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# *Escherichia coli* K-12 Envelope Proteins Specifically Required for Ferrienterobactin Uptake

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***Escherichia coli* genes specifically required for transport of iron by the siderophore enterobactin are designated *fep*. The studies reported here were initiated to identify and localize the *fepB* product. The plasmid pCP111, which consisted of an 11-kilobase *E. coli* DNA fragment containing *fepB* ligated to pACYC184, was constructed. The *fepB* gene was subcloned; in the process, complementation tests and Tn*5* mutagenesis results provided evidence for the existence of a new *fep* gene, *fepC*. The order of the transport genes in the *ent* gene cluster is as follows: *fepA fes entF fepC fepB entE*. Minicell, maxicell, and in vitro DNA-directed protein synthesizing systems were used to identify the *fepB* and *fepC* products. The *fepC* polypeptide was 30,500 daltons in standard sodium dodecyl sulfate-polyacrylamide gels. The *fepB* gene was responsible for the appearance of three or four bands (their apparent molecular weights ranged from 31,500 to 36,500) in sodium dodecyl sulfate-polyacrylamide gels, depending on the gel system employed. The largest of these was tentatively designated proFepB, since it apparently had a leader sequence. Localization experiments showed that FepC was a membrane constituent and that mature FepB was present in the periplasm. An additional polypeptide (X) was also encoded by the bacterial DNA of pCP111, but its relationship to iron transport is unknown. The results indicated that ferrienterobactin uptake is mediated by a periplasmic transport system and that genes coding for outer membrane (*fepA*), periplasmic (*fepB*), and cytoplasmic membrane (*fepC*) components have now been identified.**

The endogenous high-affinity system for iron transport of the gram-negative bacterium *Escherichia coli* utilizes the siderophore enterobactin (enterochelin) (for a review, see reference 39). Under conditions of iron deprivation, enterobactin is synthesized and released into the environment, where it binds iron; the resulting ferrienterobactin complexes are then actively transported back into the cell. The mechanism by which ferrienterobactin enters cells is not well understood and is the subject of this work.

Four genes are known to produce products that influence the passage of ferrienterobactin through the cell envelope; two of the genes (*tonB* and *exbB*) have pleiotropic phenotypes, whereas the other two, *fepA* and *fepB*, seem to be specific for ferrienterobactin transport. A functional *tonB* gene is required for all high-affinity iron transport systems, for vitamin $B_{12}$ uptake, and for sensitivity to many phages and colicins (22). Cells harboring a mutation in *exbB* are insensitive to group B colicins (14, 44), defective in vitamin $B_{12}$ transport (16), and partially defective in ferrienterobactin transport (44). Both *fep* genes map in the enterobactin gene cluster at approximately 13 min. The *fepA* gene codes for an 81,000-dalton protein that serves as the outer membrane receptor for ferrienterobactin. Cells with *fepA* mutations are unable to accomplish the initial binding step in transport and consequently lack the ability to grow in iron-poor environments. In 1983, we (42) and others (32) reported the existence of a second *fep* gene. We isolated and characterized the *E. coli* mutant DK214 and demonstrated that the mutation in this strain was in a gene distinct from *fepA*. The new gene was designated *fepB*.

In the present study, an 11-kilobase (kb) *E. coli* DNA fragment containing the *fepB* gene was cloned. Work with this plasmid and its subclones indicated that *fepB* coded for a periplasmic protein, that at least one additional gene (*fepC*) was required for ferrienterobactin transport, and that the *fepC* product was a membrane protein.

(A preliminary report of this work has been presented [J. R. Pierce, C. F. Earhart, and C. L. Pickett, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K9, p. 173].)

## MATERIALS AND METHODS

**Bacteria, plasmids, and phage.** The bacteria, all of which were *E. coli* K-12 derivatives, and plasmids used are listed in Table 1. Bacteriophage λ467, which was used as a source of Tn*5*, has the genotype *b*221 *rex*::Tn*5 c*I857 *O*am29 *P*am80 (11) and was obtained from C. Prody.

**Media and reagents.** LB (37), M9 (36), M9 depleted of iron by extraction with 8-hydroxyquinoline (Aldrich Chemical Co., Inc., Milwaukee, Wis.) (34), and MM (20) media have been described. Agar was added to a 1.5% concentration to prepare solid media. Antibiotics were added to LB medium cooled to 55°C at the following concentrations: chloramphenicol (Sigma Chemical Co., St. Louis, Mo.), 30 μg/ml; carbenicillin (Roerig), 200 μg/ml; kanamycin (neomycin) (Sigma), 25 or 200 μg/ml; and tetracycline (Sigma), 25 μg/ml. Supplements to M9 and MM media were added as described previously (42); when necessary, shikimic acid was added to a concentration of 50 μM. To render MM plates iron deficient, succinate and α,α′-dipyridyl (Sigma) were added (42). Enterobactin was isolated and deferrated as described previously (42). $^{55}FeCl_3$ and L-[$^{35}$S]methionine were purchased from Amersham Corp., Arlington Heights, Ill.

**Cloning and DNA techniques.** Cells were transformed with plasmid DNA as previously described (8). Plasmid DNA was routinely isolated by either the procedure of Birnboim and Doly (5) or that of Holmes and Quigley (18).

Large quantities of plasmid DNA, which were necessary

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

| Strain or plasmid | Genotype | Source or construction |
|---|---|---|
| *E. coli* K-12 | | |
| AB1515 | *fhuA23 purE42 proC14 leu-6 trpE38 thi-1 lacY1 mtl-1 xyl-5 rpsL109 azi-6 tsx-67* | CGSC[a] |
| DK214 | *fepB* derivative of AB1515 | 42 |
| AN102 | *fep purE*$^+$ transductant of AB1515 | 9 |
| AN260 | *fep-402 proA2 argE3 pheA1 tyrA4 trp-401 aroB351* | 27 |
| AN266 | *fep-406*, otherwise like AN260 | I. G. Young |
| AN270 | like AN260 but with a different *fep* mutation | I. G. Young |
| CSR603 | *recA uvrA phr leuB thi rpsL gyrA lacY galK ara-14 xyl-5 mtl-1 proA his-4 argE supE tsx* | CGSC |
| MC4100 | *araD Δ(argF-lac)205 flbB ptsF relA rpsL deoC* | CGSC |
| P678-54 | *thr leu thi supE lacY tonA gal mal xyl ara mtl minA minB* | J. R. Walker |
| RW193 | *fhuA*$^+$ *entA403 purE*$^+$ derivative of AB1515 | J. Neilands |
| Y1088 | *ΔlacU169 supE supF hsdR metB trpR fhuA21 proC*::Tn*5* | C. Prody |
| Plasmids | | |
| pACYC184 | Cm$^r$ Tc$^r$ | 6 |
| pCP111 | pACYC184 with an 11-kb insert into the *Eco*RI site | C. Pickett |
| pJP52, pJP154, pJP251 | *Bal* 31 deletions derived from pCP111 | This study |
| pJP1543 | *fepC*$^+$ *Bal* 31 deletion derived from pJP154 | This study |
| pJP529 | *fepB*$^+$ *Bal* 31 deletion derived from pJP52 | This study |
| pJP199 | pCP111 *fepC*::Tn*5* | This study |
| pJP414 | pCP111 *fepB*::Tn*5* | This study |
| pJP419 | pCP111 derivative with Tn*5* not in *fepB* or *fepC* | This study |

[a] *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

for *Bal* 31 digestions and in vitro transcription-translation experiments, were obtained by using the method of Katz et al. (23) and then further purified by cesium chloride-ethidium bromide equilibrium centrifugation.

Restriction endonucleases and T4 DNA ligase were used by following the directions of the suppliers (New England BioLabs, Inc., Beverly, Mass., or Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Restricted DNA was analyzed on agarose gels as previously described (31). *Hin*dIII fragments of λ DNA, the sizes of which are given in Daniels et al. (10), were used as molecular weight standards. *Bal* 31 digestions were performed by the methods of Maniatis et al. (30). After nuclease treatment, the DNA was ligated with T4 DNA ligase and then, to reduce recovery of transformants containing undigested DNA, restricted again with the enzyme first used to linearize the molecule.

**Identification of plasmid-encoded polypeptides.** Strain P678-54 was transformed with plasmids of interest, and minicells were then isolated and labeled with [$^{35}$S]methionine by the method of Meagher et al. (35). Maxicells were prepared by irradiating strain CSR603 containing appropriate plasmids. The procedure used was essentially that of Sancar et al. (46), modified to include the addition of cycloserine and the use of sulfate-free Hershey salts medium (47). In vitro DNA-directed protein synthesis was examined by using a kit (Amersham).

Three electrophoretic procedures were used to resolve plasmid-encoded proteins. In the first, proteins were solubilized (25) and then analyzed with the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system of Lugtenberg et al. (29) as modified (15). The second system was identical to the first except for the presence of 8 M urea in the separating gel. Apparent molecular weights were determined by using standard kits obtained from LKB Instruments, Inc., Rockville, Md. (molecular weight range, 12,300 to 78,000) and Bethesda Research Laboratories (high range). Two-dimensional gel electrophoresis was also used. Samples were prepared by the method of Ames and Nikaido (2); the isoelectric focusing gels were prepared as described previously (40), and SDS-PAGE was performed by the method of Lugtenberg et al. (29), with or without urea as described above. Autoradiographs of dried gels were made by using Kodak XRP-1 X-ray film.

**Cell fractionation procedures.** Total membrane fractions were isolated by the procedure of Inouye and Guthrie (21), and outer membranes were obtained by incubating total membrane in sodium lauryl sarcosinate (12). Proteins were labeled, and the periplasmic fraction of maxicells was isolated by the procedure of Koshland and Botstein (24).

**Transposon Tn*5* mutagenesis.** Two methods were used. The first procedure was a variation of that of Sasakawa et al. (48). pCP111 was transformed into Y1088, parallel cultures of independent transformants were grown overnight, and the cultures were then plated on neomycin (200 μg/ml) and tetracycline LB plates. All colonies on each plate were suspended in L broth, and plasmid DNA was isolated. Strain DK214 was transformed with this DNA; transformants were selected on neomycin and tetracycline plates. The second method used λ467 (4) and the procedures described by de Bruijn and Lupski (11).

**Assays.** The procedure of Pugsley and Reeves (44) as modified (33) was used to measure cell uptake by $^{55}$Fe-enterobactin. Protein concentrations were determined by the method of Lowry et al. (28), and enterobactin was estimated by the assay of Arnow (3) for phenolates. The ability of plasmids to complement defects in strains carrying *fep* mutations was tested on succinate-dipyridyl plates.

## RESULTS

**Plasmid pCP111.** We isolated and described the hybrid λ phage A1 (42); A1 does not complement strains bearing *fepA* mutations but does complement all other *fep* strains (DK214, AN260, AN266, AN270, and AN102) in our possession. Phage A1 was found to contain an 11-kb *Eco*RI fragment of *E. coli* DNA. Plasmid pCP111 was constructed by restricting 20 μg of A1 DNA and 4 μg of pACYC184 DNA with *Eco*RI, mixing the two DNAs, ligating, using the ligated DNA to

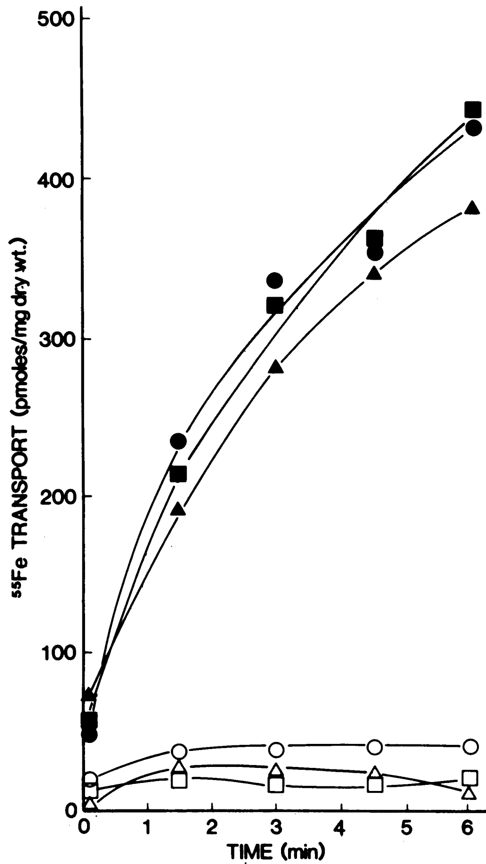


FIG. 1. Effect of plasmid pCP111 on $^{55}$Fe-enterobactin transport by whole cells. Unless otherwise indicated, all assay mixtures contained 2 μM enterobactin. △, RW193 with no added enterobactin; ▲, RW193; □, DK214; ■, DK214 transformed with pCP111; ○, AN102; ●, AN102 transformed with pCP111.

transform strain DK214, selecting for tetracycline resistance, and then screening for chloramphenicol sensitivity (C. L. Pickett, Ph.D. dissertation, University of Texas at Austin, 1983). This plasmid, as expected, had the same complementation abilities as did phage A1. Complementation was measured both by growth on low-iron solid medium and by whole cell enterobactin-dependent iron transport assays. An example of complementation of *fep* mutations by pCP111 in the latter assay is shown in Fig. 1. When DK214 (*fepB*) and AN102 were transformed with pCP111, their uptake of ferrienterobactin was similar to that of wild-type cells. The results with strain RW193 (*entA*), in addition to providing an example of uptake in cells with a normal ferrienterobactin transport system, demonstrated that the assay is enterobactin dependent.

**Restriction map, subclones, and Tn*5* mutagenesis of pCP111.** A restriction nuclease map of pCP111 is shown in Fig. 2. Subclones of pCP111 were generated by cleaving the DNA at the *Kpn*I site and then digesting with *Bal* 31 nuclease. A total of 62 of 90 Tc$^r$ transformants of DK214 failed to grow on succinate-dipyridyl plates, indicating that the *fepB* gene in these plasmids was defective. Plasmid DNA was isolated from 20 of these transformants and from 20 transformants whose plasmid retained the ability to complement *fepB*. The sizes of the plasmid deletions were roughly determined by restriction mapping with *Hin*dIII and *Eco*RI. The amount of DNA deleted from pCP111 (15 kb) was quite variable. Plasmids that complemented DK214 (*fepB*) varied in size from 7.5 to 14 kb, and plasmids that failed to complement DK214 ranged from 4 to 8.5 kb. Most of the *Bal* 31-generated plasmids had lost the *Eco*RI and *Hin*dIII sites nearest to the *Kpn*I site. Restriction maps of three of these plasmids, pJP154, pJP52, and pJP251, are shown in Fig. 2, along with an illustration of their ability to complement *fep* strains in the $^{55}$Fe-enterobactin uptake assay. Like pCP111, pJP154 complemented DK214, AN102, AN260, AN266, and AN270; pJP52 complemented only DK214; and pJP251 failed to complement any *fep* strain. These results suggest that at least two genes necessary for ferrienterobactin transport resided on pCP111.

A second set of *Bal* 31 digestions was made from the

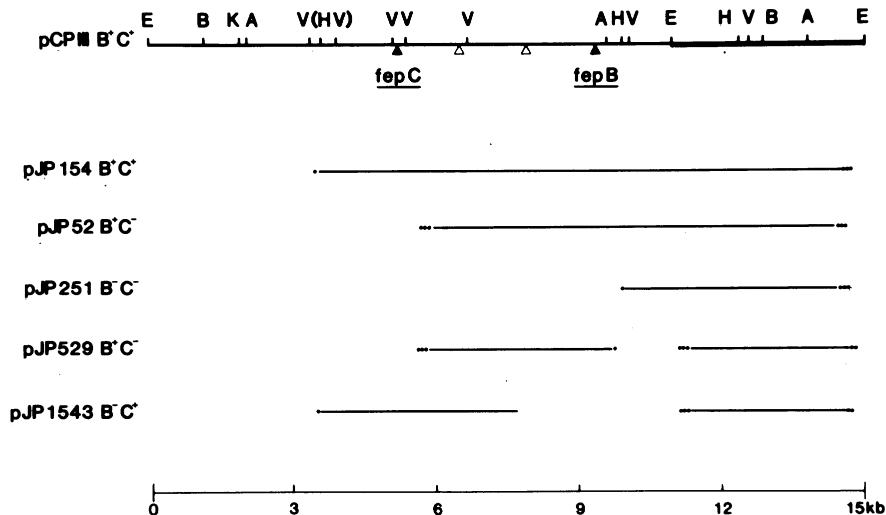


FIG. 2. Restriction map of pCP111 and several derivatives of pCP111 generated by *Bal* 31 digestion. Uncertainties regarding the extent of DNA deleted are indicated by dotted lines. △, Sites of Tn*5* insertions; ▲, insertions that resulted in inactivation of *fep* genes. The abilities of the plasmids to complement *fepB* and *fepC* mutations are indicated. Abbreviations for restriction sites are as follows: A, *Ava*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; V, *Eco*RV. The scale at the bottom is in kilobases, and the bold line at the top represents vector pACYC184.

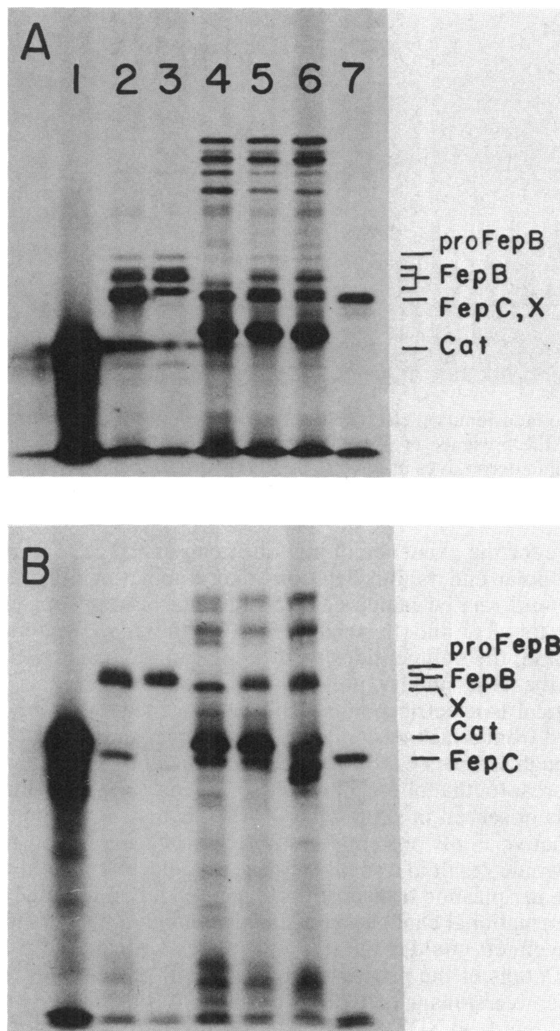


FIG. 3. Autoradiogram of $^{35}$S-polypeptides synthesized in minicells by plasmid pCP111 and several of its derivatives. SDS-PAGE was performed without (A) and with (B) urea in the separating gel. Plasmids were as follows: pACYC184 (lane 1), pCP111 (*fepB*$^+$ *fepC*$^+$) (lane 2), pJP529 (*fepB*$^+$) (lane 3), pJP414 (*fepB*::Tn*5* *fepC*$^+$) (lane 4), pJP419 (*fepB*$^+$ *fepC*$^+$ Tn*5*) (lane 5), pJP199 (*fepB*$^+$ *fepC*::Tn*5*) (lane 6), pJP1543 (*fepC*$^+$) (lane 7). Polypeptide bands are indicated on the right. Cat, Chloramphenicol acetyltransferase.

remaining *Eco*RI site in pJP52 and pJP154. The digested pJP52 and pJP154 DNAs were treated and analyzed as described above. Restriction maps and complementation properties of pJP529 and pJP1543, derivatives of pJP52 and pJP154, respectively, are shown in Fig. 2. The data provide additional evidence that pCP111 contained two *fep* genes. DK214 was designated the type strain for *fepB* mutations (42); accordingly, the *fep* gene present on pJP1543 and defective in strains AN260, AN266, AN270, and AN102 was designated *fepC*.

Tn*5* mutagenesis of pCP111 was performed to further localize *fepB* and *fepC* and to aid in the identification of their products (see below). The location of some of the Tn*5* insertions is shown in Fig. 2; the plasmids with inserts in *fepB* and *fepC* were named pJP414 and pJP199, respectively. When AN102 (*fepC*) was transformed with pJP199, it not only remained unable to transport ferrienterobactin but also, like AN102, hyperexcreted enterobactin in iron-replete medium. The locations and resulting phenotypes of the Tn*5* inserts were consistent with the map positions of *fepB* and *fepC* as determined by *Bal* 31 deletion analysis.

**Identification of polypeptides encoded by *fepB* and *fepC*.** Proteins encoded by pCP111 and several of its subclones were synthesized in minicells and examined in two SDS-PAGE systems (Fig. 3). The only detectable protein produced by pACYC184 was chloramphenicol acetyltransferase. At least five pCP111-specific bands, with molecular masses ranging from 30.5 to 36.5 kilodaltons, were resolved (Fig. 3A, lane 2). In the presence of urea (Fig. 3B), the migration of several of the polypeptides was significantly altered, and the polypeptides had apparent molecular masses varying from 23.5 to 39 kilodaltons.

The *fepC* gene appeared to direct the synthesis of a protein with an apparent molecular weight of 30,500 on standard SDS-PAGE gels and approximately 23,500 in the presence of urea. This protein was the only one observed when proteins encoded by the *fepC*$^+$ plasmid pJP1543 were examined (Fig. 3, lane 7), it was absent from the protein complement directed by pJP529 (*fepB*$^+$) (Fig. 3, lane 3), and it was lost when Tn*5* was inserted into *fepC* (pJP199) (Fig. 3, lane 6). Figure 3, lane 5, served as a control for pCP111 containing Tn*5* insertions; Tn*5* was present in pJP419, but it was not located in *fepB* or in *fepC*. Identification of the *fepB* product was more complex. As can be seen in Fig. 3, lane 3, four polypeptide bands with apparent molecular weights ranging from 31,500 to 36,500 were observed in standard gels upon examination of the products of the *fepB*$^+$ plasmid; in urea gels, a broad band subsequently shown to consist of three components (Fig. 4C and 5B, lanes 2 and 3) was observed. Tn*5* insertions into *fepB*, as in pJP414, resulted in the loss of these polypeptides (Fig. 5, lane 4).

To understand the multiplicity of polypeptides apparently associated with *fepB*, plasmid-encoded polypeptides were examined in an in vitro DNA-directed protein synthesizing system and by two-dimensional electrophoresis. In the in vitro system, *fepB* coded for just one polypeptide, which corresponded to the largest polypeptide seen in one-dimensional standard and urea gels (data not shown). This polypeptide has been tentatively designated proFepB. In the two-dimensional system used, three *fepB*-specific spots were observed (Fig. 4C). The largest polypeptide (Fig. 4C, no. 1) was well resolved from two more prevalent polypeptides (Fig. 4C, no. 2 and 3) which had the same isoelectric point. (The two more abundant spots also routinely exhibited shadowing; that is, pairs of spots appeared on either side of the main spots.) The in vitro protein synthesis results and the two-dimensional gel results provided further evidence that the multiplicity of polypeptides encoded by *fepB*$^+$ plasmids were related.

A protein designated X was also coded for by pCP111. In standard SDS-PAGE, X, FepC, and the smallest FepB band migrated similarly (Fig. 3 and 5; M. F. Elkins, unpublished observations); in urea SDS-PAGE, X migrated slightly faster than did the smallest FepB polypeptide and was well resolved from FepC (Fig. 3B, lane 4; Fig. 4A; Fig. 5B, lane 3).

The band migrating slightly slower than FepC and X (Fig. 3A, lane 4) was of unknown origin. It was not regularly observed under any conditions, and deletion analyses indicate that it cannot be coded for solely by bacterial DNA (J. R. Pierce, Ph.D. thesis, University of Texas at Austin, 1984). We suspect that it is related to the pACYC184 *tet* gene product, which is known to have unusual solubility properties and which, in minicells, can give rise to polypeptides

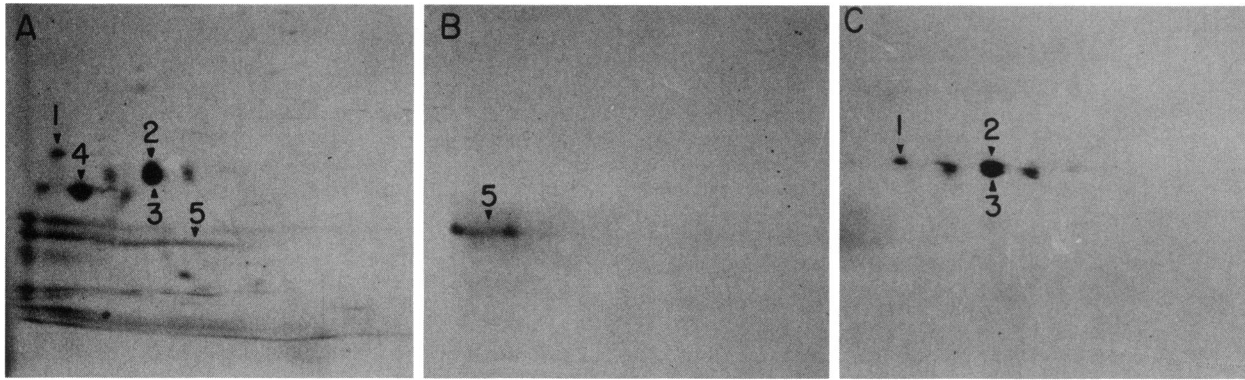


FIG. 4. Autoradiogram of plasmid-encoded polypeptides separated by two-dimensional gel electrophoresis. The proteins were synthesized in minicells and labeled with [$^{35}$S]methionine; SDS-PAGE was performed in the presence of urea. Polypeptides are identified as follows: proFepB (no. 1), mature FepB (no. 2 and 3), X (no. 4), and FepC (no. 5). Plasmid derivatives used were pCP111 (*fepB*$^+$ *fepC*$^+$) (A), pJP1543 (*fepC*$^+$) (B), and pJP529 (*fepB*$^+$) (C).

with a variety of molecular weights, including 34,000 and 26,000 (35).

**Localization of FepB and FepC proteins.** A comparison of periplasmic and membrane proteins with whole cell proteins indicated that the *fepB* product was enriched in the periplasm (Fig. 5, lane 2) and that the *fepC* product was a membrane protein (Fig. 5, lane 3). The results also indicate that X and proFepB are membrane associated. Similar results were obtained by using fractionated maxicells only. The interpretation of these results was complicated by the similarities in apparent molecular weights of the several polypeptides (see above). Samples in Fig. 5, lanes 4 through 6, were run as controls to emphasize the positions of the FepB and FepC proteins in these gel systems.

## DISCUSSION

An approximately 11-kb *Eco*RI fragment that contained the central portion of the enterobactin gene cluster has been characterized. The existence of such a fragment is consistent with previous results (13, 26). We show in this work that the fragment contained at least two genes required for ferrienterobactin transport, and previous results indicate that it also contains a portion of the *entF* gene. (Laird and Young [26], by using the plasmid pMS101, showed that cleavage of the *Eco*RI site which corresponds to the left end of pCP111 [Fig. 2] inactivates *entF*, a result confirmed by Coderre and Earhart [7] with plasmid pPC103, and λA1 was reported to contain part of *entF* [42].) We did not detect any portion of the *entF* product among the polypeptides made in minicells, maxicells, and in vitro protein synthesizing systems. This provides evidence that the *entF* promoter is not present on pCP111 and therefore that it is located counterclockwise from *entF*, as indicated by Fleming et al. (13).

The *fepB* product was identified and found to be located in the periplasm (Fig. 5). It was apparently synthesized with a signal peptide; this proprotein was seen in the membrane fraction (Fig. 5, lane 3) and was the only *fepB*-related protein seen in the in vitro system. In addition to proFepB, two (urea SDS-PAGE) or three (standard SDS-PAGE) more bands were observed in minicells programmed with the *fepB*$^+$ plasmid pJP529 (Fig. 3). Despite the fact that genes for periplasmic components of transport systems are often found to have undergone duplication (1), it is unlikely that these additional bands were the product(s) of a gene other than *fepB*. Deletion analysis, Tn*5* mutagenesis results, and in vitro DNA-directed protein synthesis results furnish evidence for the existence of just one gene on pJP529. Tn*5* can have polar effects, but deletions that eliminate all presumptive *fepB*-related bands can be obtained by digesting from both the left and the right sides of the *fepB* region. In addition, the polypeptides were similar in molecular weight, and the two smaller polypeptides seen in urea gels had identical isoelectric points. The heterogeneity possibly resulted from a lack of complete reduction of disulfide bonds, although samples were routinely solubilized by boiling in 5% β-mercaptoethanol for 5 min. Also, one of the three smaller bands observed in standard SDS-PAGE may correspond to the native FepB protein with ferrienterobactin bound to it; this would result in a small increase in molecular weight, and some periplasmic transport proteins are known to undergo a conformational change upon substrate binding (1). Either of these effects could result in polypeptides with altered mobility. Details of the processing of the FepB protein and other interconversions which are responsible for the multiplicity of FepB bands in SDS-PAGE gels remain to be determined.

The FepC protein was identified as a 30,500-dalton (standard SDS-PAGE) membrane constituent. No proFepC protein was observed in mini- or maxicells, and the in vitro product migrated identically to the in vivo product. FepC is therefore probably present in the cytoplasmic membrane, since such proteins, unlike periplasmic or outer membrane proteins, are synthesized without a leader sequence. Furthermore, periplasmic transport systems using outer membrane proteins have just one such protein and, for the enterobactin system, this role is already filled by the FepA protein.

Plasmid pCP111 contained an additional gene besides *fepB* and *fepC* that codes for protein X. The X protein is a membrane constituent and has an apparent molecular weight of 29,500 (standard SDS-PAGE). We have tentative evidence that X is entirely coded for by bacterial DNA and that the cognate gene for X is located between *fepB* and *entE* M. F. Elkins, unpublished results). There is no direct evidence linking X to ferrienterobactin transport. However, two facts suggest it may have such a role: (i) X is a membrane protein, and periplasmic transport systems generally have at least two cytoplasmic membrane polypeptides (1), and (ii) the gene for X is located in the middle of the *ent* gene cluster, and thus far only enterobactin-related genes have been detected in this region.

The data indicate that ferrienterobactin uptake requires a periplasmic protein (FepB). This observation serves to fur-

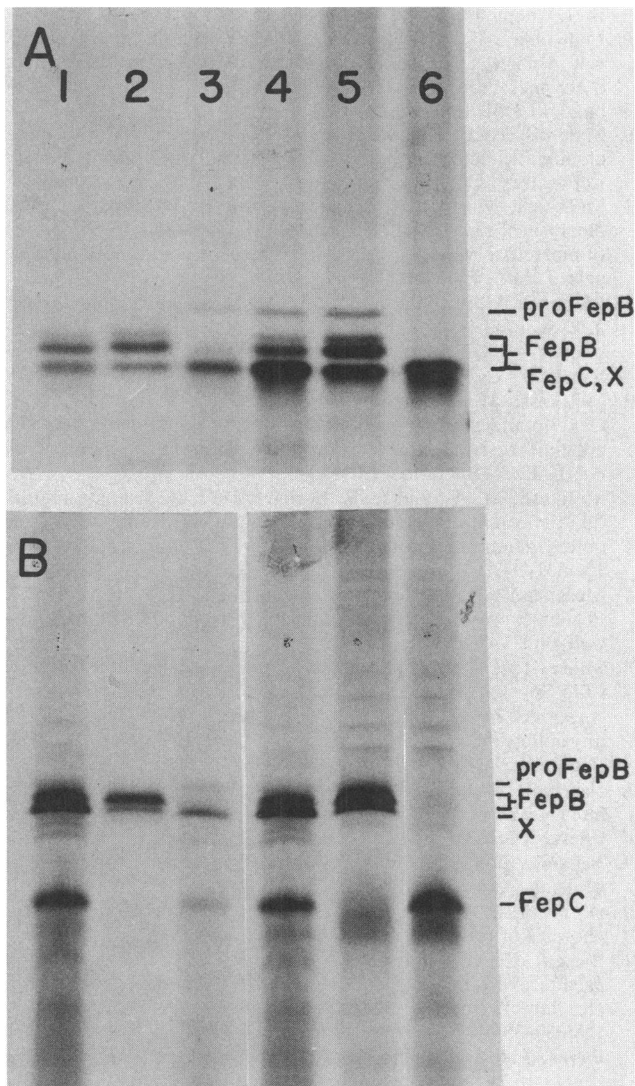


FIG. 5. Autoradiogram of SDS-PAGE separations of fractionated and unfractionated [$^{35}$S]methionine-labeled polypeptides encoded by pCP111. A standard 11% SDS-PAGE (A) and the same samples run in an 11% SDS-PAGE which included 8 M urea (B) are shown. The conditions under which the polypeptides were synthesized and the fractions were studied were as follows: pCP111-directed proteins synthesized in minicells, whole cell fraction (lane 1); pCP111-directed proteins synthesized in maxicells, periplasmic fraction (lane 2); pCP111-directed proteins synthesized in minicells, membrane fraction (lane 3); pCP111-encoded polypeptides synthesized in minicells, whole cell fraction (lane 4); pJP529 (*fepB*$^+$)-encoded proteins synthesized in minicells, whole cell fraction (lane 5); and pJP1543 (*fepC*$^+$) -encoded proteins synthesized in minicells, whole cell fraction (lane 6).

ther emphasize (19, 22) the similarities between vitamin $B_{12}$ and ferrienterobactin transport. Both systems require a functional *tonB* gene, are positively affected by the *exbB* protein, and have specific outer membrane, periplasmic, and cytoplasmic membrane components. The only other periplasmic transport system known to require an outer membrane protein is that for maltose (17); the necessity for such proteins presumably results from the fact that ferrienterobactin, $B_{12}$, and maltodextrins are all too large to readily pass through the outer membrane porin channels. Studies on the energy requirements for ferrienterobactin uptake (45) are consistent with binding protein dependent transport (17). (Iron uptake by pseudorevertants of Ent$^-$ *fepA* strains exhibits sensitivities to energy poisons similar to those of ferrienterobactin transport [41], suggesting that the pseudorevertant system also relies on a periplasmic protein.)

Previous results which failed to demonstrate ferrienterobactin transport in spheroplasts (49) are readily explained by the necessity for and location of the FepB protein. In contrast, ferric rhodotorulic acid uptake can occur in inner membrane vesicles (43), and ferrichrome uptake has been demonstrated in vesicles and spheroplasts (38, 43, 49). This suggests that there is a basic difference between the transport of iron by these hydroxamate siderophores and enterobactin, which is a cyclic catechol type of siderophore. The association of ferric iron with enterobactin is less stable than that of other iron-siderophore complexes at pH values below 7, and enterobactin itself has acid-labile ester bands. Possibly the FepB protein serves to stabilize ferrienterobactin complexes against the effects of reduced pH which would pertain on the periplasmic side of the cytoplasmic membrane. However, when other periplasmic transport system substrates such as $B_{12}$ and maltose are considered, it is clear that this would not be a typical role for a periplasmic binding protein.

In summary, the enterobactin system now appears to require at least 11 genes whose products function solely in iron assimilation; the sequence and designations of these genes are as follows: *entD fepA fes entF fepC fepB entEBG(AC)*. The genes *fepA*, *fepB* and *fepC*, which code for proteins located in the outer membrane, periplasm, and probably the cytoplasmic membrane, respectively, are required for ferrienterobactin entry into the cell interior. That ferrienterobactin uptake is accomplished by a periplasmic transport system is indicated by the location of the *fepB* product.

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