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Iron Transport in *Escherichia coli* K-12: Involvement of the Colicin B Receptor and of a Citrate-Inducible Protein

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It was shown that *feuB* mutants (defective in ferric enterochelin uptake) were unable to adsorb colicin B. In addition, they were missing one of the three outer-membrane proteins which are overproduced in strains grown in iron-deficient, extracted medium. Thus this protein (the *feuB* protein) is probably the receptor for colicin B and functions in enterochelin-mediated iron transport. The *feuB* gene was located by P1 transduction at approximately 72.5 min on the *Escherichia coli* K-12 genetic map and thus maps separately from the other genes concerned with the enterochelin system. The outer membranes of various strains grown in the presence of 1 mM citrate contained a high level of a protein which was present in very small amounts when citrate was absent from the growth medium. This protein was most easily observed in *feuB* mutants grown in the presence of citrate, since on polyacrylamide gels it ran in a similar position to the *feuB* protein, which is missing in these mutants. The relationship of this citrate-inducible protein to the inducible citrate-dependant iron uptake system is discussed.

Four iron transport systems have been described for *Escherichia coli* K-12. There are three specific, high-affinity systems which transport iron in complex with enterochelin (11), citrate (5), or ferrichrome (9). The fourth system is a low-affinity system for which no such complexing agent has been described (5). The best characterized iron uptake system is the enterochelin-mediated system (13), but even in this case our knowledge of the membrane components required for transport and the way in which they act is poor. One possible method of studying this problem is by examining mutants, defective in the various transport systems, for membrane protein alterations. Mutants in the *tonB* locus lack all three high-affinity iron uptake systems (8, 14). Davies and Reeves (4) noted that *tonB* mutants contained two additional outer-membrane proteins. Using a slab polyacrylamide gel electrophoresis system, we demonstrated that these mutants actually overproduced three outer-membrane proteins (2), which we designated the 74K, 81K, and 83K proteins according to their apparent molecular weights (K, kilo; e.g., 74K = 74,000 molecular weight). Furthermore, the appearance of these proteins could be induced in a wild-type strain by growth on iron-deficient, extracted medium (2, 7). Normal medium appeared to contain enough iron to suppress the appearance of these proteins in the wild-type strain but not in *tonB* mutants. However, even in *tonB* mutants excess iron in the growth medium suppressed the appearance of these proteins. We therefore reasoned that overproduction of these proteins was a consequence of low intracellular iron levels, rather than being specifically concerned with the *tonB* mutant defect.

It had been observed that *tonB* mutants grown in tryptone-yeast medium exhibited a strongly enhanced capacity for colicin B and I binding (8). This raised the possibility that one or more of the 74K, 81K, and 83K proteins were the receptors for colicins B and I. Recently the isolation of *feu* mutants (defective in ferric enterochelin uptake) was described (8). These mutants had essentially normal citrate- and ferrichrome-mediated iron transport systems and mapped separately from previously described iron transport mutants. They fell into two groups with respect to colicin resistance. One group (*feuA*) was resistant (as opposed to tolerant) to colicin I and was missing the 74K (*feuA*) protein (7). This demonstrated that the *feuA* protein is the receptor or a component of the receptor for colicin I and functions in enterochelin-mediated iron transport. In this paper we show that *feuB* mutants are unable to adsorb colicin B and lack the 81K (*feuB*) protein.

During this study we found that an outer-membrane protein was induced in strains grown in the presence of citrate.

MATERIALS AND METHODS

Bacterial strains. The designation *feu* has been previously used for mutants defective in enterochelin-mediated iron uptake (8). In this paper the mutants are subdivided into two groups according to their colicin resistance pattern (Table 1). The gene symbols below are those used for the new *E. coli* genetic map (1).

Most of the strains studied were previously described mutants of *E. coli* K-12 strain AB2847 (*aroB tsx thi lamB*) (8). In addition, doubly colicin-resistant mutants were isolated by streaking the *feuA* mutant strain VR42 onto a nutrient agar plate which had previously been spread with a colicin B suspension (reciprocal titer, 128). Single colonies were picked and tested for resistance or sensitivity to phages T1, T5, and ϕ80 and to colicins B and I. Two mutants, VR42/B7 and VR42/B9, which were *tonA*$^+$ and *tonB*$^+$ (i.e., sensitive to the above phages) were selected for further study. For the isolation of colicins, strains KH595 (J5-3 *pro met*, lysogenic for λ and carrying the colicin Ia-CT2 plasmid) and KH596 (J5-3 *pro met*, lysogenic for λ and carrying the colicin Ib-P9 plasmid) were kindly provided by K. Hardy, strain R2.1 (*rpsL met lamB*, lysogenic for λ and carrying a colicin B plasmid) was provided by P. Fredericq, and a wild-type *E. coli* K-12 carrying the colicin Ib-P9 plasmid was obtained from D. J. McCorquodale. Strain AT2455 (*mal thi cysG*) was provided by H. U. Schairer, whereas strain DG78-X36 *cir* (*ara lac gal try his argH rpsL mal xyl thi tonA pyr man purC cir*) (3) was received from J. K. Davies.

TABLE 1. *Sensitivity of strain AB2847 and its colicin-insensitive derivatives to colicins B, Ia, and Ib*

Strain	Sensitivity[a]		
	Colicin B	Colicin Ia	Colicin Ib
AB2847	10^4	10^4	128
VR42 *feuA*	10^4	0	0
VR27 *feuA*	10^4	0[b]	0[b]
VR42/B7	0	0	0
VR42/B9	0	0	0
BR10 *feuB*	1	10^4	128
IR20 *feuB*	1	10^4	64
IR35 *feuB*	1	10^4	32-64
P20 *tonA*	10^4	10^4	128
BR158 *tonB*	0	0	0
BR128	0	10^2	1-2

[a] The results are expressed as the reciprocal of the greatest dilution of a stock solution of each colicin which resulted in a clear zone of inhibition of a lawn of the given strain. The use of 0 signifies full insensitivity (i.e., no clear zone of inhibition).

[b] Strain VR27 was very slightly inhibited by all dilutions of colicins Ia and Ib.

Media. For the growth of strains prior to the preparation of outer membranes, iron-deficient, extracted M9 minimal medium, prepared and supplemented as described previously (2, 7), was used. When a full medium was required, nutrient broth (no. 0003-01; Difco Laboratories, Detroit, Mich.) was used. The addition of 2 and 0.65% agar, respectively, made plates and soft-agar overlays.

Colicin techniques. Colicins were prepared by growing the producing strains overnight in nutrient broth, diluting 1:50 into fresh nutrient broth, and incubating the culture with vigorous shaking at 37°C. When the culture had reached an optical density of 0.3 at 578 nm, mitomycin C (0.2 μg/ml) was added, and the culture was further incubated for 4 h at 37°C. The cells were harvested by centrifugation and resuspended in 20 ml of 0.01 M phosphate buffer (pH 7.0). They were then subjected to sonic oscillation for 1.5 to 2.0 min at setting 7 using a Sonic Power sonifier (Branson Instruments, Co., Stamford, Conn.). The suspension was centrifuged (48,000 $\times$ *g*, 45 min), and the colicin-containing supernatant was stored at −20°C.

Adsorption of colicins Ia and Ib to outer-membrane preparations has been described previously (7); however, this technique did not work for colicin B, and so a method based on that of Guterman (6) was used. Log-phase cultures of various strains grown in nutrient broth were centrifuged (5,000 $\times$ *g*, 10 min). The bacterial pellet was resuspended at an optical density of 10 at 578 nm in either nutrient broth or 0.01 M phosphate buffer (pH 7.0). The resuspension buffer had no effect on the level of adsorption. An equal volume of low-titer colicin B solution was added, and the adsorption mixture was incubated at 37°C. After 90 min, the bacteria and adsorbed colicin were centrifuged, and the supernatant was assayed for unadsorbed colicin.

Preparation of outer membranes and polyacrylamide gel electrophoresis. Strains were grown as described previously (2, 7) in iron-deficient, extracted medium. Outer membranes were prepared using the method of Osborn et al. (12). Polyacrylamide gel electrophoresis was performed by the technique of Lugtenberg et al. (10), with the exception that stock solution 1 contained only 0.3% (wt/vol) *N,N'*-methylenebisacrylamide (instead of 0.8%). Using this modification we achieved a better separation of the relevant proteins.

RESULTS

Colicin sensitivity pattern. When the sensitivity of a number of mutants of strain AB2847 to colicins B, Ia, and Ib was tested, the results shown in Table 1 were obtained. The *feuA* mutants VR42 and VR27 were fully sensitive to colicin B and insensitive to colicins Ia and Ib, although strain VR27 appeared to be slightly inhibited by colicins Ia and Ib. As will be shown below, cultures of strain VR27 contain small amounts of the *feuA* protein, which is absent in strain VR42. The *feuB* mutants BR10, IR20,

and IR35 were previously described as colicin B and I insensitive (8). However, we observed in this study that they were, at best, only slightly resistant to colicins Ia and Ib. This is probably due to the fact that these tests were performed using nutrient broth-based medium. It has been previously shown (2) that higher levels of colicin I receptor (74K protein) are made in the presence of nutrient broth than in the presence of tryptone-yeast broth, which was the medium of choice for previous experiments. The *feuB* mutants were, however, largely insensitive to colicin B, although a very high-titer colicin B solution gave a plaque on all *feuB* strains. The double mutants VR42/B7 and VR42/B9 were fully insensitive to all three colicins, as was the *tonB* mutant BR158. The *tonA* mutant P20, like the wild-type strain AB2847, was fully sensitive to the three colicins. Strain BR128, which has been previously described as a T1-sensitive *tonB* mutant (8), was partially sensitive to colicins Ia and Ib but fully resistant to colicin B.

Colicin adsorption. To differentiate between resistant and tolerant mutants among the colicin-insensitive strains, we tested the adsorption of colicins B, Ia, and Ib to the various strains (Table 2). It was shown that the *feuB* mutants BR10, IR20, and IR35 and the double mutants VR42/B7 and VR42/B9 were unable to adsorb colicin B, whereas all other strains studied adsorbed this colicin to the same extent as the wild-type strain AB2847.

Only the *feuA* mutant VR42 and its colicin B-resistant derivatives VR42/B7 and VR42/B9 were incapable of adsorbing colicins Ia and Ib. All other strains, with the exception of strain VR27, adsorbed the two colicins readily. Strain VR27 (*feuA*) appeared to be capable, to a reduced extent, of adsorbing colicins Ia and Ib, which was in accord with the colicin sensitivity and protein analytical data. The fact that colicins Ia and Ib caused slight inhibition of VR27 at all dilutions (Table 1) suggests that a percentage of cells in a given culture of strain VR27 have receptors rather than that all cells have a reduced number of receptors.

Outer-membrane protein alterations in *feu* mutants. We have observed (2, 7; Fig. 1, gel a) that strain AB2847 grown in iron-deficient, extracted medium produced high levels of three outer-membrane proteins (74K, 81K, and 83K proteins). Growth in unextracted media or in extracted medium with added iron suppressed the appearance of these proteins (2, 7; Fig. 1, gel j: as shown below, the appearance in this gel of a band near the position of the 81K protein is due to the citrate-inducible protein). We studied the outer-membrane protein patterns of various strains grown in iron-deficient, extracted medium in the presence or absence of dihydroxybenzoate. Without added dihydroxybenzoate the *aroB* strains used in this study cannot make enterochelin; however, in no case was the outer-membrane protein pattern altered by the presence of dihydroxybenzoate in the growth medium. The *feuB* mutants BR10 (Fig. 1, gel b), IR20, and IR35 were found to be lacking the 81K protein.

The *feuA* mutant VR42 lacks the 74K protein (7; Fig.1, gel f). The *cir* mutant of *E. coli* K-12, which like the *feuA* mutant is unable to adsorb colicins Ia and Ib (3), is also lacking the 74K protein. However, we are unable to conclude that the two genes involved are identical, since preliminary mapping studies have indicated that they map separately (3, 8). The 74K pro-

TABLE 2. *Colicin adsorption by strain AB2847 and some of its colicin-resistant mutants*

Strain	Mutant locus	Reciprocal titers after adsorption[a, b]		
		Colicin B	Colicin Ia	Colicin Ib
AB2847		4	<1 (0.05)	<1 (0.05)
VR42	*feuA*	8	128 (0.50)	64 (0.75)
VR27	*feuA*	4	32 (0.05)	32 (0.05)
VR42/B7	*feuA feuB*[c]	64	128 (0.25)	64 (0.25)
VR42/B9	*feuA feuB*[c]	64	128 (0.10)	64 (0.25)
BR10	*feuB*	64	1 (0.05)	<1 (0.10)
IR20	*feuB*	64	<1 (0.05)	<1 (0.05)
IR35	*feuB*	64	2 (0.03)	1 (0.03)
P20	*tonA*	4	1 (0.05)	1 (0.05)
BR158	*tonB*	4	<1 (0.05)	<1 (0.05)
BR128	—[d]	4	<1 (0.10)	<1 (0.10)
Control		64	128	64

[a] Adsorption was tested as described in Materials and Methods. After mixing whole cells or outer membranes with the colicins for 1.5 h at 37°C and in the former case removing the cells by centrifugation, the supernatant was diluted, and 0.01 ml of each dilution was spotted onto a plate seeded with the sensitive strain AB2847. Results are expressed as the reciprocal of the greatest colicin dilution still giving a clear zone of inhibition of the lawn of cells. As a control, the reciprocal colicin titer after incubation with buffer was measured.

[b] The figures in parentheses are the amounts of outer-membrane protein (mg) used in the individual assays for colicin Ia and Ib adsorption. As described in Materials and Methods, whole cells were used for the colicin B adsorption experiments.

[c] These mutants are colicin B-resistant derivatives of the *feuA* mutant VR42. The tests described in Tables 1 and 2, together with the observed outer-membrane protein patterns, show that they are genuine *feuA feuB* double mutants.

[d] This mutant was originally described as a T1-sensitive *tonB* mutant, since it maps in or close to the *tonB* gene region and has similar iron transport deficiencies similar to those of *tonB* mutants (8).

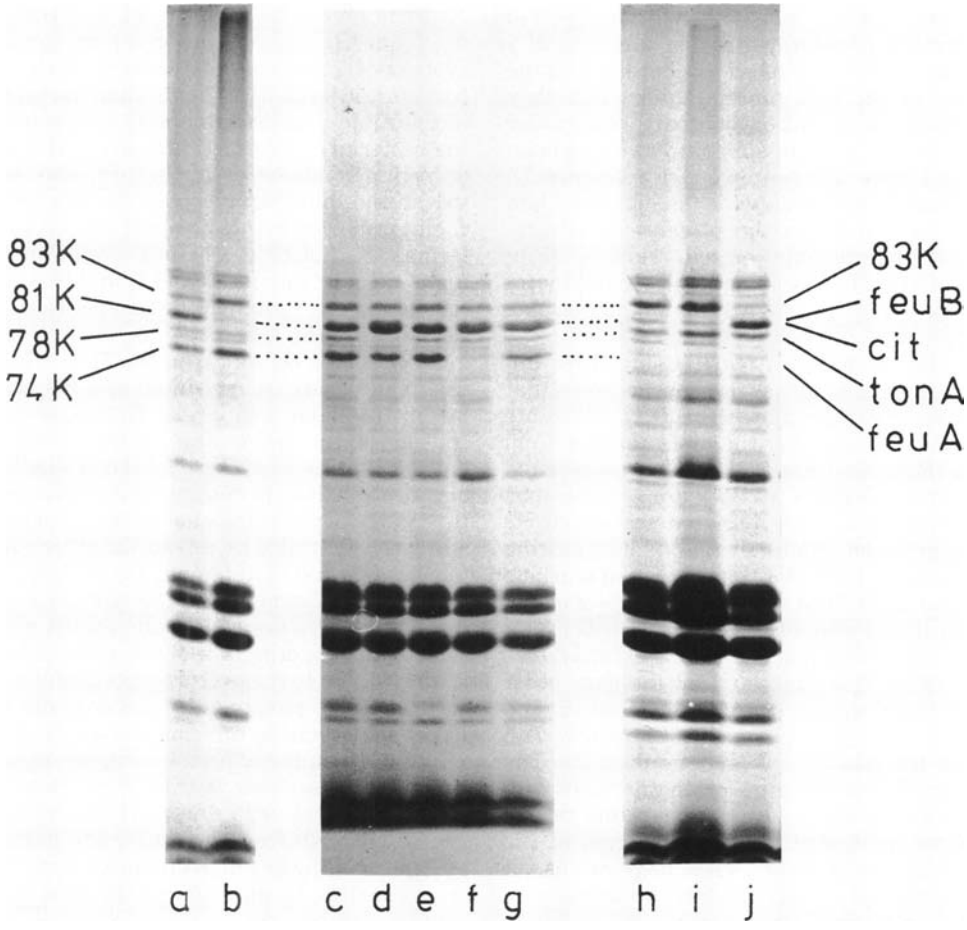

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the outer-membrane proteins of strain AB2847 and some of its colicin-resistant mutants. Cells were grown in iron-deficient, extracted medium with the indicated growth factors present to support iron uptake. Abbreviations: Gel a, strain AB2847 (wild type), no additions; b, strain BR10 (feuB), no additions; c, strain BR10 with 1 mM citrate added; d, strain P20 (tonA) with 1 mM citrate added; e, strain IR20 (feuB) with 1 mM citrate added; f, strain VR42 (feuA) with 1 mM citrate added; g, strain AB2847 with 1 mM citrate added; h, strain VR42/B9 (feuA feuB), no additions; i, strain VR42/B9, with 10 μM dihydroxybenzoate added; j, strain AB2847 with 5 μM $FeSO_4$ and 1 mM citrate added (the band near the position of the 81K protein in gel j is the citrate-inducible protein). The positions of the 74K (fueA), 78K (tonA), cit (citrate inducible), 81K (feuB), and 83K proteins are indicated. The protein standards, used for the location of the bands, were phosphorylase A (94K), transferrin (78K), bovine serum albumin (67K), ovalbumin (45K), and chymotrypsinogen A (25K).*

tein was seen to be present in two out of five outer-membrane preparations of the *feuA* mutant VR27, although when present the level was greatly reduced. This finding correlates with the colicin sensitivity and adsorption results discussed above. The colicin B-resistant mutants derived from strain VR42, strains VR42/B7 and VR42/B9, were missing both the 74K and 81K proteins (Fig. 1, gels h and i) and therefore appear to be *feuA feuB* double mutants. The *tonA* mutant P20 (Fig. 1, gel d) and *tonB* mutant BR158 produced wild-type levels of the 74K, 81K, and 83K proteins, although the latter strain required 5 μM $FeCl_3$ for growth in iron-deficient, extracted medium (2).

Observation of a citrate-inducible outer-membrane protein. When *feuB* mutants were grown in the presence of 1 mM citrate, a protein of similar, but not identical, molecular weight to the 81K protein appeared in large amounts in the outer membrane (Fig. 1, gels c and e). In contrast, in the absence of citrate there was at most only a very small residual level of this protein (Fig. 1, gel b). It was further observed

that when strains which had wild-type levels of 81K protein were grown in the presence of 1 mM citrate, they produced a wider band at the position of the 81K protein than when they were grown on dihydroxybenzoate. This wider band could be seen to split into two bands on some gel runs (for example, see reference 7). Thus there is a protein which is induced when strains are grown in the presence of citrate. This citrate-inducible protein runs slightly ahead of the 81K protein on slab gels and therefore may have a slightly lower molecular weight.

Genetic mapping of *feuB*. Using three different Hfr strains, it was previously shown that *feuB* was cotransferred with *aroB* (7). To locate *feuB* more accurately, the *cysG* marker from strain IR20 (*feuB aroB cysG*$^+$) was transferred into strain AT2455 (*feuB*$^+$ *aroB*$^+$ *cysG*) by P1 transduction. One hundred transductants which grew on minimal agar plates lacking cysteine were tested for the unselected markers *feuB* and *aroB*. The following classes of *cysG*$^+$ transductants were observed: *feuB aroB* (28%), *feuB*$^+$ *aroB* (30%), *feuB aroB*$^+$ (39%), and *feuB*$^+$ *aroB*$^+$ (3%). This suggests that the gene order of the three markers is *feuB-cysG-aroB* and that the *feuB* gene lies at approximately 72.5 min on the new *E. coli* genetic map (1). The experiment was repeated for another *feuB* mutant, BR10, with essentially the same result. Attempts to localize the map position of the *feuA* mutation in strain VR42 have not as yet been successful. When 100 transductants were selected in various strains, we were unable to demonstrate cotransduction of *feuA* with *tolC* (65 min), *argG* (68 min), *aroE* (71.5 min), *rpsL* (72 min), *cysG* (73 min), or *aroB* (73.5 min). We therefore conclude that *feuA* maps apart from *feuB*. A third type of mutant, *fep*, like the *feuA* and *feuB* mutants, is deficient in the enterochelin-mediated but not in the citrate-mediated iron uptake system. However, both mapping (1, 13) and colicin resistance (unpublished data) distinguish *fep* from our *feu* mutants.

DISCUSSION

The *feuB* mutant strains described in this paper are unable to adsorb colicin B and are missing the 81K protein. In addition, it was previously shown that *tonB* mutants have a higher level of 81K protein in their outer membranes (2) and exhibit enhanced binding of colicin B after growth in tryptone-yeast broth (8). Therefore, we conclude that this protein is the colicin B receptor or a component thereof. Similar reasoning previously led us to the conclusion that the 74K protein was the receptor or a constituent of the receptor for colicins Ia and Ib (7). Both the *feuA* and *feuB* mutants have a defect in enterochelin-mediated iron transport (8). Thus the two colicin receptors probably evolved originally as components of this iron transport system. It is likely that the proteins in their role as colicin receptors are exposed on the bacterial cell surface, and one or the other, or even a combination of the two, may act as the initial binding receptor for the enterochelin-iron complex. It has been shown by Guterman (6) that enterochelin prevents the killing of cells by colicins B and I, and this result can be simply explained by competition for a common binding site.

As discussed before (2, 7), the 74K, 81K, and 83K proteins are overproduced as a response to low intracellular iron levels. Their appearance is suppressed when the medium contains enough iron to raise these levels. A number of enzymes involved in enterochelin synthesis and the uptake of the enterochelin-iron complex are similarly controlled by intracellular iron levels (as summarized in reference 13), although the control mechanism has not as yet been elucidated. We have previously eliminated production, excretion, or uptake of enterochelin as the factor leading to the overproduction of the 74K, 81K, and 83K proteins (7), and it would seem likely that there is a common control mechanism for a number of proteins important in the enterochelin system. If this is so, then it is interesting that both the *feuA* (9) and *feuB* genes map apart from the cluster of eight genes concerned with the enterochelin system, which are located at 13 min (13) on the new *E. coli* genetic map (1).

We have also demonstrated in this paper the presence of a citrate-inducible protein. This was made possible by the use of *feuB* mutants missing the 81K protein, since on polyacrylamide gels the citrate-inducible protein ran in a very similar position to the 81K protein. Frost and Rosenberg (5) characterized the citrate-dependent iron uptake system of *E. coli* and showed that it was inducible by pregrowth of cells on 1 mM citrate. The inducibility of the citrate-dependent iron transport system may be expressed solely through the inducibility of this outer-membrane protein, which we tentatively suggest may be the receptor for the citrate-iron complex. The citrate-inducible protein is required only by the citrate-dependent iron uptake system, since the other high-affinity systems operate even when this protein is absent (i.e., they are not citrate inducible). Similarly, the *tonA* protein is required only by the ferrichrome-mediated iron transport system and the *feuA* (74K) and *feuB* (81K) proteins are required only for transport of the enterochelin-iron complex. These proteins probably recognize the different chelators rather than the iron

moiety of the chelator-iron complexes, and it is logical that there should be different receptors for the three quite different chelate structures.

It is interesting that the various proteins concerned with iron transport have very similar molecular weights. Furthermore, when solubilized with Triton X-100 plus ethylenediaminetetraacetate, they cofractionate on a DE52 cellulose column (2). It is possible that these proteins form a complex in the outer membrane which has as its specific role the initial binding and transport across the outer membrane, of chelator-iron complexes. However, it is interesting to consider what happens after the various complexes have passed through the outer membrane. The three high-affinity iron transport systems have, in addition to the specific requirements mentioned above, common requirements for the *tonB* gene product (8) and for cytoplasmic membrane-derived energy (14; our unpublished data). One possibility is that there is a transcytoplasmic membrane uptake step which is utilized by these transport systems. Clearly much work remains before the highly complex problem of iron transport through the cell envelope is properly understood.

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ADDENDUM

After this paper was submitted, two relevant papers appeared. McIntosh and Earhart (Biochem. Biophys. Res. Commun. 70:315-322) demonstrated that two polypeptides were overproduced in the outer membranes of *E. coli* B and K-12 cells grown in low-iron medium. We believe that the difference in the number of proteins observed can be explained by the greater resolution afforded us by the use of a slab polyacrylamide gel electrophoresis system, although it may conceivably be due to strain differences. The results of Wayne, Frick, and Neilands (J. Bacteriol. 126:7-12) indicate that the initial binding receptor for the enterochelin-iron complex is more likely to be the colicin B than the colicin I receptor.

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