepared from the parent cells. In the present study, the experiments reported by Davies and Reeves were repeated using the Bragg-Hou alkaline gel system (1) for electrophoresis of outer membranes from cells grown in minimal medium in the presence or absence of iron. Two high-molecular-weight polypeptides (approximate molecular weights, 85,000 and 92,000) were present in outer membranes prepared from cells of all four colicin B-resistant mutant classes, but only when grown in the absence of iron (Fig. 3). The peaks were not present in outer membranes prepared from the parent strain (AB1133) or in outer membranes of any of the other group B colicin-resistant mutants.

Supernatants of cultures used to prepare outer membranes for electrophoresis were extracted with ethyl acetate and examined for the presence of enterochelin and related compounds by thin-layer chromatography. The presence of the two new polypeptide peaks shown in Fig. 3 was correlated with the presence of enterochelin in the culture supernatants. The peaks were not present in outer membranes prepared from colicin B-resistant mutants grown in the presence of sufficient iron to suppress production of enterochelin.

TABLE 4. Titration of colicins on strain AB1133 in the presence of various iron chelators or FeCl<sub>3</sub>

Chelator	Concn (μM)	FeCl <sub>3</sub> concn (μM)	Relative colicin titer <sup>a</sup>							
			B	D	E3	H	Ib	K	M	S1
None			1	1	1	1	1	1	1	1
Enterochelin	5		0.0025	0.0025	1	0.05	0.1	1	1	0.1
Enterochelin	5	5	<10 <sup>-4</sup>	<10 <sup>-4</sup>	1	0.0025	0.05	1	1	0.05
Desferrioxamine B	100		40	40	1	10	8	1	1	8
Desferrioxamine B	100	100	1	1	1	1	1	1	1	1
Dipyridyl	100		80	80	1	40	40	1	1	40
Dipyridyl	100	100	2	2	1	1	1	1	1	1
EDTA	100		20	20	1	4	4	1	1	4
EDTA	100	100	1	1	1	1	1	1	1	1
Sodium citrate	1,000		1	1	1	1	1	1	1	1
Sodium citrate	1,000	100	0.04	0.04	1	0.4	1	1	1	1
None		100	0.04	0.08	1	0.4	1	1	1	1

<sup>a</sup> Compared with titer in absence of chelator or additional iron.

TABLE 5. Colicin resistance patterns of the mutant strains and their *aroE*<sup>-</sup> derivatives, as determined by the triple-layer plate test

Strain no.	Relevant genotype	Colicin resistance <sup>a</sup>								
		B	D	G	H	Ia	Ib	M	S1	V <sup>b</sup>
AB1133	Parent	S <sup>c</sup>	S	S	S	S	S	S	S	S
P1552	<i>aroE</i> <sup>-</sup>	S	S	S	S	S	S	S	S	S
P295	<i>cbt</i>	T <sup>d</sup>	T	S	S	S	S	S	S	S
P1553	<i>cbt, aroE</i> <sup>-</sup>	T	T	S	S	S	S	S	S	S
P535	<i>exbC</i>	T	T	T	T	S	S	T	S	S
P1554	<i>exbC, aroE</i> <sup>-</sup>	T	T	S	S	S	S	T	T	S
P575	<i>exbB</i>	T	T	T	T	T	T	T	T	P <sup>e</sup>
P1555	<i>exbB, aroE</i> <sup>-</sup>	T	T	S	S	S	S	P	S	S
P585	<i>tonB</i>	T	T	T	T	T	T	T	T	T
P1556	<i>tonB, aroE</i> <sup>-</sup>	T	T	T	T	T	T	T	T	T

<sup>a</sup> All strains were sensitive to the group A colicins: A, E1, E2, E3, K, L, N, S4, and X.

<sup>b</sup> Colicin V-CA7 may consist of two colicins.

<sup>c</sup> S, Colicin sensitive.

<sup>d</sup> T, Colicin tolerant.

<sup>e</sup> P, Partially colicin tolerant.

TABLE 6. Titration of colicins against *aroE* derivatives of the colicin B-resistant mutants grown in the presence or absence of 400 μM shikimic acid as enterochelin precursor

Strain no.	Relevant genotype	Shikimic acid	Relative colicin titer <sup>a</sup>							
			B	D	E3	H	Ib	K	M	S1
P1552	<i>aroE</i>	+	1	1	1	1	1	1	1	1
		-	32	16	1	4	4	1	1	2
P1553	<i>aroE cbt</i>	+	<10 <sup>-6b</sup>	<10 <sup>-5</sup>	1	0.5	0.5	1	1	0.5
		-	<10 <sup>-4</sup>	<10 <sup>-5</sup>	1	4	1	1	1	1
P1554	<i>aroE exbC</i>	+	<10 <sup>-4</sup>	<10 <sup>-5</sup>	1	0.0025	0.05	1	0.5	0.05
		-	<10 <sup>-4</sup>	<10 <sup>-5</sup>	1	0.25	0.5	1	1	0.5
P1555	<i>aroE exbB</i>	+	<10 <sup>-4</sup>	<10 <sup>-5</sup>	1	<10 <sup>-3</sup>	<10 <sup>-4</sup>	1	<10 <sup>-4</sup>	<10 <sup>-5</sup>
		-	<10 <sup>-4</sup>	<10 <sup>-5</sup>	1	0.25	0.25	1	<10 <sup>-4</sup>	0.5
P1556	<i>aroE tonB</i>	+	<10 <sup>-4</sup>	<10 <sup>-5</sup>	1	<10 <sup>-3</sup>	<10 <sup>-4</sup>	1	<10 <sup>-4</sup>	<10 <sup>-5</sup>
		-	<10 <sup>-4</sup>	<10 <sup>-5</sup>	1	<10 <sup>-3</sup>	<10 <sup>-4</sup>	1	<10 <sup>-4</sup>	<10 <sup>-5</sup>

<sup>a</sup> Compared with that against strain P1552 grown in the presence of shikimic acid.

<sup>b</sup> Fully resistant to the highest concentration of colicin tested.

Outer membranes of the colicin B-resistant mutants were assayed for their ability to neutralize colicins as a measure of their colicin receptor activity. Assays were performed by

method 3 (see Materials and Methods). The results shown in Table 7 indicate that outer membranes prepared from colicin B-resistant strains grown in the absence of iron have more

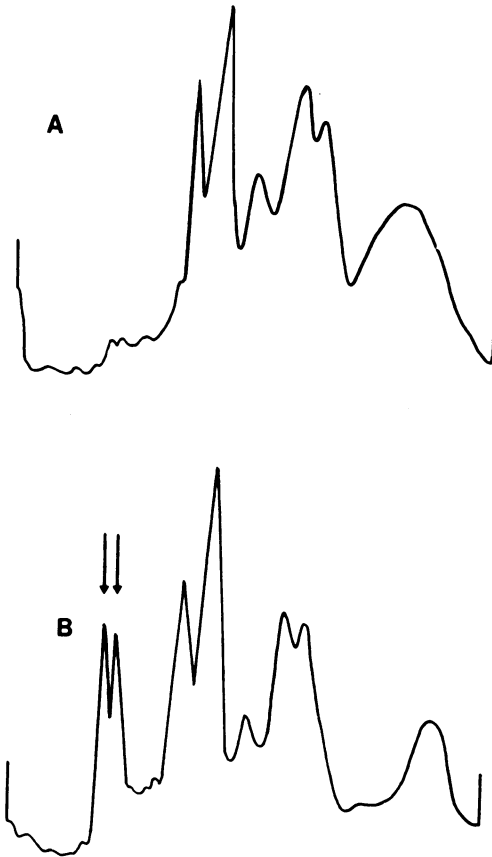


FIG. 3. Comparison by densitometry of stained bands of the outer membrane polypeptides of strains AB1133 (A) and P585 (*tonB*; B) grown in iron-free medium and examined as described in the text. Similar profiles to that shown in (B) were obtained with outer membranes from strains P295 (*cbt*), P535 (*exbC*), and P575 (*exbB*).

colicin B- and D-neutralizing activity than preparations from cells grown in the presence of iron. Similar results were obtained with outer membranes prepared from *aroE* derivatives of these mutants (see reference 22), indicating that the increased colicin-neutralizing activity is not due to enterochelin in the preparations.

**Genetic analysis of colicin B resistance.** The map locations of *tonB*, *exbB*, and *cbt* mutations have been characterized to varying extents by transduction (3, 8, 10, 13, 30), and the *exbC* locus has been very approximately mapped by Hfr crosses (3). We have confirmed or extended for our strains the transduction data for all four strains. The results, based on analysis of over 200 recombinant colonies for each transduction, are shown in Table 8. The data confirm the previously reported map locations of *tonB* and *exbB*. The co-transduction data for the *cbt* locus and the *lip*, *purE*, and *ent* loci confirm the earlier supposition (3) that *cbt* maps between *lip* (14.6 min) and *purE* (12 min). The data for the *exbC* mutation establish that this locus maps quite separately from the *exbB* locus and close to *cysC* at 52 min.

# DISCUSSION

There is now extensive evidence for the interaction of cellular iron transport systems and the early stages of group B colicin action in *E. coli* K-12 (3, 8-11). Hu et al. (12) have also presented some evidence for a similar phenomenon in the action of pesticin, a colicin-like substance originally identified as being produced by *Yersinia pestis*. In this paper, nine types of group B colicin-resistant mutants were reexamined to study in more detail the interaction between the group B colicins and the

TABLE 7. Neutralization of colicins by outer membranes of mutant strains grown in the presence or absence of iron

Strain no.	Concn of Fe in growth medium ( $\mu$ M)	$\mu$ g of protein in TIW <sup>a</sup> giving 50% neutralization of colicin killing of 1,000 cells of strain AB1133 <sup>b</sup>					
		B	D	H	Ib	M	S1
AB1133	5	20	16	8	>25	>25	>25
	<1	21	16	9	>25	>25	>25
P295 ( <i>cbt</i> )	5	21	12	10	>25	>25	>25
	<1	3.1	1.9	14	>25	14	>25
P535 ( <i>exbC</i> )	5	21	16	10	>25	>25	>25
	<1	1.5	1.2	11	>25	19	>25
P575 ( <i>exbB</i> )	5	18	11	21	>25	>25	>25
	<1	1.9	1.2	13	15	13	24
P585 ( <i>tonB</i> )	5	21	14	16	25	>25	24
	<1	1.5	0.9	14	16	13	16

<sup>a</sup> Triton-insoluble wall.

<sup>b</sup> Mean of two or more assays.



TABLE 8. Transduction data for colicin resistance genes

P1 grown on strain:	Recipient strain	Selected marker	Transduc- tants carry- ing selected and unse- lected markers (%)
P292 ( <i>cbt</i> )	AB1515 ( <i>purE</i> )	<i>pur</i> <sup>+</sup>	8.8
	H lip22 ( <i>lip</i> )	<i>lip</i> <sup>+</sup>	9.8
AN156 ( <i>entA</i> )	P292 ( <i>cbt</i> )	<i>cbt</i> <sup>+</sup> <sup>a</sup>	86
P1243 ( <i>exbB</i> )	P1561 ( <i>metC</i> )	<i>met</i> <sup>+</sup>	96
	PL8-31 ( <i>serA glc</i> )	<i>ser</i> <sup>+</sup>	1.4
P585 ( <i>tonB</i> )	χ478 ( <i>trpE</i> )	<i>glc</i> <sup>+</sup>	66
P535 ( <i>exbC</i> )	AT2427 ( <i>cysC</i> )	<i>trp</i> <sup>+</sup>	46
	PA-2-18 ( <i>argA</i> )	<i>cys</i> <sup>+</sup>	10.5
	AB2874 ( <i>aroF</i> )	<i>arg</i> <sup>+</sup>	0
		<i>aro</i> <sup>+</sup>	0

<sup>a</sup> Selected for ability to utilize enterochelin for iron transport.

siderochrome enterochelin. Four of the mutant classes secreted enterochelin when grown in medium containing sufficient iron to suppress enterochelin production in wild-type cells and are shown to be defective in enterochelin-mediated iron transport (22).

Enterochelin clearly accounted for most, if not all, of the resistance of the *exbC* and *exbB* mutants to colicins G, H, Ia, Ib, S1, and V (Tables 5 and 6). Not only is the action of these colicins inhibited by enterochelin, but also *aroE* derivatives of the two mutants became sensitive to these colicins, presumably because enterochelin is not secreted. However, the *exbB* and *exbC* mutants did not produce zones of inhibition of colicins Ib and S1. This may be because enterochelin is a far less effective inhibitor of these colicins than of colicins B, D, and H and was not present in sufficient concentration in the overlay. The enterochelin that protects the *exbB* and *exbC* mutants themselves from colicins Ib and S1 is presumably cell bound or in high concentrations near the cells.

Resistance of all four mutant classes to colicins B and D was absolute (to the levels of colicin we were able to test) in the presence or absence of enterochelin synthesis. This confirms and extends the observation of Wookey (quoted by Frost and Rosenberg [6]) with a *tonB* mutant. In this study we confirm the earlier observation (2) that resistance to colicins B and D in all our mutants is due to tolerance rather than to loss of receptor. This is so even for *aroE* derivatives, thus excluding the possibility that enterochelin is interfering in the assay. Colicin B and D resistance is therefore due to a block after binding of colicin to receptor.

The results in Table 6 show that ability to

synthesize enterochelin may affect the sensitivity of the parent strain to colicins B and D, as was suggested by Guterman (8). The increased sensitivity to colicins B and D in strain P1552 (*aroE*) compared with strain AB1133 (*aro*<sup>+</sup>) is probably due to the presence of enterochelin or related compounds attached to surface receptors of the *aro*<sup>+</sup> cells.

Additional evidence for an effect of ferrienterochelin on the sensitivity to colicins B and D comes from our observations on colicin killing in the presence of strong iron-chelating agents. Desferrioxamine B, 2,2'-dipyridyl, and EDTA all increase the activity of colicins B and D on sensitive *aro*<sup>+</sup> cells. Timmis and Hedges (32) have reported that EDTA enhanced the activity of colicin D on sensitive cells and attributed this to increased permeability of the cell membrane, permitting increased penetration of the colicin D molecule (31). Since desferrioxamine B and 2,2'-dipyridyl are both highly specific chelators of iron (see reference 16) and since none of these reagents had any effect on colicin action on colicin-sensitive *aroE* cells, a more likely explanation is that these reagents remove iron, which normally inhibits killing by the colicins. The iron-mediated inhibition of colicin B and D action presumably involves enterochelin in some way, since iron will not protect cells that are unable to synthesize enterochelin and yet considerably enhances the colicin inhibitory activity of enterochelin. A possible explanation for these phenomena is that ferrienterochelin inhibits colicin B and D action by blocking colicin adsorption in a manner similar to that by which vitamin B12 blocks colicin E3 adsorption (5) and ferrichrome blocks colicin M and phage φ80 (11, 14, 33) adsorption; that is, that ferrienterochelin and colicins B and D have a common cell surface receptor component. Desferrioxamine B, 2,2'-dipyridyl, and EDTA may compete for iron with the enterochelin, thereby leaving some enterochelin free of iron and possibly freeing ferrienterochelin receptors. Desferrienterochelin was shown to be a much less effective inhibitor of colicin action than ferrienterochelin, and thus one would expect there to be less inhibition of killing by colicins B and D under these circumstances. As noted above, there is no evidence that any of our mutants are defective in colicin receptor activity, although the *cbt* mutant may be defective in ferrienterochelin receptor activity (see reference 22).

The appearance of substantially increased amounts of two polypeptides in outer membranes of iron-deprived colicin B-resistant mutants is correlated with an increase in neutral-

izing activity for colicins B and D in the outer membrane. We have not yet demonstrated whether or not colicins B and D bind to either of these polypeptides, but it is interesting to speculate that they form part of the colicin B and D receptor complex. We hope to learn more of this interaction after purifying the two polypeptides.

The mechanism of enterochelin-mediated inhibition of colicins G, H, Ia, Ib, S1, and V is not understood at present. Ferrichrome has been reported to inhibit killing by colicins B and V as well as by colicin M (14). This inhibition presumably occurs at some stage of colicin action other than binding, and this may also be the case for enterochelin-mediated inhibition of colicins G, H, Ia, Ib, S1, and V. To test this hypothesis, one could use a ferrienterochelin receptor mutant to determine whether ferrienterochelin could protect this strain from the action of these colicins. Results of experiments described elsewhere (22) suggest that strain P1553 (*cbt*, *aroE*) may be such a mutant. The *cbt* mutant resembles strains carrying the *fep* mutation (control of ferrienterochelin uptake) described by Cox et al., both in ability to transport ferrienterochelin (22) and in location of the *cbt* mutation (2, 25, 30). Enterochelin did not protect this mutant from the action of colicins Ib and H (unpublished data), suggesting that a functional ferrienterochelin transport system is necessary for protection of sensitive cells against these colicins.

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