INCREASED PRODUCTION OF THE OUTER MEMBRANE RECEPTORS FOR COLICINS B, D AND M BY ESCHERICHIA COLI UNDER IRON STARVATION

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Growth of E. coli K-12 under severe iron stress results in increased production of the outer membrane receptors for colicins B, D, Ib and M. The increase in colicin receptor activity coincides with the appearance of large amounts of two high molecular weight proteins in the outer membrane of the cells. These proteins are identified as the outer membrane receptors for colicins B and D and for colicin M. Mutants lacking a functional outer membrane receptor for colicins B and D are defective in the uptake of iron complexed with the siderochrome enterochelin, and are thus comparable with tonA mutants which lack a functional receptor for colicin M and are defective in the uptake of iron complexed with ferrichrome (6). The colicin B and D receptor may therefore function in the uptake of ferri-enterochelin.

Proteins of the outer membrane of Escherichia coli K-12 may be divided into two types on the basis of their relative abundance in the cell wall. None of the so-called "major" proteins (1) have a well defined function, although a role in recipient ability during conjugation has been attributed to one of these proteins (2) and some of the "major" proteins are known to act as surface receptors for certain bacteriophages (2, 3). Many of the "minor" proteins of the E. coli outer membrane have also been identified by their roles as cell surface receptors for bacteriophages and for colicins and several of these proteins have subsequently been shown to function in the transport of nutrients across the outer membrane (4, 5, 6).

We have previously demonstrated that two normally undetectable outer membrane proteins appear in large amounts in certain colicin resistant mutants of E. coli (7) and when E. coli are grown under severe iron stress (8). A correlation observed between production of these proteins and increased receptor activity for colicins B and D and for ferri-enterochelin, an iron chelate used for iron uptake by E. coli, indicated that the proteins may function as a colicin and ferri-enterochelin receptor complex (8). In this paper we describe the purification and partial characterisation of these pro-

teins, which are referred to herein as proteins P and Q in order of decreasing molecular weight.

MATERIALS AND METHODS

Strain P1552 (E. coli K-12; thr, leu, proA, his, argE, thi, aroE, supE, str, spc (8)) was used in all experiments. This strain does not produce enterochelin unless it is supplied with shikimic acid or 2,3-dihydroxybenzoic acid. Cells were grown at 37° with good aeration in minimal medium 56 (9) without $FeSO_4$ and supplemented with amino acids (0.1 g/l), thiamine (0.01 g/l), 4-aminobenzoic acid (0.007 g/l) and 4-hydroxybenzoic acid (0.007 g/l). 2,2'dipyridyl (100 μM) was added to minimal media to complex free iron into a form which cannot be utilized by strain P1552. Glucose (5 g/l) was used as carbon source. Nutrient agars were as used previously (7).

Colicins were prepared after mitomycin C induction (0.4 μg/ml nutrient broth culture) of strains T20 (colB-K260), UB1082 (colD-CA23), M32.T19 (colM-K260, P9 (colIb-P9), K235 (colK-K235) and K-12.CA38 (colE3-CA38) by sonicating the cells at 4°, precipitating the colicin released with ammonium sulphate and dissolving the crude colicins in 10 mM phosphate buffer (Na^+, pH 7.1). Colicin receptor assays were performed by mixing 0.1 ml of buffer containing 20 arbitrary units of colicin (see ref. 10) with an equal volume of a dilution of the putative receptor, incubating for 30 min at 37° and spotting aliquots of the mixture onto a lawn of 3×10^6 cells of strain P1552 in soft nutrient agar. Plates were incubated at 37° and receptor titres (colicin receptor units, CRU) were the reciprocal of the last dilution of material giving complete neutralisation of colicin activity.

Outer membranes were prepared as previously described (7, 8) and solubilised in 2% Triton-X100 in tris EDTA buffer (TTE) for chromatography on DE52 (diethylaminoethyl) cellulose (Whatman) as described by Sabet and Schnaitman (11) except that 2% Triton was used for all stages of chromatography. Protein solubilised in TTE was determined by the method of Wang and Smith (12). Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl

Table 1. Titration of colicin receptor activities in the outer membrane of strain P1552 after growth in minimal medium containing 100 μM $FeCl_3$ or 100 μM 2,2'-dipyridyl (DP).

Growth Conditions	Colicin receptor activities (CRU/mg outer membrane protein)[a]					
	B	D	E3	Ib	K	M
100 μM $FeCl_3$	20	20	80	40	400	20
100 μM DP	320	320	80	100	400	400

[a] Outer membranes were suspended in distilled water to a final concentration of 10 mg/protein/ml.

sulfate was as described previously (7, 8) using the alkaline Bragg-Hou system.

RESULTS AND DISCUSSION

Outer membranes prepared from cells of strain P1552 grown in minimal medium containing 100 μM $FeCl_3$ or 100 μM 2,2'-dipyridyl were assayed for colicin receptor activities (Table 1). Receptor activities for colicins B, D and M were substantially greater in outer membrane prepared from iron-starved cells. A slight increase in colicin Ib receptor activity was also noted under these conditions. The increase in colicin receptor activity coincided with the appearance of large amounts of proteins P and Q. Outer membrane from iron-starved cells of strain P1552 was solubilised in TTE and chromatographed on DE52 cellulose (Fig. 1). Receptor activities for colicins E3, K and Ib were separated by this procedure, but receptor activities for colicins B, D and M were eluted together. Receptor activity for colicin Ib was apparently inactivated in TTE and was only detected after removal of Triton by precipitating the protein in cold ethanol (11).

Fractions 110-138, which contained over 90% of the colicin B, D and M receptor activities eluted from the DE52 column, were pooled, concentrated by ethanol precipitation (11) and a sample was examined by gel electrophoresis (Fig. 3D). The material contained proteins P and Q together with small

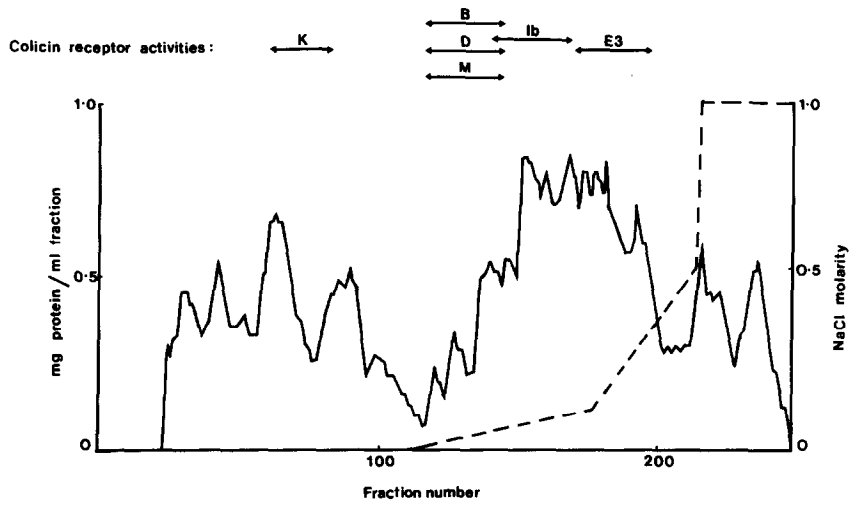

Figure 1. DE52 cellulose chromatography of TTE-solubilised outer membrane of strain P1552 grown in the presence of 100 μM 2,2'-dipyridyl. Fractions were eluted from the column (50 x 2.5 cm) with 1000 ml TTE followed by linear gradients of 0 - 0.1 M NaCl (600 ml) and 0.1 - 0.5 M NaCl (600 ml) and a step of 1 M NaCl (300 ml), all in TTE. Fractions were examined for colicin receptor activities and for protein content (———) and for NaCl molarity (------).

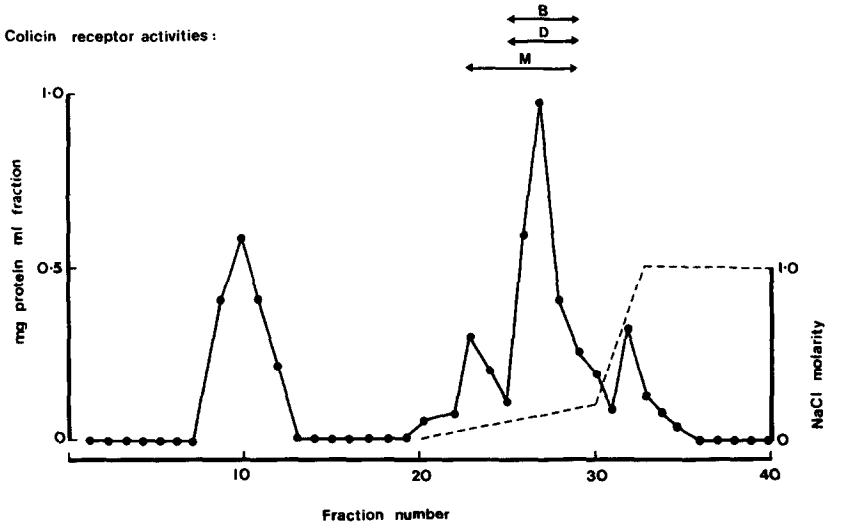

Figure 2. DE52 cellulose chromatography of material containing receptor activity for colicins B, D and M shown in Figure 1 (fractions 110 - 138). Fractions were eluted from the column (40 x 1.2 cm) with 100 ml TTE followed by a linear gradient of 0 - 0.14 M NaCl (50 ml) and a step of 1 M NaCl (50 ml), both in TTE. Fractions were examined for colicin receptor activities and for protein content (●———●) and for NaCl molarity (-----).

amounts of a third protein (identified as protein 3a (II*) by its receptor activity for bacteriophage K3 (1, 2 and P. Manning, personal communication) and a few minor contaminants. Only small amounts of proteins P and Q were identified in fractions after number 138.

The remainder of the precipitated pool was solubilised in TTE and rechromatographed on a second DE52 column (Fig. 2). Receptor activity for colicin M was eluted from this column slightly ahead of that for colicins B and D. Pooled fractions containing only colicin M receptor activity (fractions 22 and 23) were found to contain much larger amounts of protein Q than protein P when pooled and examined by gel electrophoresis (Fig. 3E). The molecular weight of protein Q (based on its position in the polyacrylamide gel, Fig. 3) is similar to that reported for purified colicin M receptor (85,000, ref. 13), and this material containing mainly protein Q has receptor activity for bacteriophages T5 and Ø80 (data not shown) which are reported to share the same receptor as colicin M (13).

Fractions 24-28 from the second DE52 column contained receptor activities for colicins B, D and M and when pooled were found by gel electrophoresis to contain both proteins P and Q, although enriched for protein P, together with four other proteins detectable only in overloaded gels (Fig. 3F). Protein Q is therefore the receptor for colicin M, while protein P is presumably the receptor for colicins B and D. A summary of the protein yield and colicin B, D and M receptor activities in the outer membrane preparations at different stages of purification is shown in Table 2.

Further separation of proteins P and Q on the basis of molecular weight was not possible, both because the two proteins have very similar molecular weights (Fig. 3) and because colicin B and D receptor activities are destroyed in sodium dodecyl sulphate, the detergent normally used to solubilise E. coli outer membrane proteins during their separation by gel filtration or polyacrylamide gel electrocphoresis (1). Proteins P and Q chromatographed as a single protein peak on Biogel P150 (100-200 mesh, 120 x 1.6 cm) in the presence of TTE.

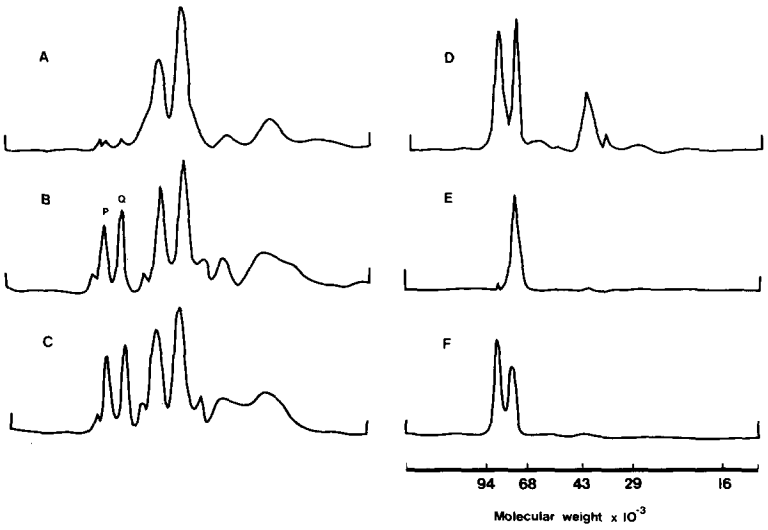

Figure 3. Densitometry scans of polyacrylamide gel electrophoretograms of outer membrane preparations of strain P1552 run in the alkaline Bragg-Hou system.
A. Crude outer membrane prepared from cells grown in the presence of 100 μM $FeCl_3$. B. Crude outer membrane prepared from cells grown in the presence of 100 μM 2,2'-dipyridyl. Note the appearance of large amounts of outer membrane proteins P and Q under these growth conditions.
C. TTE-soluble extract of material shown in B. D. Pooled fractions 110 - 138 from DE52 chromatography of material shown in C. (see Figure 1).
E. Pooled fractions 22 - 23 from DE52 chromatography of material shown in D. (see Figure 2). F. Pooled fractions 24 - 28 from DE52 chromatography of material shown in D. (see Figure 2).
All gels were run from left to right, and the scale shows the position of molecular weight markers under the same conditions of electrophoresis.

The derepression of the synthesis of the colicin B and D receptor when cells are starved of iron strongly suggested that this protein might be involved in iron uptake (8). We have isolated over thirty independent colicin B and D-resistant mutants of strain P1552 under various conditions of iron starvation or in the presence of ferric citrate or 2,3-dihydroxybenzoic acid. Thirty mutants which were resistant to colicins B and D only of the 20 different colicins used in this laboratory (7) were selected for further study. Three of these mutants were found to be of the previously described cbt class (7), being colicin tolerant (i.e., they retain a functional colicin B and D receptor but are resistant to killing by these colicins). The

Table 2. Titration of receptor activities for colicins B, D and M in outer membrane material from iron-starved cells of strain P1552 at various stages of the purification of proteins P and Q.

Material	Protein recovery (mg)	Colicin receptor activities (CRU/mg protein) B	D	M
Crude outer membrane	580	320	320	400
Outer membrane in TTE	410	320	320	400
Pooled fractions 110-138 from 1st DE52 column [a]	43	4000	4000	6400
Pooled fractions 22-23 from 2nd DE52 column [b]	2.8	0	0	12800
Pooled fractions 24-28 from 2nd DE52 column [c]	9.2	12800	12800	8000

[a] See Figs. 1 and 3D

[b] See Figs. 2 and 3E

[c] See Figs. 2 and 3F

remaining 27 mutants (referred to as cbr) lacked a functional colicin B and D receptor but were shown by gel electrophoresis to retain a protein with the same electrophoretic mobility as protein P which could be derepressed by iron starvation. TonA mutants, which lack a functional M receptor, have also been reported to retain an outer membrane protein with the same electrophoretic mobility as the functional colicin M receptor (13), and we have extended this observation with twenty independent tonA mutants selected as bacteriophage T5-resistant under the same conditions as the colicin B and D-resistant mutants. All tonA mutants retained a protein with the same electrophoretic mobility as protein Q and which could again be derepressed by growth under iron starvation. It therefore appears that loss of receptor activities for colicin B and D or colicin M is not lethal, but the absence of mutants lacking the receptor proteins themselves may indicate that their loss would be lethal. Alternatively, the tonA and cbr genes may in some way control the colicin receptor activities of their respective proteins but are not the structural genes for these proteins.

Cbr mutants were found to be phenotypically similar to the colicin B-tolerant mutants (cbt) described previously (7, 8). The cbr mutants were defective in the uptake of ferri-enterochelin but retained functional transport systems for iron complexed with ferrichrome, citrate and rhodoturulic acid (data not shown, see ref. 8). The colicin B and D receptor may therefore be an integral part of the outer membrane transport system for ferri-enterochelin in E. coli K-12 which is derepressed by iron starvation. This situation parallels that for tonA mutants which lack a functional receptor for colicin M and for bacteriophages T5 and Ø80 and which are specifically defective in the uptake of iron complexed with ferrichrome (6, 13). However, in contrast to our data on colicin M receptor activity (Table 1), Luckey, Wayne and Neilands (14) have shown that receptor activity for bacteriophage Ø80 is not derepressed by iron starvation. The reason for this discrepancy is not understood at present. In addition, the role of the colicin Ib receptor in iron transport ia also yet to be determined.

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