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Genetic Organization of Multiple *fep* Genes Encoding Ferric Enterobactin Transport Functions in *Escherichia coli*

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Three genes were shown to provide functions specific for ferric enterobactin transport in *Escherichia coli*: *fepA* encoded the outer membrane receptor, *fepB* produced a periplasmic protein, and the *fepC* product was presumably a component of a cytoplasmic membrane permease system for this siderophore. A 10.6-kilobase-pair *E. coli* chromosomal *Eco*RI restriction fragment containing the *fepB* and *fepC* genes was isolated from a genomic library constructed in the vector pBR328. Both cistrons were localized on this clone (pITS24) by subcloning and deletion and insertion mutagenesis to positions that were separated by approximately 2.5 kilobases. Within this region, insertion mutations defining an additional ferric enterobactin transport gene (*fepD*) were isolated, and polarity effects from insertions into *fepB* suggested that *fepD* is encoded downstream on the same transcript. A 31,500-dalton FepC protein and a family of FepB polypeptides ranging from 34,000 to 37,000 daltons were identified in *E. coli* minicells, but the product of *fepD* was not detectable by this system. Another insertion mutation between *entF* and *fepC* was also shown to disrupt iron transport via enterobactin and thus defined the *fepE* locus; *fepE* weakly expressed a 43,000-dalton protein in minicells. It is proposed that these newly identified genes, *fepD* and *fepE*, provide functions which act in conjunction with the *fepC* product to form the ferric enterobactin-specific cytoplasmic membrane permease. An additional 44,000-dalton protein was identified and shown to be expressed from a gene that is situated between *fepB* and *entE* and that is transcribed in the direction opposite that of *fepB*. Although the function of this protein is uncharacterized, its membrane location suggests that it too may function in iron transport.

The acquisition of iron for metabolic purposes is of critical importance to microorganisms. Because Fe^{3+} is generally insoluble in an oxygen environment at a neutral pH (38) or is complexed by host proteins such as lactoferrin or transferrin (46), microorganisms have developed an array of complex systems for high-affinity iron assimilation (9, 38). The primary system used by *Escherichia coli* consists of the components required to synthesize, excrete, and retrieve the catechol siderophore enterobactin (also called enterochelin). In *E. coli*, the genes for this complex system are clustered on a contiguous stretch of the chromosome near 13 min (4). These genes are strictly regulated (5, 18, 25), with both biosynthetic and transport functions derepressed by iron-limiting conditions.

Bacterial transport systems for many essential metabolites, including ferric enterobactin, consist of an outer membrane receptor, a periplasmic shuttle protein, and a cytoplasmic membrane permease complex (for a review, see reference 2). The initial recognition of ferric enterobactin occurs at the outer membrane receptor, FepA (34), and is followed by internalization involving the periplasmic protein, FepB (40). A third protein, FepC, likely located in the inner membrane, is also required for active transport (40). The genes for each of these proteins are situated among the enterobactin biosynthetic genes. In addition to the enterobactin-specific transport proteins, internalization requires the pleiotropic *tonB* and *exbB* products, which have unspecified roles in the energy-dependent steps of a variety of transport systems (26, 42, 47).

The functions and organization of enterobactin transport genes have only recently been examined at the molecular level. The receptor, FepA, like other outer membrane proteins, is translated as a precursor containing a signal peptide which is subsequently cleaved to form the mature protein (19, 33). It serves as the receptor for colicins B and D, as well as ferric enterobactin (24, 41). The *fepA* gene is located toward the left side of the enterobactin gene system (see Fig. 1) between *entD* and *fes*. Like FepA, FepB appears to be initially translated as a precursor with an amino-terminal signal peptide but is subsequently processed to several observable polypeptides, all of which are released by osmotic shock and assumed to function within the periplasmic space (40). Many bacterial transport systems, including those for vitamin B_{12}, histidine, and maltose, require periplasmic binding proteins (15, 28, 42). These systems also have multiprotein inner membrane permease complexes, and the enterobactin system is expected to be analogous. One cytoplasmic membrane component, FepC, was recently described (40). The genes encoding FepB and FepC were mapped clockwise from *fepA* at the locus originally described as *fep* (12, 30).

This report describes the genetic identification of two additional transport loci required for ferric enterobactin uptake. The *fepD* gene was mapped between *fepB* and *fepC*, and polarity effects resulting from insertion mutations within or near the *fepB* gene suggest that *fepD* is transcribed with *fepB*. A transposon insertion mutation defining the *fepE* locus was mapped between *fepC* and the biosynthetic gene *entF*. These newly identified transport genes are assumed to encode additional components of the cytoplasmic membrane permease system which participate with the *fepC* product in the final step of ferric enterobactin retrieval.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. All *E. coli* strains used in this report are described in Table 1. All recombinant plasmid constructions (designated pITS [plasmids containing iron transport system genes]) are in the

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TABLE 1. Bacterial strains

Strain	Genotype	Reference or source
AB1515	*thi trpE purE proC leuB lacY mtl xyl rpsL azi fhuA tsx supA*	12
AN42	*thi his proA argE pheA tyrA trp rpsL entD*	32
AN90	*purE*$^+$ *entD* derivative of AB1515 by P1 transduction from AN42	12
AN102	*purE*$^+$ *fep-401* derivative of AB1515	12
AN102-60	Δ*recA* derivative of AN102	This study
AN260	*proA argE pheA trp aroB fepC402*	31
AN261	*purE*$^+$ *fepC* derivative of AB1515 by P1 transduction from AN260	31
AN261-60	Δ*recA* derivative of AN261	This study
AN266	*fepC406*, otherwise like AN260	40
AN270	*fepC409*, otherwise like AN260	40
HB101	*thi pro leu rpsL lac his gal recA*	*E. coli* Genetic Stock Center
MC4100	*araD* Δ(*lacIOPZYA*) U169 *rpsL thiA*	10
MC4160	Δ*recA* derivative of MC4100	This study
MK1	*entF*::mini-*kan-1* derivative of AB1515	This study
MK9	*fepE*::mini-*kan-9* derivative of AB1515	This study
MK39	*fepD*::mini-*kan-39* derivative of AB1515	This study
MM383	*polA12 thy rha lac rpsL*	*E. coli* Genetic Stock Center
χ984	*minA minB pdxC purE his rpsL xyl ilv met*	20
W1485	F$^+$	*E. coli* Genetic Stock Center
JC10284	*srlR*::Tn*10 srlC srlD metB mtl gatC gatA malA xyl rpsL sup* Δ(*srlR-recA*)	13

vector pBR328 (43) and are diagrammed in Fig. 1 and 2. The λ1105 vector containing the p*tac*–mini-*kan* insertion element (45) was a kind gift of Nancy Kleckner via George Smith. Mini-*kan* contains the Tn*903* Km^r gene flanked by 70 base pairs (bp) of IS*10*R and IS*10*L from Tn*10* (45). The Tn*10* transposase gene is also present in λ1105 to provide the necessary *trans*-active transposition function. P1 transduction experiments to transfer relevant genetic markers between strains were carried out by the method of Miller (36). The *recA* deletion derivatives of each strain were constructed by isolating P1 transductants which carried the *srl*::Tn*10* and Δ*recA* markers from JC10284. Spontaneous loss of Tn*10* was selected (7), and retention of the *recA* deletion mutation was screened by sensitivity to UV radiation.

Media, chemicals, and enzymes. Tris-glucose minimal medium containing 200 μM 2,2′-dipyridyl was used as the selective iron-depleted medium. The concentration of supplements added has been previously described (18, 35). Luria broth (LB) was utilized as complete medium (36). Antibiotics were used at the following concentrations: ampicillin (Ap), 25 μg/ml; chloramphenicol (Cm), 30 μg/ml; tetracycline (Tc), 10 μg/ml; and kanamycin (Km), 50 μg/ml. Restriction nuclease enzymes were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md.; International Biotechnologies, Inc., New Haven, Conn.; New England BioLabs, Inc., Beverly, Mass.; and Promega Biotec, Madison, Wis. T4 DNA ligase and DNA polymerase I large (Klenow) fragment were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. DNA restriction and modification enzymes were used under the conditions recommended by the supplier. [^{35}S]methionine was purchased from New England Nuclear Corp., Boston, Mass. Triton X-114 was purchased from Sigma Chemical Co., St. Louis, Mo.

Plasmid isolation and transformation. Plasmids were isolated by the alkaline lysis procedure of Birnboim and Doly (6). $CaCl_2$-treated cells were transformed as described by Cohen et al. (11).

Electrophoresis. DNA was analyzed on horizontal agarose slab gels containing 0.5 μg of ethidium bromide per ml in TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA [pH 8.2]). Proteins were separated on vertical sodium dodecyl sulfate-polyacrylamide (10 to 18% continuous gradient) gels in 3% urea by the method of Laemmli (29). Samples were boiled for 5 min in buffer (62.5 mM Tris hydrochloride [pH 6.8], 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% [vol/vol] glycerol, 3% urea, 0.001% bromophenol blue) before loading. Proteins were visualized with Coomassie brilliant blue R-250, and [^{35}S]methionine-labeled polypeptides were detected by autoradiography on XAR-2 film (Eastman Kodak Co., Rochester, N.Y.).

Mini-*kan* and Tn*1000* mutagenesis. For mutagenesis with the mini-*kan* transposition element (45), strain HB101 (pITS24) was infected with λ1105 at a multiplicity of infection of 1. Cells were grown for 90 min to allow for mini-*kan* transposition and expression of the kanamycin resistance gene. Cells were then diluted in LB supplemented with 40 mM sodium citrate and were grown in the presence of high levels (300 μg/ml) of kanamycin. Plasmid DNA was isolated and used to transform MC4160, and Ap^r Tc^r Km^r transformants were selected on LB agar.

pITS24 was mutagenized with Tn*1000* by the procedure of Guyer (23). Briefly, pITS24 was transformed into strain W1485. The recombinant plasmid was mobilized by the F plasmid containing Tn*1000* during mating with the recipient MK1 (Km^r) at a 1:1 donor-to-recipient ratio. Ap^r Km^r transconjugants were selected on LB agar. Plasmid DNA was isolated, and the insertion mutations were localized by restriction endonuclease digestion with the use of the published restriction map of Tn*1000* (22).

Deletion mutagenesis. *Eco*RV- or *Hpa*I-digested fragments were randomly deleted from pITS24 by partial restriction endonuclease digestion, which was followed by gel purification and ligation of the resulting fragments. Plasmid DNA (15 μg) was treated with 10 U of *Eco*RV or 3 U of *Hpa*I at 37°C for 15 min in buffer provided by the supplier (Bethesda Research Laboratories). The partially digested DNA was electrophoresed in a 0.5% low-melting-temperature agarose gel, and appropriately sized linear DNA fragments corresponding to the deletion derivatives (including vector sequences) described in Fig. 2 were excised and extracted. Each isolated DNA fragment was treated with T4 DNA ligase and transformed into HB101. The structures of the recovered plasmids were defined by restriction endonuclease cleavage analysis.

Marker exchange experiments. Pertinent mini-*kan* insertion mutations were transferred to the chromosome by homologous recombination as described by Monk and Kinross (37). Briefly, pITS24 containing a mini-*kan* insertion was transformed into strain MM383 [*polA*(Ts)]. A single transformant was transferred to LB and grown at 42°C, the

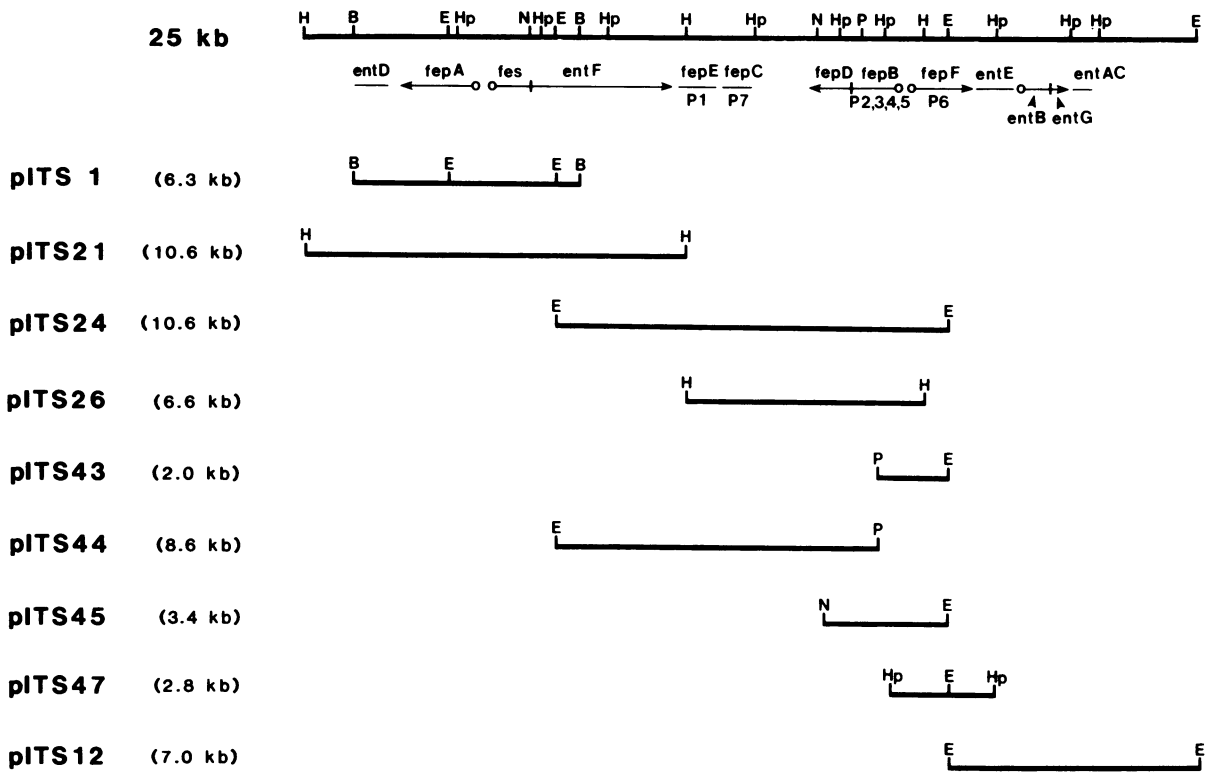

FIG. 1. Physical map of the enterobactin region contained on recombinant plasmids. The top line represents the genetic organization of the enterobactin system. Remaining lines refer to DNA fragments inserted into pBR328 at the corresponding restriction sites to create the specified recombinant plasmids. The pITS1 insert has been described previously (19), and pITS12 contains a 7.0-kb *Eco*RI fragment encoding *entE entB entG entAC* (M. Nahlik, unpublished). The pITS43 and pITS44 inserts were cloned into *Eco*RI-*Pst*I-digested pBR328. The pITS45 fragment was cloned into *Eco*RI-*Eco*RV-digested pBR328. The pITS47 insert was cloned into *Eco*RV-digested pBR328. The parenthetical sizes refer to the length of the cloned DNA fragments and do not include the vector length. The arrows indicate direction of transcription, where known, and the designations P1 to P7 represent proteins which are expressed in minicells from the pITS24 plasmid, as described in the text. Sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; N, *Nru*I; P, *Pst*I.

nonpermissive temperature. The culture was passaged three times by subculturing a 1% inoculum into fresh LB, which was followed by overnight growth at 42°C; cells were then streaked on an LB-kanamycin agar plate. Colonies were replica plated on LB-kanamycin agar and LB-ampicillin agar to select for recombinants. Km^r Ap^s clones were further examined for the inability to grow on iron-depleted medium. The insertion mutations were transduced into strain AB1515 with phage P1.

Minicell isolation and localization of plasmid-encoded proteins. Strain χ984 was transformed with the various recombinant plasmids, and minicells were isolated as previously described (20). Minicell preparations were labeled for 60 min with 1 mCi of [^{35}S]methionine per ml at 37°C in iron-deficient medium, washed with nonradioactive medium, and boiled for 5 min in sample buffer. The procedure of Bordier (8) for Triton X-114 partitioning of proteins was performed on minicells first disrupted by sonication. Treatment of minicells with chloroform was performed as described by Ames et al. (3). Periplasmic proteins were also released by osmotic shock as described by Neu and Heppel (39).

RESULTS

Cloning of the *fepB* region. We isolated a 6.3-kilobase-pair (kb) *Bam*HI fragment which could genetically complement the enterobactin biosynthesis mutant AN90-60(*entD*). This clone, designated pITS1, was selected from an *E. coli* genomic library constructed in pBR328 (19) and encodes the *fepA* and *fes* genes, as well as *entD* (Fig. 1). The 600-bp *Eco*RI-*Bam*HI fragment from pITS1 was used as a radiolabeled probe to search *Hin*dIII and *Eco*RI libraries constructed in pBR328 for the adjacent genomic sequences. Positive clones containing a 10.6-kb *Eco*RI insert (pITS24) or a 10.6-kb *Hin*dIII insert (pITS21) were selected (Fig. 1). The two internal *Hin*dIII sites, 6.6 kb apart in pITS24, were used to construct the subclone pITS26. Since previous reports have shown that the genetic region between *entF* and *entE* encodes proteins required for iron transport by the enterobactin system (30, 40), a number of transport-defective mutants were examined for complementation by pITS21 (which contains the outer membrane receptor gene *fepA*), pITS24, or pITS26. Each mutant was transformed with these plasmids and examined for the ability to grow on iron-depleted medium and to transport enterobactin (Table 2). All of the transport mutants were complemented by pITS24 but not pITS21, indicating that essential functions for ferric enterobactin transport distinct from those provided by the outer membrane receptor FepA are localized in this region. All of these mutants, except MK9 (described below), could also be complemented by the subclone pITS26.

Genetic organization of pITS24. Examination of the proteins expressed by *E. coli* minicells containing pITS24 identified a minimum of seven insert-specific polypeptides (Fig. 3). To delineate the gene boundaries for these products, *Pst*I and *Nru*I sites within pITS24 were used to construct addi-

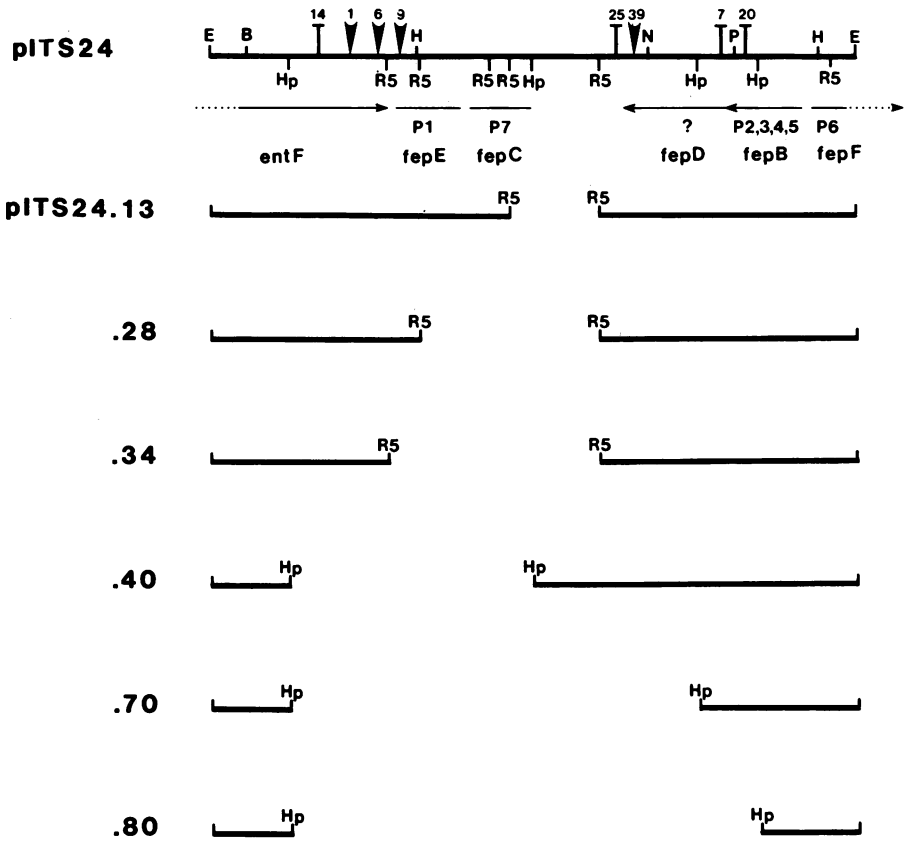

FIG. 2. Insertion and deletion derivatives of pITS24. pITS24 was mutagenized with the mini-*kan* insertion element (45) or Tn*1000* as described in Materials and Methods, and the location of individual insertions was determined by restriction cleavage site mapping. Mini-*kan* (➤) and Tn*1000* (⊤) insertions are denoted on the DNA map. Deletion derivatives of pITS24 were constructed by partial digestion of the plasmid with restriction enzyme *Eco*RV or *Hpa*I, which was followed by religation. The proteins designated by P1 to P7 are described in the text; the protein product of *fepD* is unknown and denoted by a question mark. Sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; N, *Nru*I, P, *Pst*I; R5, *Eco*RV. Arrows show direction of transcription.

TABLE 2. Genetic complementation

Plasmid	Growth ability of recipient strain[a]:			
	AN261 *fepC*[b]	AN102 *fepD*	MK39 *fepD*	MK9 *fepE*
pITS21	−	−	−	−
pITS24	+	+	+	+
pITS26	+	+	+	−
pITS44	+	−	−	+
pITS45	−	−	−	−
pITS24-M9	+	+	+	−
pITS24-M39	+	−	−	+
pITS24-T20	+	−	−	+
pITS24-T7	+	−	−	+
pITS24-T25	+	+	+	+
pITS24.40 (Δ*fepE fepC*)[c]	−	+	+	−

[a] Complementation was measured as the ability (+) or inability (−) to grow on Tris-glucose minimal medium agar containing 200 μM 2,2′-dipyridyl (iron-deficient conditions) or to utilize ferric enterobactin as an iron source in feeding assays on the same medium. A *recA* derivative of each recipient strain was transformed with the indicated plasmid and evaluated for complementation of enterobactin transport defects.

[b] The *fepC* mutants AN266 and AN270 gave results identical to those obtained with AN261.

[c] Deletion derivative of pITS24 as described in Fig. 2.

tional subclones. The 2.0- and 8.6-kb *Pst*I-*Eco*RI fragments and the 3.4-kb *Nru*I-*Eco*RI fragment were each cloned into pBR328 (pITS43, pITS44, and pITS45, respectively; Fig. 1). When protein expression from these plasmids and pITS26 was examined, the various proteins were assigned to specific positions on the DNA map. The internal 6.6-kb *Hin*dIII fragment represented by pITS26 encoded P2, P3, P4, P5, and P7 but lacked P1 and P6 (Fig. 3, lane 3). pITS44, which contains the left 8.6-kb *Eco*RI-*Pst*I fragment of pITS24, encoded only P1 and P7 (Fig. 3, lane 5). These data and the observation that *entF*, a large enterobactin biosynthesis gene, extends to approximately 400 bp to the left of the left-most *Hin*dIII site in pITS24 (G. S. Pettis and M. A. McIntosh, submitted for publication) suggest that the gene for P1 must span that *Hin*dIII site. The P7 gene is located adjacent to that for P1 (see below) and corresponds to the previously described *fepC* gene (40).

pITS45 (Fig. 3, lane 6) contains the coding regions for P2, P3, P4, P5, and P6, which localizes these products to the right end of pITS24. Since P6 was not expressed from pITS26, it must be located near the *Eco*RI end. The 2.0-kb *Pst*I-*Eco*RI insert in pITS43, which is 1.4 kb shorter than the cloned fragment in pITS45, yielded altered P2, P3, and P4 polypeptides; P5 was not detected (Fig. 3, lane 4). The polypeptides originating from pITS43 migrate slower than do the corresponding proteins from pITS24 or pITS45 but maintain a consistent size relationship, indicating that the

altered migration rates result from fusion of their coding regions to flanking sequences in the vector. This suggests that P2, P3, and P4 (and probably P5) might be related products from a single gene that is transcribed right to left through the *Pst*I site. The observations confirm the previous finding that this gene, *fepB*, expresses multiple, related polypeptide products (40).

The increase in apparent size associated with the protein corresponding to P6 expressed from pITS43 must also reflect a fusion event resulting from the construction of this recombinant molecule and indicates that the P6 structural gene must be transcribed left to right through the *Eco*RI site. P6, in its truncated form (expressed from pITS24 and pITS45), corresponds to the previously described protein X (40). To characterize the product of the intact structural gene for P6, a recombinant plasmid (pITS47) containing the 2.8-kb *Hpa*I fragment that spans the *Eco*RI site was constructed. The 1.6-kb *Hpa*I-*Eco*RI fragment from the right end of pITS24 was ligated to the 1.2-kb *Eco*RI-*Hpa*I fragment from the left end of pITS12, a recombinant plasmid containing the adjacent 7.0-kb *Eco*RI chromosomal fragment (M. Nahlik, unpublished results) which encodes *ent* biosynthetic genes

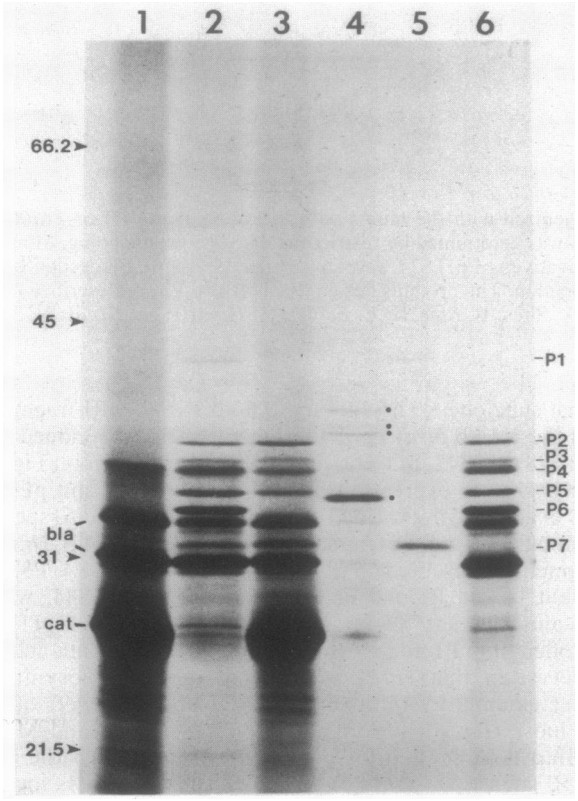

FIG. 3. Autoradiogram of ^{35}S-labeled proteins expressed from plasmid-containing minicells. Minicells containing the specified plasmids were isolated and labeled as described in Materials and Methods. Labeled proteins were separated on a 10 to 18% continuous gradient polyacrylamide-3% urea gel in the presence of sodium dodecyl sulfate and were visualized by exposing the dried gel to Kodak XAR-2 film. The positions of protein products expressed by pITS24 are indicated as P1 to P7. β-Lactamase (bla) and chloramphenicol acetyltransferase (cat) expressed by the vector are denoted as well. Migration positions of molecular weight standards are shown in thousands in the left margin. The altered products expressed by pITS43 are indicated by dots alongside lane 4. Lanes: 1, pBR328; 2, pITS24; 3, pITS26; 4, pITS43; 5, pITS44; 6, pITS45.

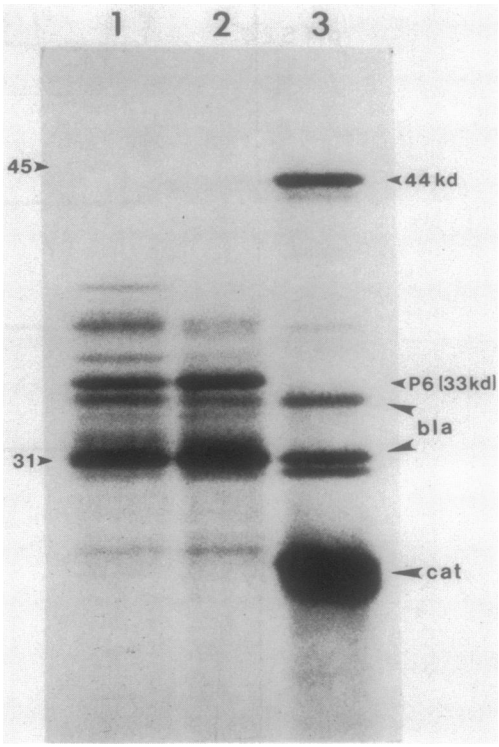

FIG. 4. Identification of full-length P6 protein. Strain χ984 was transformed with the indicated plasmids. Minicells were isolated, and proteins were prepared and separated as described in the legend to Fig. 3. The migration positions of P6 (33 kilodaltons [kd]) and its full-length equivalent (44 kd), as well as those of β-lactamase (bla) and chloramphenicol acetyltransferase (cat), are indicated. Migration positions of molecular weight standards are shown in thousands in the left margin. Lanes: 1, pITS24; 2, pITS24.80 (Fig. 2); 3, pITS47.

(Fig. 1); the mixture was then cleaved with *Hpa*I to eliminate those molecules joined at the *Hpa*I ends. From this reaction, linear 2.8-kb molecules were isolated and cloned into the *Eco*RV site of pBR328 to produce pITS47. Examination of the proteins expressed from this plasmid (Fig. 4) revealed that the P6 protein (which migrates with an apparent molecular weight of 33,000 in this gel system) that was detected with pITS24 and its deletion derivative pITS24.80 (see Fig. 2) was not observed with pITS47. Instead, a larger 44,000-dalton protein, which represents the native product of the P6 gene, was detected (Fig. 4, lane 3), confirming its transcriptional orientation. Although the function of this 44,000-dalton protein is unknown at present, an insertion mutation mapped in or near its structural gene resulted in growth inhibition under iron-deficient conditions and an inability to transport ferric enterobactin (data not shown). Production of the siderophore was not impaired. These observations suggest that the 44,000-dalton protein is encoded by another *fep* locus. Further analysis of this gene is in progress.

Insertion and deletion mutations were constructed in pITS24 to probe the genetic organization of the region. Insertion mutations were created with either the mini-*kan* insertion element (45) or Tn*1000* (23). The eight insertions studied extensively are shown in Fig. 2. Each plasmid containing an insertion was transformed into χ984 for analysis of expression products (Fig. 5). The insertion mutations in pITS24-M1, pITS24-M6, and pITS24-T14 are located

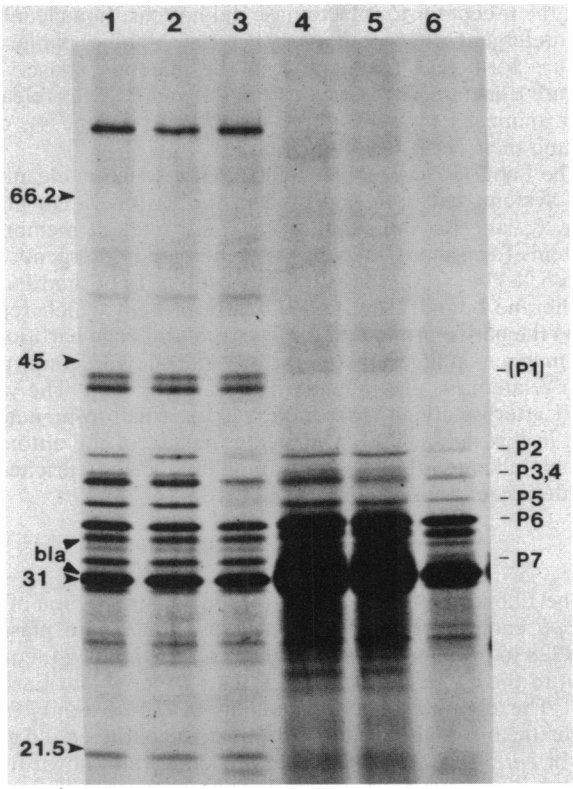

FIG. 5. Autoradiogram of ^{35}S-labeled proteins expressed by pITS24 carrying mini-*kan* or Tn*1000* insertions. Strain χ984 was transformed with various pITS24 insertion derivatives. Minicells were isolated, and proteins were prepared and separated as described in the legend to Fig. 3. The migration positions of P1 to P7 are indicated. P1 is parenthetical because it comigrates with a Tn*1000*-encoded protein (lanes 1 to 3) and is poorly expressed and therefore weakly detectable in lanes 4 and 5. Migration positions of β-lactamase (bla) and molecular weight standards (in thousands) are shown in the left margin. Lanes: 1, pITS24-T14; 2, pITS24-T7; 3, pITS24-T20; 4, pITS24-M1; 5, pITS24-M6; 6, pITS24-M9.

within the *entF* gene and do not affect the expression of any of the transport region proteins. The insertions in pITS24-M39 and pITS24-T25 are located in the region between *fepC* (P7) and *fepB* (P2, P3, P4, and P5) and also demonstrate no observable change in the protein pattern (data not shown). The mini-*kan* insertion in pITS24-M9 is located 300 bp to the left of the *Hin*dIII site previously predicted to be located in the P1 structural gene; this mutation eliminated P1 expression (Fig. 5, lane 6). It should be noted that P1 was consistently detected at a very low level in this system; whether this is the result of poor transcription or translation or a deficiency of methionine residues in the protein is at present unknown. The insertion mutation in pITS24-T20 defines the gene encoding the P2-P3-P4 complex and demonstrates that P5 must be an additional polypeptide originating from this transcript; each of these products was eliminated by the insertion (Fig. 5, lane 3). pITS24-T7, with a Tn*1000* insertion located only 400 bp to the left of the insertion in pITS24-T20, expressed all proteins (Fig. 5, lane 2), providing further evidence that P2, P3, P4, and P5 represent related polypeptides originating from a single gene, *fepB*.

This interpretation was confirmed with a series of deletions constructed from pITS24 after partial digestion with *Eco*RV or *Hpa*I (Fig. 2). Protein expression in minicells (data not shown) indicated that deletion of the internal 1.3-kb *Eco*RV fragment (pITS24.13) resulted in the loss of only P7. Further deletions to the left (pITS24.28 and pITS24.34) eliminated P1 in addition to P7. Deletion of the 4.0-kb *Hpa*I fragment (pITS24.40) or concomitant deletion of the 4.0 and the 2.7-kb *Hpa*I fragments (pITS24.70) resulted in the loss of only P1 and P7. The additional deletion of the 1.0-kb *Hpa*I fragment (pITS24.80) eliminated the P2, P3, P4, and P5 group, and only P6 was expressed. The insertion and deletion mutation data, in conjunction with the subclone results, allowed the genes for P1 and P7 (*fepC*) to be mapped to a region on the left of pITS24, spanning approximately 2.5 kb from just left of the *Hin*dIII site to the central *Hpa*I site, and the genes for P6 and P2 through P5 (*fepB*) to be located on the extreme right side covering the 2.5 kb to the second *Hpa*I site (Fig. 2). No polypeptides which originate from the central 2.5 kb of this region were detected in these experiments.

Mutant construction and analysis. Previously characterized enterobactin transport mutations were mapped to the *fepB* and *fepC* cistrons by genetic complementation studies (40) with cloned DNA fragments spanning the region contained within pITS24. Since the insertion mutations in pITS24-M39 and pITS24-M9 did not map to either of these two loci, marker exchange experiments were performed and resulted in the definition of two additional gene loci involved in ferric enterobactin transport. Homologous recombination events resulting in transfer of insertion mutations from pITS24-M1, pITS24-M9, or pITS24-M39 to the chromosome were selected by transforming the mutant plasmid into MM383, diluting out the plasmids by growth at 42°C, and isolating Km^r survivors. Each mutation was then transduced from the MM383 genetic background to strain AB1515 by using phage P1. The resulting mutants, MK1, MK9, and MK39, were examined for enterobactin biosynthesis and transport phenotypes. Synthesis of the siderophore was detected by bioassay with the *entD* mutant strain AN90 as an indicator on iron-depleted medium (19). MK1 was unable to crossfeed AN90, indicating that this strain is defective in enterobactin biosynthesis and confirming the location of the insertion within the *entF* structural gene. MK9 and MK39 were able to produce enterobactin but were themselves unable to grow on iron-depleted medium. The addition of purified enterobactin to the medium did not promote the growth of these mutants, suggesting that MK9 and MK39 contain defects in the ferric enterobactin transport system. The loss of transport was especially interesting in the case of MK39, since this insertional mutation does not eliminate any polypeptide detected by minicell analysis. MK39 and the *fepC* mutant AN261 (40) were transformed with relevant insertion derivatives of pITS24 and with the subclones pITS24.40, pITS43, pITS44, and pITS45 (Table 2). All of these plasmids which expressed P7 complemented the *fepC* mutant AN261. pITS24-M9, pITS24-T25, and pITS24.40 were able to complement the transport lesion in MK39. pITS24-M39, pITS24-T7, and pITS24-T20 each lacked the product required for growth of the mutant on iron-depleted medium. Since the Tn*1000* insertion in pITS24-T20 is located in *fepB* and eliminated its family of polypeptide products (Fig. 5), the lack of genetic complementation of MK39 by pITS24-T20 can be explained by a polarity effect of the insertion on a downstream gene. The absence of a complete *fepB* gene and its promoter would then explain why the subclone pITS44 was unable to complement the mutation in MK39. It was also noted that MK39 hyperexcretes entero-

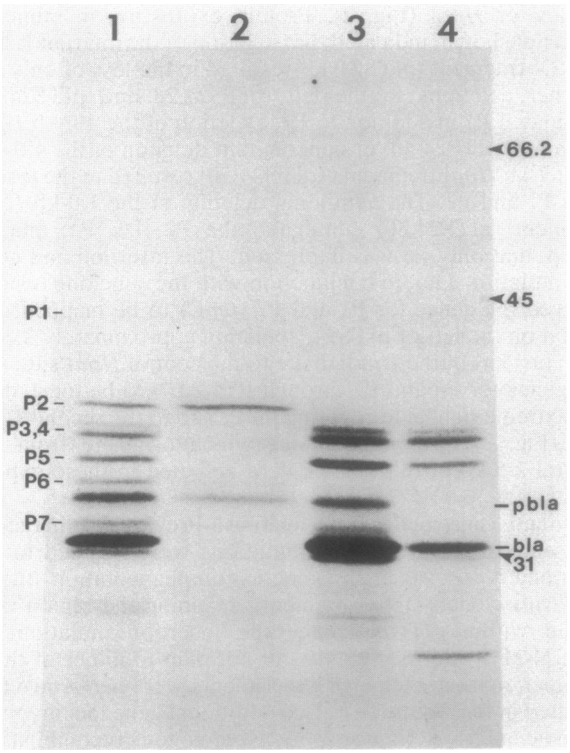

FIG. 6. Cell fractionation analysis of pITS24 products. Minicells containing pITS24 were labeled with [^{35}S]methionine and then treated with Triton X-114 or chloroform as described in Materials and Methods. Proteins were prepared and separated as described in the legend to Fig. 3. The positions of P1 to P7 are indicated. The migrations of molecular weight standards (in thousands) and β-lactamase (bla) and the precursor to β-lactamase (pbla) are also indicated. Lanes: 1, untreated; 2, Triton X-114 detergent fraction; 3, Triton X-114 aqueous fraction; 4, chloroform shock fluid.

bactin, a trait observed in the transport mutant AN102 (12). When AN102 was transformed with these various plasmids, complementation results identical to those obtained with MK39 were observed (Table 2). The gene defined by the lesions in AN102 and MK39 was designated *fepD*, which presumably is cotranscribed with *fepB*, and its 3′ end is located between the mutations defined by pITS24-M39 and pITS24-T25 (Fig. 2). The enterobactin transport lesion defined by the insertion mutation in pITS24-M9, which eliminated the expression of P1, identifies an additional transport locus which is situated between *entF* and *fepC*. The transcriptional orientation of this cistron, designated *fepE*, is unclear at present.

Localization of the Fep proteins. Phase separation in the presence of nonionic detergents has been used to partition hydrophilic proteins from integral membrane proteins which are amphiphilic in nature (8). When proteins expressed in minicells containing pITS24 were phase partitioned in the presence of Triton X-114, distinctive subpopulations of these proteins could be identified (Fig. 6). P2 partitioned exclusively in the detergent phase (Fig. 6, lane 2), whereas its related polypeptides P3, P4, and P5 were hydrophilic and found exclusively in the aqueous phase (Fig. 6, lane 3). This observation is highly supportive of the previous suggestion (40) that P2 represents the precursor (signal peptide-containing) form of FepB and that P3, P4, and P5 represent various periplasmic forms of this protein. It is interesting that the precursor to β-lactamase did not partition cleanly in the detergent phase but is rather amphiphilic, while the mature form was found only in the aqueous phase. The mature β-lactamase, as well as P3, P4, and P5, was released from minicells by chloroform shock (Fig. 6, lane 4), confirming their periplasmic location (3).

The FepC (P7) and P6 proteins did not separate cleanly in this system, but both appeared in the Triton X-114 phase (Fig. 6, lane 2). Considering that only integral membrane proteins form mixed micelles with nonionic detergents like Triton X-114 (8), these results suggest that FepC and P6 are membrane bound. The 44,000-dalton protein which represents the native product of the P6 structural gene partitioned in a manner similar to that of P6 (data not shown), confirming their relatedness and integral membrane location. The yield of P1 after detergent extraction was too small to be detectable in this figure, but prolonged exposure of the autoradiograph indicated that P1 is also amphiphilic and enriched in the detergent phase (data not shown).

DISCUSSION

The 10.6-kb *Eco*RI fragment from the central region of the *E. coli* enterobactin gene cluster was cloned into plasmid pBR328 to produce the recombinant plasmid pITS24, equivalent to the clone pCP111, described by Pierce and Earhart (40). The chromosomal fragment in pITS24 extends from within the biosynthetic gene *entF* to an uncharacterized gene left of *entE* (Fig. 1). This plasmid encodes all known functions specific for ferric enterobactin transport other than those provided by *fepA*, the gene encoding the outer membrane receptor.

Gel electrophoresis analysis of plasmid-specified proteins expressed in minicells indicated that seven polypeptides were specific to pITS24. Identification of the products originating from a series of subclones, insertions, and deletions derived from pITS24 localized the genes corresponding to each protein on the DNA map (Fig. 1). The *fepB* gene, which is transcribed from right to left, encodes a family of polypeptides ranging in apparent molecular weights from 34,000 to 37,000. The coding region extends from inside the rightmost *Hin*dIII site in pITS26 to the region between the mutations defined by the Tn*1000* insertions in pITS24-T20 and pITS24-T7 (Fig. 2). A smaller protein designated protein X (which corresponds to the present polypeptide P6) is encoded by the region between *fepB* and *entE* (40), and a role for protein X in membrane transport of enterobactin was proposed. Analysis of proteins expressed from pITS47, however, revealed that protein X (P6) is a truncated form of a larger 44,000-dalton protein that represents the true gene product. Its structural gene is therefore transcribed from left to right and extends across the *Eco*RI site that defines the right end of pITS24 and pCP111. The close proximity of this structural gene to *fepB* and their opposing transcriptional orientations provide an opportunity for coordinated expression of both cistrons from a single genetic locus, as has been described for the opposing *fepA* and *fes-entF* transcripts on the left end of the enterobactin gene cluster (G. S. Pettis and M. A. McIntosh, submitted). Although this 44,000-dalton protein has not yet been functionally characterized, the observations that it is localized in the membrane fraction and that a preliminarily identified Tn*5* insertion mutation in this genetic region eliminates ferric enterobactin transport suggest that this protein plays a role in the active transport system for this catechol siderophore and therefore represents another *fep* gene product.

A protein (P7) of 31,500 apparent molecular weight represents the previously described FepC gene product (40). This protein was absent in minicells containing the small deletion derivative pITS24.13 (Fig. 2), localizing *fepC* to a position approximately 2 kb to the right of the 3′ end of *entF*. In the region between these two cistrons, a single insertion mutation, pITS24-M9, was isolated which, when recombined into the chromosome, led to a defect in siderophore transport. A previously undescribed polypeptide of 43,000 approximate molecular weight is weakly detectable but consistently observed in our gel system; this protein is absent in pITS26 and is eliminated by the insertion mutation pITS24-M9, which disrupts enterobactin uptake, providing strong evidence that the structural gene for P1 (*fepE*) encodes a component of enterobactin permease. The difficulty in detecting membrane-bound transport proteins is common to a number of other characterized systems, including those for the ferric hydroxamate uptake system (17) and vitamin B_{12}-uptake (15), and results from their presence in low quantity in most cells and the general insolubility of membrane-bound proteins.

These problems may also be relevant to the inability to observe a *fepD* product. No polypeptides were seen to originate from the region bounded by *fepB* and *fepC*, a span of approximately 2.5 kb. A strain (MK39) carrying an insertion mutation in this region which eliminated a critical transport function was isolated and was unable to be genetically complemented by insertion derivatives pITS24-T7 and pITS24-T20. The mutant strain could be complemented by pITS24-T25. This evidence allowed the *fepD* gene to be mapped precisely within the *fep* cluster and suggested that it is cotranscribed with *fepB* and extends to a point between the mutations defined by pITS24-M39 and pITS24-T25. Nucleotide sequencing data (B. A. Ozenberger, unpublished) corroborated these results, and no strong transcription termination structure was observed to follow the *fepB* coding region. It should be pointed out that the transport lesion in strain AN102 was previously mapped to the *fepC* gene (40). Complementation data obtained in this study with the *fepC* deletion plasmid pITS24.40 and the insertion derivatives pITS24-T7, pITS24-T20, and pITS24-M39, however, do not support that conclusion. The reasons for the discrepancies in genetic definition of this transport lesion are not presently clear.

Osmotic-shock-sensitive permease systems, including those for histidine uptake (27), high-affinity phosphate transport (1, 44), and maltose and vitamin B_{12} uptake (14–16, 21), commonly include an inner membrane permease system consisting of three distinct proteins. This striking uniformity may also include the enterobactin system, with FepC, FepD, and FepE constituting a cytoplasmic membrane permease specific for ferric enterobactin in a manner analogous to that of other high-affinity uptake complexes. The interactions among these proteins and with the periplasmic protein, FepB, as well as the possible involvement of another Fep protein (the 44,000-dalton polypeptide), await further investigation.

The multiplicity of *fepB* products observed in this study and by Pierce and Earhart (40) is an intriguing phenomenon. The slowest migrating form is clearly the unprocessed polypeptide with an attached signal peptide, as shown by its propensity to partition with the membrane and by in vitro translation studies in which only the single product was found (40). Recent DNA sequence analysis confirmed the presence of a 26-amino-acid hydrophobic signal peptide at the amino terminus of FepB (M. Elkins and C. F. Earhart, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, K93, p. 218). The three remaining polypeptides are all released by chloroform or osmotic shock and could each represent various conformations of FepB, as has been observed in other periplasmic transport proteins (2).

Almost all of the DNA extending from the *entD* gene clockwise for over 20 kb to the *entAC* gene on the circular *E. coli* chromosome has now been genetically characterized. The genetic organization of this region is as follows: *entD fepA fes entF fepE fepC fepD fepB fepF entE entB entG entAC* (Fig. 1). The inclusion of *fepF* is based upon the identification of a 44,000-dalton membrane-associated protein encoded by this region and the preliminary characterization of a single insertion mutation between *fepB* and *entE* that disrupts ferric enterobactin transport. The transport functions beyond the outer membrane receptor are clustered together on at least three transcripts in the center of the region. The structural organization of the ferric enterobactin permease complex appears to reiterate the uniformity of bacterial high-affinity transport systems. *fepB* encodes the periplasmic transport protein, and the *fepC*, *fepD*, and *fepE* (and perhaps *fepF*) gene products are proposed to form the cytoplasmic membrane permease. With our increased understanding of the genetic organization of ferric enterobactin transport, structural and functional interactions of the various products can now be more easily explored.

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LITERATURE CITED

1. **Amemura, M., K. Makino, H. Shinagawa, A. Kobayashi, and A. Nakata.** 1985. Nucleotide sequence of the genes involved in phosphate transport and regulation of the phosphate regulon in *Escherichia coli*. J. Mol. Biol. **184**:241–250.
2. **Ames, G. F.** 1986. Bacterial periplasmic transport systems: structure, mechanism, and evolution. Annu. Rev. Biochem. **55**:397–425.
3. **Ames, G. F., C. Prody, and S. Kustu.** 1984. Simple, rapid, and quantitative release of periplasmic proteins by chloroform. J. Bacteriol. **160**:1181–1183.
4. **Bachmann, B. J.** 1983. Linkage map of *Escherichia coli* K-12, edition 7. Microbiol. Rev. **47**:180–230.
5. **Bagg, A., and J. B. Neilands.** 1985. Mapping of a mutation affecting regulation of iron uptake systems in *Escherichia coli* K-12. J. Bacteriol. **161**:450–453.
6. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
7. **Bochner, B. R., H. Huang, G. L. Schieven, and B. N. Ames.** 1980. Positive selection for loss of tetracycline resistance. J. Bacteriol. **143**:926–933.
8. **Bordier, C.** 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. **256**:1604–1607.
9. **Braun, V.** 1985. The unusual features of the iron transport systems of *Escherichia coli*. Trends Biochem. Sci. **10**:75–78.
10. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: in vivo probe for transcriptional control sequences. Proc. Natl. Acad. Sci. USA **76**:4530–4533.
11. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69**:2110–2114.

12. **Cox, G. B., F. Gibson, R. K. J. Luke, N. A. Newton, I. G. O'Brien, and H. Rosenberg.** 1970. Mutations affecting iron transport in *Escherichia coli*. J. Bacteriol. **104:**219–226.
13. **Csonka, L. N., and A. J. Clark.** 1979. Deletions generated by the Tn*10* in the *srl recA* region of the *Escherichia coli* K-12 chromosome. Genetics **93:**321–343.
14. **Dassa, E., and M. Hofnung.** 1985. Sequence of gene *malG* in *E. coli* K-12: homologies between integral membrane components from binding protein-dependent transport systems. EMBO J. **4:**2287–2293.
15. **DeVeaux, L. C., D. S. Clevenson, C. Bradbeer, and R. J. Kadner.** 1986. Identification of the BtuCED polypeptides and evidence for their role in vitamin B_{12} transport in *Escherichia coli*. J. Bacteriol. **167:**920–927.
16. **DeVeaux, L. C., and R. J. Kadner.** 1985. Transport of vitamin B_{12} in *Escherichia coli*: cloning of the *btuCD* region. J. Bacteriol. **162:**888–896.
17. **Fecker, L., and V. Braun.** 1983. Cloning and expression of the *fhu* genes involved in iron(III)-hydroxamate uptake by *Escherichia coli*. J. Bacteriol. **156:**1301–1314.
18. **Fleming, T. P., M. S. Nahlik, and M. A. McIntosh.** 1983. Regulation of enterobactin iron transport in *Escherichia coli*: characterization of *ent*::Mu *d*(Ap^r *lac*) operon fusions. J. Bacteriol. **156:**1171–1177.
19. **Fleming, T. P., M. S. Nahlik, J. B. Neilands, and M. A. McIntosh.** 1985. Physical and genetic characterization of cloned enterobactin genomic sequences from *Escherichia coli* K-12. Gene **34:**47–54.
20. **Frazer, A. C., and R. Curtiss III.** 1975. Production, properties and utility of bacterial minicells. Curr. Top. Microbiol. Immunol. **69:**1–84.
21. **Froshauer, S., and J. Beckwith.** 1984. The nucleotide sequence of the gene for *malF* protein, an inner membrane component of the maltose transport system of *Escherichia coli*. J. Biol. Chem. **259:**10896–10903.
22. **Guyer, M. S.** 1978. The γδ sequence of F is an insertion sequence. J. Mol. Biol. **126:**347–369.
23. **Guyer, M. S.** 1983. Uses of the transposon γδ in the analysis of cloned genes. Methods Enzymol. **101:**362–369.
24. **Hancock, R. E. W., K. Hantke, and V. Braun.** 1976. Iron transport in *Escherichia coli* K-12: involvement of the colicin B receptor and of a citrate-inducible protein. J. Bacteriol. **127:** 1370–1375.
25. **Hantke, K.** 1984. Cloning of the repressor protein gene of iron-regulated systems in *Escherichia coli* K-12. Mol. Gen. Genet. **197:**337–341.
26. **Hantke, K., and L. Zimmermann.** 1981. The importance of the *exbB* gene for vitamin B_{12} and ferric iron transport. FEMS Microbiol. Lett. **12:**31–35.
27. **Higgins, C. F., P. D. Haag, K. Nikaido, F. Ardeshir, G. Garcia, and G. F. Ames.** 1982. Complete nucleotide sequence and identification of membrane components of the histidine transport operon of *S. typhimurium*. Nature (London) **298:**723–727.
28. **Hobson, A. C., R. Weatherwax, and G. F. Ames.** 1984. ATP-binding sites in the membrane components of histidine permease, a periplasmic transport system. Proc. Natl. Acad. Sci. USA **81:**7333–7337.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
30. **Laird, A. J., D. W. Ribbons, G. C. Woodrow, and I. G. Young.** 1980. Bacteriophage Mu-mediated gene transposition and in vitro cloning of the enterochelin gene cluster of *Escherichia coli*. Gene **11:**347–357.
31. **Langman, L., I. G. Young, G. E. Frost, H. Rosenberg, and F. Gibson.** 1972. Enterochelin system of iron transport in *Escherichia coli*: mutations affecting ferric-enterochelin esterase. J. Bacteriol. **112:**1142–1149.
32. **Luke, R. K. J., and F. Gibson.** 1971. Location of three genes concerned with the conversion of 2,3-dihydroxybenzoate into enterochelin in *Escherichia coli* K-12. J. Bacteriol. **107:**557–562.
33. **Lundrigan, M. D., and R. J. Kadner.** 1986. Nucleotide sequence of the gene for the ferrienterochelin receptor FepA in *Escherichia coli*. J. Biol. Chem. **261:**10797–10801.
34. **McIntosh, M. A., S. S. Chenault, and C. F. Earhart.** 1979. Genetic and physiological studies on the relationship between colicin B resistance and ferrienterochelin uptake in *Escherichia coli* K-12. J. Bacteriol. **137:**653–657.
35. **McIntosh, M. A., and C. F. Earhart.** 1977. Coordinate regulation by iron of the synthesis of phenolate compounds and three outer membrane proteins in *Escherichia coli*. J. Bacteriol. **131:**331–339.
36. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Monk, M., and J. Kinross.** 1972. Conditional lethality of *recA* and *recB* derivatives of a strain of *Escherichia coli* K-12 with a temperature-sensitive DNA polymerase I. J. Bacteriol. **109:**971–978.
38. **Neilands, J. B.** 1982. Microbial envelope proteins related to iron. Annu. Rev. Microbiol. **36:**285–309.
39. **Neu, H. C., and L. A. Heppel.** 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. **240:**3685–3692.
40. **Pierce, J. R., and C. F. Earhart.** 1986. *Escherichia coli* K-12 envelope proteins specifically required for ferrienterobactin uptake. J. Bacteriol. **166:**930–936.
41. **Pugsley, A. P., and P. Reeves.** 1976. Characterization of group B colicin B-resistant mutants of *Escherichia coli* K-12: colicin resistance and the role of enterochelin. J. Bacteriol. **127:**218–228.
42. **Reynolds, P. R., G. P. Mottur, and C. Bradbeer.** 1980. Transport of vitamin B_{12} in *Escherichia coli*. J. Biol. Chem. **255:**4313–4319.
43. **Soberon, X., L. Covarrubias, and F. Bolivar.** 1980. Construction and characterization of new cloning vehicles. IV. Deletion derivatives of pBR322 and pBR325. Gene **9:**287–305.
44. **Surin, B. P., H. Rosenberg, and G. B. Cox.** 1985. Phosphate-specific transport system of *Escherichia coli*: nucleotide sequence and gene-polypeptide relationships. J. Bacteriol. **161:**189–198.
45. **Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner.** 1984. New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene **32:**369–379.
46. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42:**45–66.
47. **Wookey, P.** 1982. The *tonB* gene product in *Escherichia coli*. FEBS Lett. **139:**145–153.