THE ROLE OF COLICIN RECEPTORS IN THE UPTAKE OF FERRIENTEROCHELIN BY ESCHERICHIA COLI K-12

A.P. Pugsley and P. Reeves, Department of Microbiology and Immunology, University of Adelaide, Adelaide, South Australia 5001.

Received November 15,1976

The colicin B-D receptor in the outer membrane of Escherichia coli K-12 is shown to function also as the receptor for the iron-siderophore complex ferrienterochelin. The outer membrane receptors for colicins I and M are not involved in ferrienterochelin uptake.

Several workers have noted interactions between the siderophore-mediated uptake of iron and the action of a number of colicins in E. coli K-12. Certain siderophores have been shown to protect cells from the action of colicins in group B (1,2), and some group B colicin-resistant mutants are defective in siderophore-mediated iron uptake (3). In many cases, the nature of the lesions which block colicin action and siderophore uptake are not fully understood (3), but in others, colicin resistance and defective siderophore uptake are thought to be due to the loss of a common, specific outer membrane receptor protein. Thus, the receptor for colicin M (and for bacteriophages T5 and Ø80) is considered also to be the receptor for ferrichrome-iron complexes (2,4,5), and is under the control of the tonA gene. The receptors for colicins B and D and for colicin I have been implicated in the uptake of iron complexed with enterochelin, the siderophore secreted by E. coli (1,3,6,7).

It has also been reported that E. coli grown under iron stress hyperproduces several relatively high molecular weight outer membrane proteins (1,3,7,8). In a previous report we tentatively identified two of these proteins as the receptors for colicins B and D, which probably have similar or identical receptor recognition sites (9), and for colicin

M (10). Other evidence has suggested that the receptor for colicin I may also be hyperproduced by growth under iron stress (7,10,11). In this report we further examine the role of these colicin receptor proteins in the uptake of ferrienterochelin.

MATERIALS AND METHODS

Strains of *E. coli* K-12 used in this study are listed in Table 1; colicinogenic bacteria were as listed previously (10). Minimal medium was as used previously, and where necessary was treated with 8-hydroxyquinoline and chloroform to remove iron (3).

Ferrienterochelin uptake was measured as described previously (3) using cells grown in iron-depleted minimal medium in the absence of iron chelators.

Outer membranes were prepared as previously (1) and were examined by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate using the Bragg-Hou gel system (1), or the method of Lugtenberg *et al.*, (12) with 150 x 100 x 1.5 mm slab gels. In the latter system, the gels were loaded with 12.5 µg outer membrane protein, (determined as previously; 3), and were stained after electrophoresis by the method of Fairbanks *et al.*, (13). Assays of ferrienterochelin binding to outer membranes were performed in dialysis chambers similar in design to those used by Furlong and Weiner (14). All reagents were suspended or dissolved in a solution containing 50 mM NaCl, 10 mM Na^+ phosphate buffer, pH 7.0 and 100 µM nitrilotriacetic acid. 0.5 ml volumes of ^{55}Fe(III)-ferrienterochelin (40 ng ^{55}Fe(III)/ml, giving approximately 30,000 cpm; see reference 3) and dilutions of outer membrane protein (10 - 0.2 mg/ml) were separated by a boiled dialysis membrane (Visking) and allowed to dialyse to equilibrium (24 h) at 4° on a vertical rotating turntable. Samples (0.1 - 0.5 ml) were removed from both chambers after dialysis, emulsified in a Triton X-100-based scintillation fluid (15) and counted as previously (3). The concentrations of outer membrane used were adjusted to give up to 15% binding of radioactivity at equilibrium, and the amount of ferrienterochelin bound (expressed as ng ^{55}Fe(III)/mg outer membrane protein) was calculated as described by Furlong and Weiner (14).

Hfr gradient mapping was performed by the method described by Verhoef and de Haan (16) with initial selection for *thr*$^+$ (ability to grow in the absence of threonine) in the recipient strain P1852. Streptomycin (100 µg/ml) was used for counter selection. The recipient strain P1853 carried a mutation in the *ompA* gene which reduced its ability to act as a recipient in conjugation (19). *Thr*$^+$ recombinants were therefore detected at lower than normal frequencies.

RESULTS AND DISCUSSION

Mutants lacking the receptors for colicin M and bacteriophages T1, T5 and Ø80 (*tonA*), for colicin I (*cir*) and for colicins B and D

TABLE 1. *E. coli* K-12 strains

Strain number	Relevant genotype
P1552	F^-, *thr*, *leu*, *proA*, *his*, *argE*, *aroE*, *thi*, *spc*, *str*
P1795	as P1552 but *cbr*
P1796	as P1552 but *cir*
P1797	as P1552 but *tonA*
AT2472	HfrH, *thi*, *aroE*
P1798	as AT2472 but *cbr*
P1799	as AT2472 but *cir*
P1855	as AT2472 but *tonA*
P1852	F^-, *thr*, *leu*, *proA*, *argE*, *thi*, *str*, *tsx*, *ompA*, *tonA*.

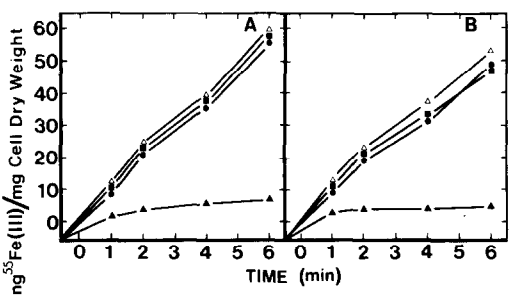

FIGURE 1. Uptake of ferrienterochelin by colicin resistant mutants of *E. coli* K-12. A. Δ = P1552 (parent), ▲ = P1795 (*cbr*), ● = P1796 (*cir*), ■ = P1797 (*tonA*). B. Δ = AT2472 (parent), ▲ = P1798 (*cbr*), ● = P1799 (*cir*), ■ = P1855 (*tonA*). Uptake mixtures contained 100 μM nitrilotriacetic acid, 10 μM enterochelin and 5 μM $^{55}FeCl_3$.

(*cbr*) were independently isolated as bacteriophage T5, colicin Ib-P9 and colicin B-K260 resistant derivatives respectively of strains P1552 and AT2472. All mutants showed identical colicin and bacteriophage resistance patterns to previously described *tonA*, *cir* and *cbr* mutants (1,10,17). Outer membranes from these mutants did not neutralise the colicins M, Ib and B and D respectively, and they were therefore considered true colicin receptor mutants. However, all *cir* mutants were tolerant to colicin V-CA7 in the triple layer plate test, as reported previously (17).

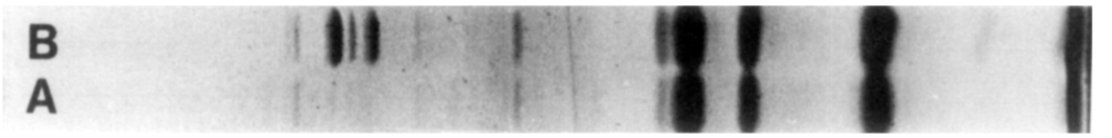

FIGURE 2. Outer membranes of strain P1552 grown in minimal medium containing 25 μM $FeCl_3$ (A) or 100 μM 2,2'-dipyridyl (B) examined by the Lugtenberg PAGE system. The direction of electrophoresis was from left to right.

Representatives of each class of receptor mutants (Table 1) were assayed for ability to take up ferrienterochelin. As shown in Fig. 1, only the cbr mutants were defective in ferrienterochelin uptake. A total of 15 independently isolated cir mutants were assayed in this way, and all strains had a fully functional ferrienterochelin uptake system. We were thus unable to show an interaction between the colicin I receptor and ferrienterochelin uptake, a finding which conflicts with the earlier data of Braun and co-workers (6,7).

Outer membranes were prepared from representatives of the three classes of colicin resistant mutants and the two parent strains (Table 1) grown in minimal medium containing 25 μM $FeCl_3$ (iron-supplemented) or 100 μM 2,2'-dipyridyl (iron-stressed; 3). In the Bragg-Hou gel system, only 2 outer membrane proteins were shown to be derepressed by iron-stress as reported previously (1,3,10). Better resolution of the outer membrane proteins was achieved by using the Lugtenberg PAGE system. As shown in Fig. 2, three outer membrane proteins were produced in increased amounts in strain P1552 as a result of growth under iron stress. These proteins have molecular weights in the range 74-84,000 daltons, as calculated from the positions of molecular weight markers run under identical conditions, and are referred to hereafter as the outer membrane iron-stress proteins.

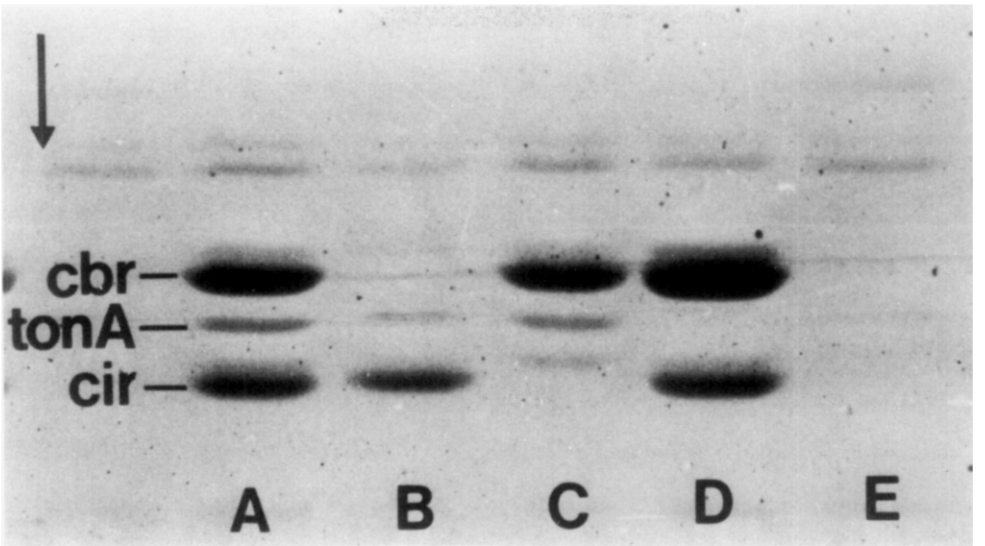

FIGURE 3. Detail of outer membrane iron stress proteins of strains P1552 (parent; A), P1795 (cbr; B), P1796 (cir; C) and P1796 (tonA; D) grown in minimal medium containing 100 μM 2,2'-dipyridyl. E = outer membrane from strain P1552 grown in minimal medium containing 25 μM $FeCl_3$. All samples were examined by the Lugtenberg PAGE system. The direction of electrophoresis is shown by the arrow.

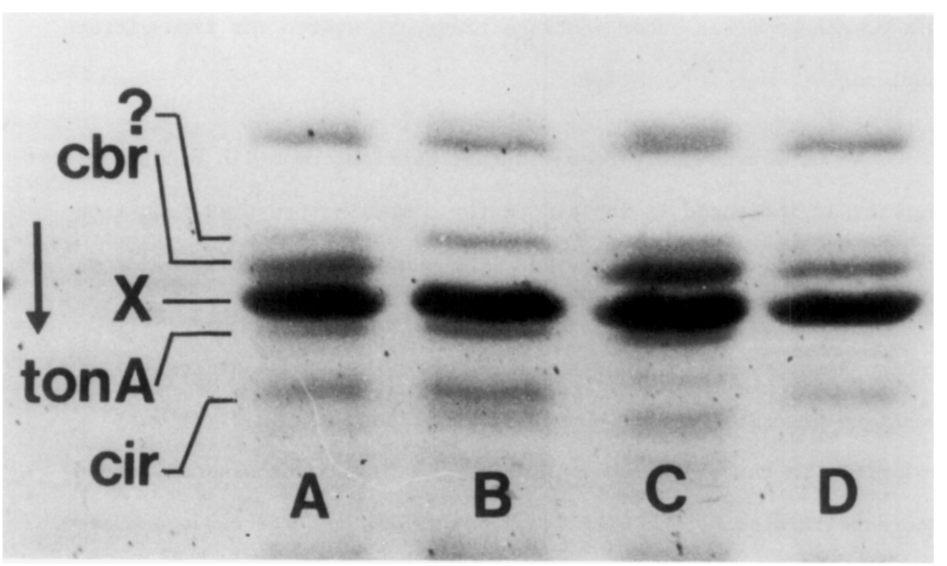

FIGURE 4. Detail of outer membrane stress proteins of strains AT2472 (parent; A), P1798 (cbr; B), P1799 (cir; C) and P1855 (tonA; D) grown in minimal medium containing 100 μM 2,2'-dipyridyl+ 500 μM sodium citrate. All samples were examined by the Lugtenberg PAGE system, and the direction of electrophoresis is indicated by the arrow.

We were previously unable to detect the absence of outer membrane proteins in either cbr or tonA mutants which could be correlated with the loss of outer membrane receptor activities for colicins B and D or colicin M respectively. These alterations could, however, be demonstrated by using the Lugtenberg PAGE system. As shown in Fig. 3 B-D, cbr, cir and tonA mutants each lacked one of the three outer membrane iron-stress proteins. Similar results were obtained with iron stressed cells of strain AT2472 and its colicin resistant mutants. However, a fourth outer membrane protein was found to be hyperproduced by all of this set of strains under these conditions. The role of this fourth iron stress protein (labelled ? in Fig. 4) remains unknown. In addition, we have identified a fifth outer membrane protein, again within the iron stress cluster, which is hyperproduced by all strains (whether derived from P1552 or AT2472) when grown in minimal medium containing 100 μM 2,2'-dipyridyl + 500 μM sodium citrate. We therefore tentatively identify this protein (labelled X in Fig. 4) as a component of an outer membrane transport system for iron-citrate complexes.

We have previously reported that ferrienterochelin binding activity is increased in isolated outer membranes prepared from iron-starved E. coli K-12 (3). However, the membrane filtration binding assay used was unsatisfactory, and gave unreliable results. The binding assays were considerably improved by using the dialysis technique described here. Non-specific binding of $^{55}Fe(III)$ was negligible in this technique. As shown in Table 2, increased ferri-enterochelin binding activity is absent from outer membranes prepared from the cbr mutant P1795 grown under iron stress, indicating that ferrienterochelin receptor activity, like the colicin B and D receptor activity, is absent in cbr mutants.

TABLE 2. Binding of $^{55}Fe(III)$-enterochelin to isolated outer membranes of E. coli K-12 strains P1552 and P1795 (cbr) grown in the presence or absence of available iron.

Outer membrane from strain	ng Fe(III)/mg outer membrane protein[a] from cells grown in	
	25 μM $FeCl_3$	100 μM 2,2'-dipyridyl
P1552	0.21	16.96
P1795 (cbr)	0.34	0.38

a mean of several independent experiments.

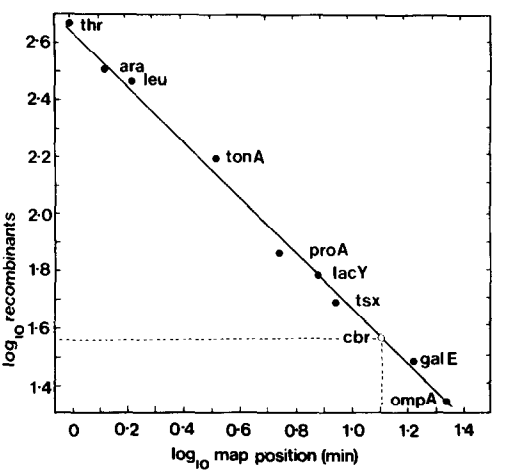

FIGURE 5. Hfr transfer gradient obtained by crossing strains P1798 (Hfr, cbr) and P1852 (F^-, cbr^+). Thr^+ recombinants in strain P1852 were scored for receipt of the following markers from the donor strain:- ability to grow in the absence of leucine (leu) or proline (proA), ability to ferment arabinose (ara), lactose (lacY) or galactose (galE), sensitivity to bacteriophages T5 (tonA), T6 (tsx) and K3 (ompA, called tolG in Backmann et al., (18), see reference 19) and resistance to colicin B-K260 (cbr).

In previous reports we have described a colicin B and D-tolerant mutant, cbt, which is phenotypically similar to the cbr mutants described here (1,3). The cbt gene is located within the ent-fep gene complex controlling enterochelin production and uptake (1). In order to determine whether the cbr and cbt mutations are located in the

same region of the chromosome, the position of the cbr mutation in strain P1798 was located by means of a Hfr gradient conjugation experiment (Fig. 5). The cbr gene was located between the tsx and galE genes at approximately 13.0 min on the recalibrated E. coli K-12 chromosome (18), and is therefore likely to be within the ent-fep gene cluster and close to, or identical with, the cbt gene. We believe that the cbt mutant carries an alteration in the ferrienterochelin receptor protein which does not permit ferrienterochelin binding, but which allows colicins B and D to bind normally without being able to proceed to later stages of colicin action (1,3). It has recently come to our attention that another, different gene has been given the mnemonic cbt (18), and we therefore wish to replace our previous designation with cbr. It may be preferable to change this name at a later date to indicate the primary role of the cbr gene product in ferrienterochelin binding, but we do not wish to do this at present since there is still insufficient evidence to differentiate the cbr and fep (ferrienterochelin permease) genes (1,3).

ACKNOWLEDGEMENTS. We wish to thank A. Richardson and C. Dearden for technical assistance.

REFERENCES.

1. A.P. Pugsley and P. Reeves. J.Bacteriol. 127, 218-228 (1976)
2. R. Wayne, K. Frick and J.B. Neilands. J.Bacteriol. 126, 7-12 (1976)
3. A.P. Pugsley and P. Reeves. J.Bacteriol. 126, 1052-1062 (1976)
4. K. Hantke and V. Braun. F.E.B.S. Lett. 49, 301-305 (1975)
5. R. Wayne and J.B. Neilands. J.Bacteriol. 121, 497-503 (1975)
6. K. Hantke and V. Braun. F.E.B.S. Lett. 59, 277-281 (1975)
7. R.E.W. Hancock and V. Braun. F.E.B.S. Lett. 65, 208-210 (1976)
8. M.A. McIntosh and C.F. Earhart. Biochem. Biophys. Res. Comm. 70, 315-322 (1976)
9. A.P. Pugsley and P. Reeves. Antimicrobial Agents & Chemotherapy, (February 1977)
10. A.P. Pugsley and P. Reeves. Biochem. Biophys. Res. Comm., 70, 846-853 (1976)
11. J. Konisky, S. Soucek, K. Frick, J.K. Davies and C. Harmond. J.Bacteriol. 127, 249-257 (1976)

12. B. Lugtenberg, J. Meijers, R. Peters, P. van der Hoek and L. von Alphen. F.E.B.S. Lett. 58, 254-258 (1976)
13. G. Fairbanks, T.L. Steck and D.F.H. Wallach. Biochemistry, 10, 2606-2617 (1971)
14. C.F. Furlong and J.H. Weiner. Biochem. Biophys. Res. Comm. 38, 1076-1083 (1970)
15. M.S. Patterson and P.C. Greene. Anal. Chem. 37, 854-857 (1965)
16. G. Verhoef and P.G. de Haan. Mut. Res. 3, 101-110 (1966)
17. J.K. Davies and P. Reeves. J.Bacteriol. 123, 96-101 (1975)
18. B.J. Bachmann, K.B. Low and A.L. Taylor. Bacteriol. Rev. 40, 116-167 (1976)
19. P.A. Manning and P. Reeves. J.Bacteriol. 127, 1070-1079 (1976)