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Genetics of the Iron Dicitrate Transport System of *Escherichia coli*

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***Escherichia coli* B and K-12 express a citrate-dependent iron(III) transport system for which three structural genes and their arrangement and products have been determined. The *fecA* gene of *E. coli* B consists of 2,322 nucleotides and encodes a polypeptide containing a signal sequence of 33 amino acids. The cleavage site was determined by amino acid sequence analysis of the unprocessed protein and the mature protein. For the processed form a length of 741 amino acids was calculated. The mature FecA protein in the outer membrane contains at the N terminus the "TonB box," a pentapeptide, which has hitherto been found in all receptors and colicins which functionally require the TonB protein. In addition, the dyad repeat sequence GAAAA TAATTCTTATTTCG is proposed to serve as the binding site of the Fur iron repressor protein. The *fecB* gene was mapped downstream of *fecA* and encodes a protein with an apparent molecular weight of 30,000. It was synthesized as a precursor, and the mature form was found in the periplasm. The *fecD* gene follows *fecB* and was related to a membrane-bound protein with an apparent molecular weight of 28,000. In Mu dl insertion mutants upstream of *fecA*, the *fec* genes were not inducible by iron limitation and citrate, indicating a regulatory region, termed *fecI*, which controls *fec* gene expression.**

Five iron(III) transport systems have been identified in *Escherichia coli* (2, 3). The systems use strong iron-complexing compounds of low molecular weight called siderophores. Each system requires an outer membrane receptor protein and additional genetically defined functions which have been tentatively assigned to the cytoplasmic membrane (2, 28, 36). For all iron(III) transport systems, the *tonB* gene product and, to a lesser and variable extent, the *exbB* gene product are additionally required (2, 10, 20). The regulation of all iron transport systems depends on the iron supply mediated by the Fur repressor protein. All genes are derepressed under iron-limiting growth conditions, and the transport activities are increased (18, 19).

The *fec* genes, located at 7 min on the *E. coli* K-12 chromosome between *proAB* and *lac*, encode the iron(III) dicitrate transport system (51). The *fecA* gene encodes the outer membrane receptor protein. Mutations in genes outside *fecA* have been termed *fecB* (49). Transduction experiments with phage P1 revealed that both loci are closely linked (25, 53), although only *fecA* mutations were cotransducible with *argF* (51, 53). The region between *proAB* and *lac* is difficult to study genetically. It contains an accumulation of insertion sequence elements which, by inversions and deletions, can cause changes in the order of and distances between genes (16). The *argF* gene and the citrate-dependent iron transport system are lacking in *E. coli* W and *Salmonella typhimurium* (13).

The peculiarity of the iron dicitrate transport system in comparison with the other iron(III) transport systems is the existence of two regulatory controls: regulation by the iron level mediated by the Fur protein and induction by citrate plus iron (25, 53). A minimal concentration of 0.1 mM iron(III) dicitrate in the medium induces the system. Because the amount of iron and citrate in the cytoplasm can be 10 to 100 times higher without inducing the system, an exogenous induction has been postulated. For induction, iron transport via citrate is not required. Fluorocitrate and phosphocitrate are strong inducers, but they transport iron very poorly (25, 53). Transport-deficient *fecB* mutants retained inducibility (53). Mutations which prevent expression of the FecA receptor protein also prevent the induction. Moreover, for induction the TonB and the ExbB protein have to be active (53). Citrate does not serve as a carbon or energy source in *E. coli*, and it is not transported under aerobic growth conditions (17, 40). We propose that the iron(III) dicitrate complex must enter the periplasm where it binds to a transmembrane protein, which regulates directly, or via a further inductor, transcription of the *fec* genes.

This paper communicates cloning of genes responsible for the iron dicitrate transport, their order and products, and the locations of the gene products in the cell. The *fecB* region can now be subdivided into at least two genes. Furthermore, the nucleotide sequence of the *fecA* gene of *E. coli* B and the corresponding amino acid sequence were determined.

MATERIALS AND METHODS

Bacterial strains and media. The *E. coli* B and K-12 strains used are listed in Table 1. Tryptone-yeast extract (TY) medium and nutrient broth (NB) medium were used as described previously (10). Ampicillin (25 μg/ml), chloramphenicol (40 μg/ml), and streptomycin (200 μg/ml) were added to cultures of cells that contained the respective resistance markers. Growth on ferric dicitrate was tested on Fec agar plates (containing nutrient agar, 0.2 mM 2,2'-dipyridyl, and 1 mM citrate). Dipyridyl and citrate bind the iron in the medium and prevent growth of *fec* mutants.

Standard procedures. Isolation of outer membranes and gel electrophoresis were performed as previously published (18, 31). Preparation of periplasmic proteins followed the method of Ames et al. (1).

Recombinant DNA techniques. Isolation of chromosomal DNA and plasmids, use of restriction enzymes, ligation, and agarose gel electrophoresis were as described by Maniatis et al. (33). For cosmid cloning, chromosomal DNA of *E. coli* K-12 and B was digested with the endonuclease *Sau*3A and size fractionated by centrifugation through a sodium chloride gradient. DNA of the 40-kilobase fraction was ligated with the same molar amount of cosmid pHC79 cleaved with *Bam*HI (6). In vitro packaging (7, 24) was performed with a

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TABLE 1. *E. coli* strains used

Strain	Genotype[a]	Source or reference
E. coli K-12		
H1443	*aroB araD lac thi rpsL*	21
ZI367	*aroB malT tsx thi lac*::Tn*10*	53
H799	As H1443, but *fecB*::Mu d1	53
ZI418	As H799, but *fecB*::Mu d1X[b]	This study
ZI314	As H1443, but *fecB zag*::Tn*10*	This study
ZI640	As H1443, but *fecA zag*::Tn*10*	This study
ZI331	As ZI367, but *fecI*::Mu d1	53
ZI332	As ZI367, but *fecI*::Mu d1	53
ZI333	As ZI367, but *fecI*::Mu d1	53
ZI335	As ZI367, but *fecI*::Mu d1	53
ZI337	As ZI367, but *fecI*::Mu d1	53
ZI334	As ZI367, but *fecA*::Mu d1	53
ZI336	As ZI367, but *fecA*::Mu d1	53
ZI338	As ZI367, but *fecA*::Mu d1	53
ZI379	As ZI367, but *fecA*::Mu d1	53
ZI381	As ZI367, but *fecA*::Mu d1	53
CR63	F^+	This institute
DS410	*minA minB rpsL lacY xyl mtl thi*	9
ZI813	As H1443, but *fepA*	This study
ZI814	As ZI640, but *fepA*	This study
ZI815	As ZI814, but containing pST18	This study
ZI816	As ZI814, but containing pLZ30	This study
ZI817	As ZI814, but containing pST25	This study
E. coli B	Wild type	This institute
ZI801	As *E. coli* B, but *aroB*	This study
ZI812	As ZI801, but *fepA*	This study
BL21 (DE3)	F^- *hsdS gal*	47

[a] Mutants which expressed the FecA protein but were unable to grow on iron(III) dicitrate as the sole iron source were termed *fecB*.

[b] In Mu d1X mutants the killing functions of the prophage are eliminated (4).

kit from Amersham-Buchler (Braunschweig, Federal Republic of Germany). For insertion mutagenesis with the transposon Tn*1000* (15), the F^+ strain CR63 was transformed with plasmid pLZ30. Cointegrates between the plasmids F and pLZ30 were transferred into the *fecB* mutant ZI418 by conjugation. Streptomycin- and chloramphenicol-resistant colonies were selected on TY plates. Tn*1000* insertions in the *fec* genes of pLZ30 were tested on Fec agar plates. Plasmids from conjugates that could not grow on the Fec screening plates were isolated and cleaved with the enzymes *Bam*HI, *Bgl*I, *Cla*I, *Eco*RI, *Hin*dIII, and *Sal*I to determine the insertion sites and the orientation of Tn*1000*.

For Southern DNA-DNA hybridization, restriction enzyme digests of plasmids and chromosomal DNA were separated on agarose gels overnight with Tris-acetate buffer (33). After transfer of the DNA fragments onto GeneScreen Plus (New England Nuclear Chemicals, Dreieich, Federal Republic of Germany), the ^{35}S-labeled probe was hybridized to bound DNA in the presence of formamide as recommended by the manufacturer. Labeling of DNA fragments with [α-^{35}S]dATP (Amersham) was performed as described by Feinberg and Vogelstein (11).

Expression and localization of plasmid-encoded proteins. Minicells were isolated from a 300-ml overnight culture of *E. coli* DS410, transformed with one of the constructed plasmids, in TY medium supplemented with 0.1% glucose and chloramphenicol or ampicillin. They were purified by three cycles of sucrose gradient (10 to 30%) centrifugation. A sample (0.5 ml) adjusted to 10^{10} cells per ml was incubated for 1 h at 37°C with 740 kBq of [^{35}S]methionine in 50 μl of methionine assay medium (Difco Laboratories, Detroit, Mich.). After 1 h cells were centrifuged, suspended in 0.05 ml of sample buffer, heated for 5 min at 95°C, and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (31). Proteins were stained with Coomassie blue before they were subjected to autoradiography. Isolation of the total membrane fraction of minicells was performed as previously published (10).

Sequencing. DNA fragments from plasmid pST18, carrying parts of the *fecA* gene, were prepared by digestion with *Bam*HI, *Bgl*II, *Pst*I, *Bgl*I, or *Pvu*II and cloned either directly or after treatment with *Sau*3A, *Hpa*II, or *Taq*I into phages M13 mp18 and mp19 (34, 52). Nucleotide sequences were determined by the dideoxy method (42) with [α-^{35}S]dATP (M13 sequencing kit purchased from Amersham). For the sequencing of G+C-rich regions showing band compression, dGTP was replaced by 7-deaza-dGTP (Boehringer, Mannheim, Federal Republic of Germany). The DNA fragments were separated with the Macrophor-Electrophoresis-System 2010 of LKB (Graefelfing, Federal Republic of Germany).

Protein sequencing. The 6.8-kilobase (kb) *Bam*HI-*Hin*dIII fragment of pST18, containing the *fec* region, was cloned into plasmid pT7-5 (48), leading to plasmid pST28. A 250-ml culture of strain BL21(DE3) (47) containing plasmid pST28 was shaken at 37°C to an optical density of 0.5. IPTG (isopropyl-β-D-thio-galactopyranoside) was added to a concentration of 0.5 mM, and the culture was shaken for 45 min. Rifampin (5 to 15 μg/ml) was added, and shaking was continued for 2.5 h. Cells were harvested by centrifugation, and outer membranes were isolated. Outer membrane proteins were separated by preparative gel electrophoresis with 8% polyacrylamide gels. Protein bands were visualized with 4 M sodium acetate (23). The FecA and ProFecA bands were excised, and proteins were electroeluted. Eluted proteins were precipitated (50), and about 150 pmol of each protein was sequenced with an Applied Biosystems Protein Sequencer 477A coupled to a PTH Analyzer 120.

RESULTS

Cloning of the *fec* locus of *E. coli* K-12. About 9,000 cosmids were constructed after partial digestion of chromosomal DNA of strain H1443 with endonuclease *Sau*3A. No cosmid could be obtained that complemented *fecA* mutants. Another cosmid gene bank of *E. coli* K-12 from the laboratory of J. Collins (7) showed the same result. Only one cosmid of the latter collection complemented *fecB* mutants. From this cosmid clone a 8-kb *Hin*dIII fragment was subcloned into pACYC184, and the resulting plasmid was designated pLZ30. All *fecB* mutants transformed with pLZ30 grew well on Fec agar plates. From a previously constructed plasmid pool (18), two additional pACYC184 derivatives, pUP40 and pLZ41, were obtained which complemented the *fecB* mutant ZI314.

Characterization of recombinant plasmids. The physical map of the *fec* plasmids pLZ30, pUP40, and pLZ41 is shown in Fig. 1. Since all plasmids complemented the *fecB* mutant ZI314, we concluded that the corresponding gene is located on the 2.3-kb *Pvu*I-*Bgl*I fragment common to all inserts. To localize the *fecB* gene and to identify additional genes on plasmid pLZ30, insertion mutagenesis with the transposon Tn*1000* was performed. Of 15 Tn*1000* insertions which did not complement the *fecB*::Mu d1 mutant ZI418, all were located within a 5.4-kb *Eco*RI-*Ava*I fragment (Fig. 1). Further complementation analyses subdivided the former *fecB* mutants into two groups. One group showed no complemen-

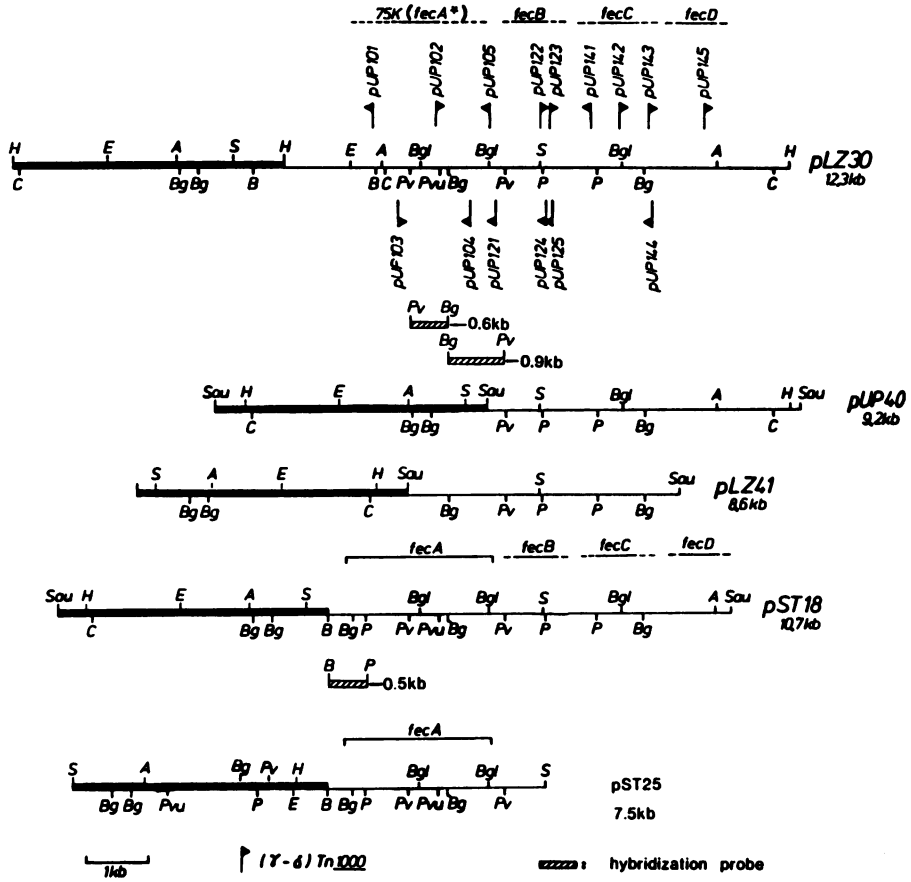

FIG. 1. Physical map and restriction sites of the constructed plasmids. The thick lines represent the vector DNA of pACYC184 (pLZ30, pUP40, pLZ41, pST18) and pBR322 (pST25); the thin lines show the inserted chromosomal DNA fragments. The flags indicate Tn*1000* insertion sites and their orientation: ⚑ means orientation of the transposon from γ to δ. Restriction endonucleases: *Ava*I (A), *Bam*HI (B), *Bgl*I (Bg), *Bgl*II (Bgl), *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), *Pvu*I (Pv), *Pvu*II (Pvu), *Sal*I (S), and *Sau*3A (Sau). Fragments of pLZ30 and pST18 used for hybridization are indicated (▨). For the sake of space, the Tn*1000* insertion sites were drawn above and below the line.

tation with all Tn*1000* plasmids, whereas the second group (ZI314 for example) could grow on Fec agar plates after transformation with the plasmids pUP141, pUP142, pUP143, pUP144, and pUP145 containing Tn*1000* insertions in the right-hand part of pLZ30. This result implies the existence of more genes in the former *fecB* region. Whereas strain ZI314 is mutated in *fecB* and shows only a FecB$^-$ phenotype, Mu dl insertions in *fecB* (for example, in strain ZI418) exert a polar effect on downstream *fec* genes. Plasmid pLZ41, with a deletion to the right, failed to complement strain ZI418, which also indicates an extension of the *fec* locus to the right of *fecB*.

Cloning of the *fec* locus of *E. coli* B. The failure to clone the total *fec* locus of *E. coli* K-12 prompted cloning of the corresponding region of *E. coli* B. *E. coli* B contains an iron dicitrate transport system, but the citrate-dependent induction is lower than that in *E. coli* K-12 (Fig. 2A). Starting with DNA of strain ZI801, a cosmid was obtained that complemented all *fec* mutants. The *fec* region on a *Sau*3A fragment was cloned into pACYC184. Of 24 *fec*$^+$ clones examined, plasmid pST18 contained the smallest insert of 6.6 kb. Expression of the FecA receptor protein by pST18 was determined by comparing gel patterns of outer membrane proteins isolated from *E. coli* B ZI812 (Fig. 2A), *E. coli* K-12 ZI813 (Fig. 2B), the *E. coli* K-12 *fecA* mutant ZI814 before (Fig. 2C) and after (Fig. 2D) transformation with pLZ30, and

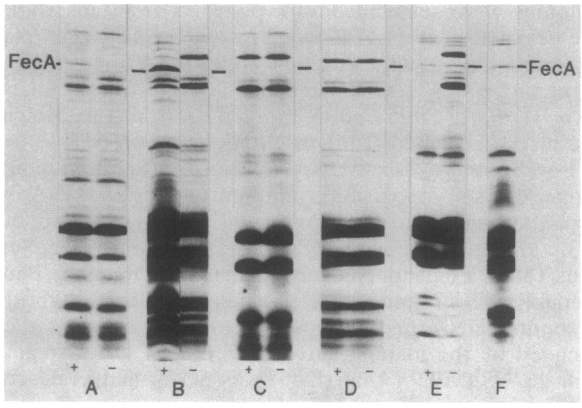

FIG. 2. Protein pattern of outer membranes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Strains: (A) ZI812 (*E. coli* B *aroB fepA*); (B) ZI813 (*E. coli* K-12 *aroB fepA*); (C) ZI814 (*E. coli* K-12 *aroB fepA fecA*); (D) ZI816 (strain ZI814, plasmid pLZ30); (E) ZI815 (strain ZI814, plasmid pST18); (F) ZI817 (strain ZI814, plasmid pST25). For A through E, cells were grown in NB medium (−) or in NB medium supplemented with 1 mM citrate (+). The concentration of citrate in NB medium was not sufficient to induce the *fec* system. Strain ZI817 (F) was grown in TY medium.

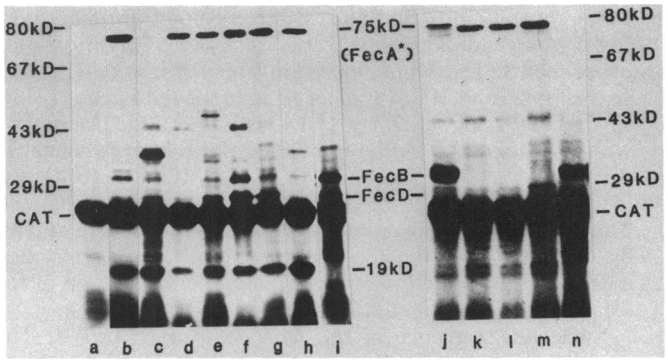

FIG. 3. Autoradiograph of a sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of [^{35}S]methionine-labeled proteins in minicells derived from strain DS410. The minicells contained the following plasmids: (a) pACYC184; (b) pLZ30; (c) pUP102; (d) pUP123; (e) pUP142; (f) pUP143; (g) pUP144; (h) pUP145; (i) pUP40; (j) pUP121; (k) pUP122; (l) pUP124; (m) pUP125; (n) pLZ41. All plasmids are derivatives of pACYC184 and expressed chloramphenicol transacetylase. The positions of the standard proteins human transferrin (80 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa) are indicated. The 75-, the 30-, 28-, and 19-kDa proteins are marked in the middle. Lanes a through i and lanes j to n were from different gels.

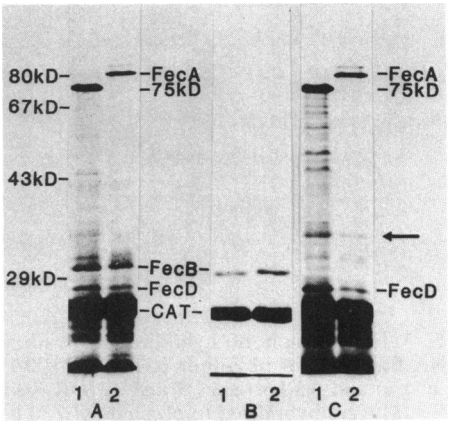

FIG. 4. Autoradiograph of [^{35}S]methionine-labeled proteins in minicells. (A) whole cells, (B) periplasmic proteins, and (C) membrane proteins. The minicells contained plasmid pLZ30 (lanes 1) or pST18 (lanes 2).

the pST18 transformant ZI815 (Fig. 2E). Mutants with deletions in *fepA* were used, since the FepA and FecA proteins have similar electrophoretic mobilities. The *fecA*$^+$ *E. coli* B and K-12 strains and the pST18 transformant, which is chromosomally *fecA*, expressed an outer membrane protein of identical molecular weight (80,500) which was absent in the untransformed *fecA* mutant. The physical map of the plasmid pST18 is shown in Fig. 1. To localize the *fecA* gene, a 3.4-kb *Bam*HI-*Sal*I fragment was subcloned from pST18 into pBR322. The resulting plasmid, pST25 (Fig. 1), encoded the FecA protein (Fig. 2F). In plasmids pST18 and pST25 the expression of FecA was not citrate dependent.

Polypeptides expressed by pLZ30 and its derivatives. The minicell-producing strain DS410 containing plasmid pLZ30 expressed four proteins of 75, 30, 28, and 19 kilodaltons (kDa) (Fig. 3, lane b). The 28-kDa protein was weakly expressed and poorly separated from chloramphenicol transacetylase. Minicells containing the plasmid pUP40 and pLZ41 did not synthesize the 75-kDa protein (Fig. 3, lanes i and n). Therefore, the corresponding gene must be located in the region on the left of the pLZ30 insert (Fig. 1). This conclusion was supported by the lack of the 75-kDa protein in minicells containing the Tn*1000* insertion plasmid pUP102. Instead, a 32-kDa protein band appeared which was considered to be a truncated form of the 75-kDa protein (Fig. 3, lane c). Plasmid pUP40 expressed both the 28- and 30-kDa proteins (Fig. 3, lane i), and plasmid pLZ41 expressed only the 30-kDa protein (lane n). Because both plasmids complemented *fecB* mutants, we concluded that the 30-kDa protein represents FecB. Plasmids pUP122, pUP123, pUP124, and pUP125 contained Tn*1000* insertions in *fecB* (Fig. 1). Minicells programmed with these plasmids showed no FecB protein (Fig. 3, lanes k, d, l, and m). Therefore, the *fecB* gene must be localized in the region flanking the *Sal*I restriction site (Fig. 1). In plasmid pUP121, Tn*1000* is presumably located between *fecB* and the locus encoding the 75-kDa protein. In minicells containing pUP121, the FecB protein was expressed very strongly. Instead of the 75-kDa protein, a protein band with a slightly higher molecular size appeared (Fig. 3, lane j).

Only plasmids pLZ41 and pUP145 failed to synthesize the 28-kDa protein (Fig. 3, lanes n and h). The gene encoding this protein is located around the right-hand *Ava*I restriction site of pLZ30 (Fig. 1) and was designated *fecD*. The expression of *fecB* and *fecD* was not impaired by the Tn*1000* insertions between the two genes in pUP142, pUP143, and pUP144 (Fig. 3, lanes e, f, and g). Because these plasmids showed no complementation of *fecB* and *fecD* mutants, another *fec* gene (*fecC*) could be located between *fecB* and *fecD*. No protein could be identified which was determined by this region. Localization of *fecB* and *fecD* and of the locus for the 75-kDa protein was confirmed by additional plasmids. Plasmid pUP46, bearing the left *Hin*dIII-*Sal*I fragment of pLZ30 (data not shown), expressed only the 75-kDa protein in minicells, whereas in the presence of the right *Sal*I-*Hin*dIII fragment of pUP47 (data not shown) the 28-kD FecD protein appeared. Both plasmids did direct the synthesis of the FecB protein, supporting the localization of *fecB* at the *Sal*I site. The 19-kDa protein was only expressed by pLZ30 and the Tn*1000* insertion plasmids. It is not required to complement *fecB fecD* mutants and therefore is unlikely to be related to the Fec system. As shown below, the 75-kDa protein is a truncated FecA protein.

Plasmids pUP121 (Fig. 3, lane j) and pLZ41 (lane n) gave rise to a 30-kDa FecB double band, indicating the existence of a precursor. Addition of 8% ethanol during the [^{35}S] methionine labeling of the minicells (37) resulted in a stronger upper band, supporting the notion of a precursor form (data not shown).

Subcellular localization of *fec*-determined proteins. Membrane (10) and periplasmic proteins, the latter released after chloroform treatment (1), were isolated from minicells carrying the plasmids pLZ30 and pST18. The FecA, FecB, and FecD proteins were expressed in the presence of pST18 (Fig. 4A, lane 2). The 30-kDa FecB protein was found in the periplasm (Fig. 4B). FecA, the 75-kDa protein, and FecD were in the membrane fraction (Fig. 4C). The presence of FecB protein in the periplasm was studied additionally in vegetative cells. Proteins released by the chloroform shock procedure (1) were examined by sodium dodecyl sulfate-gel electrophoresis (data not shown). Plasmids pLZ30 and pUP40 gave rise to the 30-kDa protein, which was absent in strains with Tn*1000* insertions in the *fecB* gene (pUP122, pUP123, and pUP124). In the membrane fraction an addi-

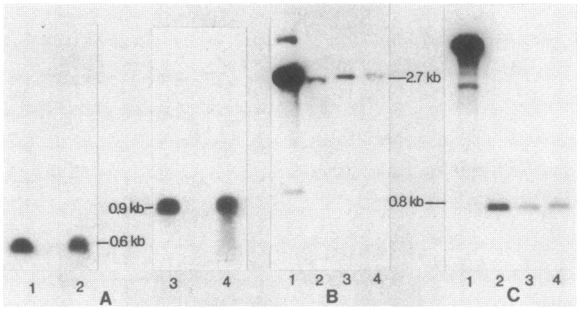

FIG. 5. Autoradiograph of hybridizations with [^{35}S]dATP-labeled DNA fragments of pLZ30 or pST18. (A) ^{35}S-labeled 0.6-kb (lanes 1 and 2) and 0.9-kb (lanes 3 and 4) *Bgl*I-*Pvu*I fragment of pLZ30 (Fig. 1) were hybridized to plasmids pLZ30 (lanes 1 and 3) and pST18 (lanes 2 and 4), digested with *Bgl*I-*Pvu*I, and separated on agarose gels. (B) ^{35}S-labeled 0.6-kb *Bgl*I-*Pvu*I fragment of pLZ30 was hybridized to pST18 (lane 1) and chromosomal DNA of *E. coli* B ZI801 (lane 2), *E. coli* K-12 AB2847 (lane 3), and ZI331 (lane 4), digested with *Pst*I, and separated on agarose gels. (C) ^{35}S-labeled 0.6-kb *Bam*HI-*Pst*I fragment of pST18 was hybridized against pST18 (lane 1) and chromosomal DNA of ZI801 (lane 2), AB2847 (lane 3), and ZI331 (lane 4), digested with *Pst*I, and separated on agarose gels.

tional 35-kDa protein can be seen (Fig. 4C, arrow); we are presently investigating whether it represents the FecC protein.

DNA homology between the *fec* region of *E. coli* K-12 and B. The restriction maps of pLZ30 and pST18 agreed in a region of about 5 kb defined by the left *Pvu*I site and the right *Ava*I site (Fig. 1), including the locus which determined the 75-kDa protein of pLZ30. The smaller molecular size of this protein compared with the 80.5-kDa FecA protein indicated a truncated, inactive form of FecA. Therefore DNA homology between these two loci was studied by hybridization experiments. The 0.6- and 0.9-kb *Bgl*I-*Pvu*I fragments of pLZ30 (Fig. 1) were nick translated with [^{35}S]dATP. The autoradiograph in Fig. 5A shows strong hybridization to the two *Bgl*I-*Pvu*I fragments of pLZ30 and pST18, indicating homology. This implied that the 75-kDa locus was a defective *fecA* gene. Either this gene was a construction artifact already present in the *Sau*3A cosmid bank used, or it is naturally contained in the chromosome of *E. coli* K-12. In the second case the intact *fecA* gene must be localized outside the 8-kb *Hin*dIII insert of pLZ30. Hybridization experiments with *Pst*I-digested chromosomal DNA of *E. coli* K-12 revealed only one fragment homologous to the 0.6-kb *Bgl*I-*Pvu*I segment of *fecA* (Fig. 5B, lane 3). The *Hin*dIII-*Sal*I fragment from the 5′ end of this gene hybridized to a different *Pst*I fragment (data not shown). We therefore concluded that the 75-kDa locus was a cloning artifact. The 0.5-kb *Bam*HI-*Pst*I fragment of pST18 (Fig. 1) hybridized with a 0.8-kb chromosomal *Pst*I fragment of *E. coli* K-12 and B (Fig. 5C, lanes 2 and 3), showing identity in this part of the *fecA* gene of both strains.

Characterization of *fecA*::Mu d1 mutants. Strain ZI331 was originally termed a *fecA*::Mu d1 mutant because it expressed no FecA receptor protein (53). Interestingly, as shown by the results in Fig. 5B and C, lanes 4, phage Mu d1 is inserted not in but upstream of the *fecA* gene. Further hybridization experiments with chromosomal DNA of strain ZI331 cleaved with *Eco*RI plus *Sal*I and *Bgl*II revealed Mu d1 to be inserted close to *fec* (data not shown). This result suggested an additional locus which participates in the expression of the FecA receptor protein. To test this assumption, nine more *fecA*::Mu d1 mutants (53) were examined by hybridization experiments. The Mu d1 insertion sites fell into two groups. In the first group Mu d1 was inserted in the *fecA* gene (ZI334, ZI336, ZI379, ZI338, ZI381), always in the same orientation with the *lacZ* gene on the right side of the Mu prophage. In the second group (ZI332, ZI333, ZI335, ZI337), Mu d1 was inserted to the left of *fecA* within a 4.6-kb *Bgl*II fragment. This region was designated *fecI*. The insertions were at least 0.5 kb apart from the *fecA* gene. The lack of a restriction map of this uncloned region prevented determination of the orientation and the exact localization of Mu d1.

Nucleotide sequence of the *fecA* gene of *E. coli* B. The nucleotide sequence of the *fecA* gene region of *E. coli* B present on plasmid pST18 was determined. The DNA from the *Bam*HI site to the *Sau*3A site 223 base pairs to the right of the second *Bgl*II site was sequenced completely in both directions and across all restriction sites. The nucleotide sequence of the *fecA* region is presented in Fig. 6.

Amino-terminal amino acid sequence of FecA and proFecA. For determination of the amino-terminal amino acid sequences of the unprocessed and mature FecA protein, FecA was overproduced by using the T7 expression system. Plasmid pST28 with the *fec* genes under control of the phage T7 gene 10 promoter was transformed into strain BL21 (DE3), which has the T7 RNA polymerase gene inserted into the chromosome under the control of the *lac*UV5 promoter. In addition, this strain carries the plasmid pLysS with the T7 lysozyme gene cloned into pACYC184 (A. H. Rosenberg, J. J. Dunn, and F. W. Studier, personal communication). T7 lysozyme binds low levels of T7 RNA polymerase expressed before induction and thus increases tolerance for toxic gene products (35).

Overproduction of FecA and the ratio of unprocessed to mature FecA could be regulated by the amount of rifampin added after induction of T7 RNA polymerase with IPTG. With no rifampin added only a slight overproduction was observed (Fig. 7, lane 3), which may be due to a rather ineffective translation initiation at the quite poor ribosomal binding site in front of *fecA*. A 10-fold overproduction resulting in about equal amounts of proFecA and mature FecA was achieved when 5 μg of rifampin per ml was added (Fig. 7, lane 2). When the concentration of rifampin was raised to 15 μg/ml or more, high amounts of proFecA accumulated (Fig. 7, lane 1).

The purified proteins (Fig. 7, lanes 4 and 5) were sequenced by automatic Edman degradation. Seven amino acids from the amino-terminal end of the mature FecA and four amino acids of proFecA were determined. The sequences obtained were identical to those predicted from the nucleotide sequence and confirmed the cleavage site of the signal peptidase between residues 33 and 34.

DISCUSSION

The nucleotide sequence of the *fecA* region of *E. coli* B contains only a single long open reading frame of 2,322 nucleotides, coding for a polypeptide of 774 amino acids with a molecular weight of 85,302. The region preceding the open reading frame shows only partial complementarity to the 3′ end of 16S rRNA (46), for example, GGA (bases 222 through 224). Only weak affinity of the *fecA* mRNA to the ribosome is expected from this rather poor binding site. Further upstream, two regions with a strong homology to the −10 region of the consensus sequence for bacterial promoters (41) were found: TAAAAT and AATAAT, starting with

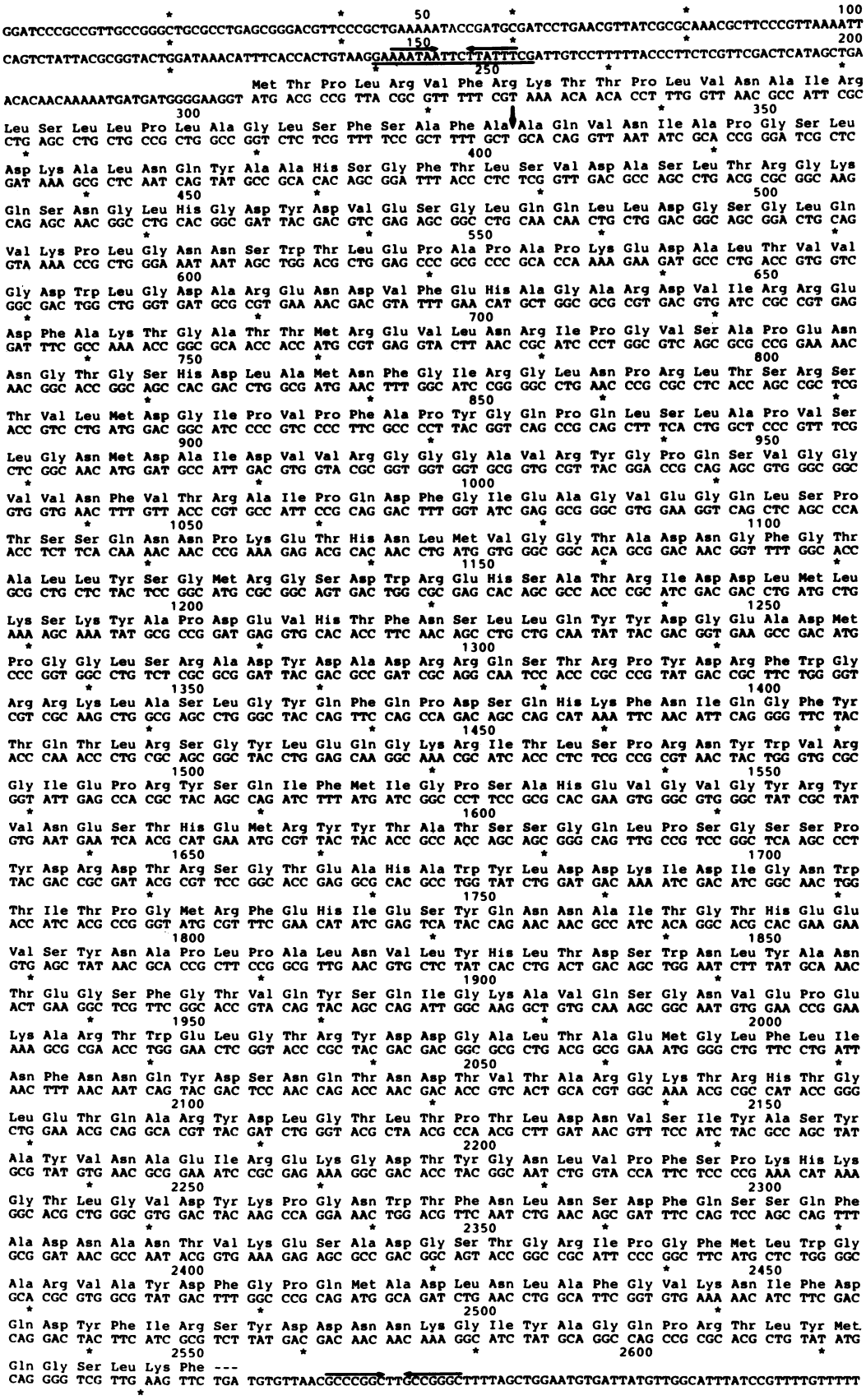

FIG. 6. Nucleotide sequence of the *fecA* gene with the deduced amino acid sequence of the longest open reading frame. The potential binding site of the Fur repressor, including an inverted repeat, is underlined (see Discussion). The inverted repeat around base 2570 marks the potential terminator. Dashes indicate the stop codon. The cleavage site of the leader peptidase is shown by the vertical arrow.

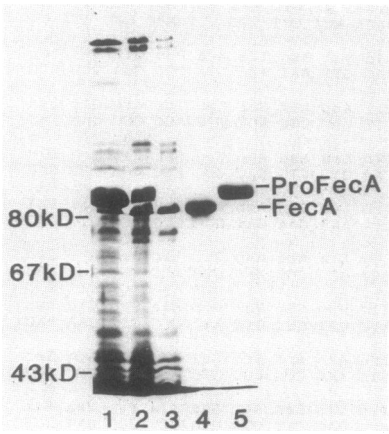

FIG. 7. Overproduction and isolation of FecA. FecA was overproduced by using the T7 expression system as described in Materials and Methods. Lanes 1 through 3 show the effect of different concentrations of rifampin during induction of T7 RNA polymerase on the amount of FecA and proFecA in the outer membrane. Lanes: 1, rifampin at 15 μg/ml; 2, rifampin at 5 μg/ml; 3, no rifampin added; 4, isolated FecA; 5, isolated proFecA.

nucleotides 94 and 146, respectively. Both corresponding −35 regions show only very poor homology to the consensus sequence, which may indicate that an additional regulatory factor is required for transcription initiation of the *fec* genes. Search for a potential binding site of the Fur iron repressor revealed two DNA regions. Based on the originally proposed consensus sequence GATAATGATAATCATTATC (5, 30), the *fec* sequence with 63% identity reads TGTAAGGAAAA-TAATTCTT, starting with base 138. Since the nucleotide sequences of more iron-regulated genes with potential Fur-binding sites have been published, the consensus sequence GAT(AT)ATGAT(AT)AT(CT)ATTTTC is proposed, taking into account all the sequence information presently available for *fhuA*, *fepA*, *fur*, *sltA*, *fhuE*, *tonB*, *cir*, and *fecA* (5, 14, 30, 39, 43, 44). The *fec* sequence closest to the latter starts with nucleotide 144 (Fig. 6) and reads GAAAATAATTCT-TATTTCG. It shows 73% identity to the improved consensus sequence and 57% identity to the original sequence. Both potential Fur-binding sites overlap the second proposed −10 region. In contrast to the first site, the second site includes a 6-base-pair inverted repeat which was previously found in some Fur-binding sites (5, 30). Therefore, we propose the second sequence as the binding site of the Fur repressor. The UGA termination codon is followed by a potential rho-independent terminator consisting of a 7-base-pair inverted repeat ending in a run of T's.

FecA as an outer membrane protein is synthesized as a precursor and processed between the two alanine residues 33 and 34. The amino acid sequence Ala-Phe-Ala-Ala at the signal peptidase cleavage site is exactly the same as that in the FhuE outer membrane receptor protein (43). These rather large outer membrane proteins with molecular weights at 80,000, including the FhuA protein, are synthesized with rather large signal peptides comprising 33, 36, and 33 amino acids. However, for the enterochelin receptor FepA of a similar size, a signal peptide of 22 amino acids has been proposed (32). The signal peptide of FecA is unusual, since it contains an arginine residue within the sequence of hydrophobic amino acids which follows the positively charged amino acids at the amino-terminal end.

The mature FecA protein consists of 741 amino acids with a molecular weight of 81,718. This agrees well with the molecular weight of 80,500 for the FecA protein determined previously (49) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2). Twenty percent of the residues of the mature FecA are charged, resulting in a net charge of −18. The mean hydropathy value, calculated according to Kyte and Doolittle (29), is −0.57. These values are very similar to those found for other outer membrane proteins, especially for the iron and the vitamin B_{12} receptor proteins (32).

Previously, only four regions with similar amino acid sequences were identified in the iron receptors FepA, FhuA, and IutA and the vitamin B_{12} receptor BtuB (32). All four regions are also present in FecA at equivalent positions. The first region is found at the amino terminus (starting with amino acid 23 of the mature protein) and consists of the pentapeptide Phe-Thr-Leu-Ser-Val. Besides the proteins cited above, a similar sequence was found in the receptor for ferric coprogen and rhodotorulic acid and colicins B, M, and I (26, 43, 45). Uptake of the substrates via these receptors and uptake of the three colicins are dependent on the TonB protein. In addition, the *btuB451* mutant (with a Leu-to-Pro exchange in this region) binds, but does not transport, vitamin B_{12} (22). Taken together, these data suggest that this region, which we term the TonB box, is involved in the interaction of the receptor proteins and of the colicins with the TonB protein. The amino acids Thr and Val at the second and fifth position of the pentapeptide are invariant, whereas residues 3 and 4 are less conserved. Surprisingly, the position of Phe in FecA is in all the other receptor and colicin consensus sequences occupied by the acidic amino acid Glu or Asp. This considerable difference does not seem to affect the proper interaction of FecA with TonB. The second region of similarity starts with amino acid 128. It consists of some highly conserved amino acids which are spaced by regions of weaker homology: Pro-Gly-Val-18AA-Ile-Arg-Gly-13AA-Asp-Gly (AA indicates variable amino acids). The third similarity box is found at residue 193: Asp-Val-Val-Arg-Gly-5AA-Tyr-Gly-4AA-Gly-Gly-Val-Val-Asn-Phe-Val-Thr-Arg. The last region shows very strong similarity and is located near the carboxy terminus starting with residue 707: Asn-Ile-Phe-Asp-Gln. Instead of Gln in FecA the other sequences contain a positively charged amino acid, either Lys or Arg. Nothing is known about possible functions of the last three conserved regions. No other substantial homology could be detected.

A part of the chromosomal *fec* region of *E. coli* K-12 was cloned on plasmid pLZ30. By means of Tn*1000* mutagenesis four loci could be identified and localized. Three genes were associated with proteins: a 75-kDa protein (which represents a defective FecA protein), the 30-kDa FecB protein, and the 28-kD FecD protein. FecB was synthesized as a precursor and localized in the periplasm, whereas FecD could be associated with the membrane fraction. Tn*1000* insertions in the fourth locus located between *fecB* and *fecD* caused the loss of complementation of *fecB* and *fecD* mutants, although both proteins were expressed in these cases (Fig. 3, lanes e, f, and g). This indicates the existence of one additional *fec* gene (*fecC*) which is mutated in the *fecB fecD* mutants used for complementation assays.

The location of FecB in the periplasm suggests that the iron dicitrate system falls into the category of the so-called binding-protein-dependent transport systems. They contain soluble proteins in the periplasm which bind the substrates and deliver them to the transport components in the cytoplasmic membrane. The amino acid and sugar transport

systems are composed of three or four proteins (12). By analogy, one can expect the *fec* system, aside from the FecD protein, to have one additional protein in the cytoplasmic membrane which is probably encoded in the region between *fecB* and *fecD*. Identification of such proteins is usually difficult because they are weakly expressed, and they are often very hydrophobic (12). The nucleotide sequence of the corresponding DNA region must permit the identification of these proteins. A periplasmic protein was also found for the iron(III) enterochelin and for the vitamin B12 transport system (8, 38).

Hybridization and sequencing data revealed that the 75-kDa protein encoded by pLZ30 represents an N-terminal shortened FecA protein. Apparently, during cosmid cloning two unrelated *Sau*3A fragments were fused at the left *Pvu*I (*Sau*3A) restriction site. Thus, the 75-kDa protein consists of the C-terminal portion of FecA and a 25-kDa polypeptide of unknown origin.

The pLZ30-derived plasmids pUP101, pUP102, pUP103, pUP104, and pUP105 (Fig. 1), which carry Tn*1000* insertions in the *fecA* fusion gene, could not complement *fecB fecD* mutants. The transposons apparently exhibited polar effects on the *fec* genes located downstream. Sequencing of the *fecA* gene of *E. coli* B showed that *fecB* and *fecD* are located downstream of *fecA*. Therefore we concluded that the most efficient promoter for the genes *fecA*, *fecB*, and *fecD* is located in front of *fecA*. Presumably, citrate induction also takes place there. Expression of the *fecB* gene cloned in both orientations in the *Bam*HI site of PACYC184 (Fig. 1; pUP40 and pLZ41) suggests the existence of an additional promoter upstream of *fecB*.

Attempts to clone the intact *fecA* gene of *E. coli* K-12 were unsuccessful. Restriction maps of pLZ30 and pST18 imply that the arrangement of the genes *fecA*, *fecB*, and *fecD* is the same in both *E. coli* strains. Since *fecB* immediately follows *fecA*, the exclusive cotransduction of *fecA* with *argF* (51, 53) cannot be understood. A Tn*10* insertion (*zag*::Tn*10*) was previously localized by phage P1 transduction studies between *fecA* and *fecB* (53). Since this insertion does not inhibit the expression of *fec* genes, in contrast to the Tn*1000* insertions characterized more precisely in this paper (Fig. 1), the transposon must be located outside of the *fec* region. The higher cotransduction frequency with *fecB* (63%) than with *fecA* (50%) points to a location of *zag*::Tn*10* at the *fecD* side.

The restriction map of the *fec* region (Fig. 1) corresponds neither with the recently published restriction map of the *E. coli* chromosome (27) nor with the previously published map of the region between *proAB* and *purE* (16). Complementation experiments with some of the F plasmids used at that time confirmed that the *fec* genes are absent (53). This indicates that cloning of the *fec* genes is difficult, presumably because of the known accumulation of insertion sequence elements and inversions in this region. Another explanation may be the presence of genes near the *fec* region which are deleterious for the cell.

Hybridization experiments with Mu d1 insertion mutants revealed two *fec* regions which are necessary for expression of the FecA receptor protein, the *fecA* gene itself and a region upstream of *fecA* designated *fecI*. Either the *fecI* locus codes for a regulatory protein of the Fec system or it represents an operater-promoter region for *fecA*, *fecB*, and *fecD*.

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