JOURNAL OF BACTERIOLOGY, Oct. 1993, p. 6212–6219
0021-9193/93/196212-08$02.00/0

Vol. 175, No. 19

Characterization of the Ferrous Iron Uptake System of *Escherichia coli*

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Received 12 April 1993/Accepted 26 July 1993

***Escherichia coli* has an iron(II) transport system (*feo*) which may make an important contribution to the iron supply of the cell under anaerobic conditions. Cloning and sequencing of the iron(II) transport genes revealed an open reading frame (*feoA*) possibly coding for a small protein with 75 amino acids and a membrane protein with 773 amino acids (*feoB*). The upstream region of *feoAB* contained a binding site for the regulatory protein Fur, which acts with iron(II) as a corepressor in all known iron transport systems of *E. coli*. In addition, a Fnr binding site was identified in the promoter region. The FeoB protein had an apparent molecular mass of 70 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was localized in the cytoplasmic membrane. The sequence revealed regions of homology to ATPases, which indicates that ferrous iron uptake may be ATP driven. FeoA or FeoB mutants could be complemented by clones with the *feoA* or *feoB* gene, respectively.**

Since iron(III) is practically insoluble at neutral pH, many aerobic microorganisms secrete siderophores, iron(III) chelating compounds, for their iron supply. Six different siderophore-iron(III) transport systems in *Escherichia coli* have been sequenced and analyzed, and many more in other gram-negative bacteria have been characterized (4). These transport systems share a common structure. A ferric siderophore-specific receptor in the outer membrane delivers its substrate in an energy-dependent mechanism to the periplasm. The energy is provided by the TonB-ExbB-ExbD complex (4). Even heme (33) and transferrin iron (6) are taken up in a TonB-dependent manner. With the help of a binding protein-dependent transport system, the ferric siderophore crosses the cytoplasmic membrane (4).

Much less is known about the uptake of ferrous iron, although there are some microorganisms which mainly or exclusively use ferrous iron for their iron supply. The best-studied examples are *Bifidobacterium bifidum* (3), which is one of the early colonizers of the intestine in breast-fed infants. For the intracellular pathogen *Legionella pneumophila*, no siderophores have been found, and it is assumed that iron(III) reduced by the bacterium to iron(II) is the main source of iron (14). The odontopathogen of human dental caries, *Streptococcus mutans*, has been postulated to use only ferrous iron furnished by reductants at the cell surface (10). In yeast cells, an iron-regulated ferric reductase which is assumed to supply ferrous iron for an uncharacterized iron(II) uptake system has been cloned and sequenced (7). All these organisms live in oxygen-restricted environments where ferrous iron may be available.

A major habitat of the facultative anaerobe *E. coli* is the gut, where it helps to maintain anaerobic conditions. From this point of view, it is not astonishing that *E. coli* also possesses a ferrous iron transport system. Mutants of *E. coli* in the ferrous iron uptake system (*feo*) have been isolated (12). Recently, it was shown that they are severely impeded in their ability to colonize the mouse intestine (31).

Here we present the first characterization of an iron(II) transport system in *E. coli*.

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MATERIALS AND METHODS

Strains and plasmids, constructions, and growth conditions. Strains are listed in Table 1. The media used were TY (8 g of tryptone, 5 g of yeast extract, 5 g of NaCl per liter) and nutrient broth (8 g of nutrient broth and 5 g of NaCl per liter). P1 transductions have been described by Miller (17). β-Galactosidase was determined by the method of Miller (17). One unit was defined as 1 nmol of *ortho*-nitrophenol produced per min per mg (dry weight). Representative values from three experiments are given.

Standard methods for plasmid DNA isolation, restriction endonuclease analyses, and ligations were carried out as described by Sambrook et al. (25) or according to the instructions of the suppliers. Phage and plasmid inserts are shown in Fig. 1. In addition, the vectors pWKS30 (37) and pBSK (Stratagene) were used for cloning or sequencing. Plasmids not shown in Fig. 1 are pUH30, which contained the 2.1-kb *Xho*I-*Sal*I fragment with the end of Tn*5* and *feoA'* from phage lambda 4, and pUH20, in which the 4.2-kb *Pst*I-*Kpn*I fragment of pUH18 was deleted, which resulted in a truncated *feoB'* gene.

A chromosomal *feoB-lacZ* operon fusion was constructed by inserting the 1-kb *Kpn*I-*Eco*RV fragment of *feoB* into pGP704 (18). *lacZ* was moved with *Not*I from pUJ8 (8) into pBSK. With *Bam*HI, the *lacZ*-containing fragment was cloned into the *Bgl*II site behind *'feoB'*. Orientation of *lacZ* was checked by restriction analysis. This plasmid was moved from strain SM10 lambda *pir* into MC4100 under selection for ampicillin and streptomycin resistance, and strain H5107 was obtained. P1 transduction of *feoB*::Tn*5* into H5107 showed removal of the ampicillin resistance and the *lac* marker, indicating that the insertion site was in *feoB* as expected. *fnr-250* was introduced by cotransduction with *zcj-637*::Tn*10* from strain RK5288 (28). H5108 was one of the transductants which proved to be an *fnr* mutant on KNO_3-MacConkey plates (29). Both strains were always kept in the presence of ampicillin to prevent loss of the insert.

Expression and localization of plasmid-encoded proteins. Expression of proteins has been described (34). DNA fragments were cloned into plasmid pT7-5, pT7-6, or pBSK+ and transcribed by the phage T7 RNA polymerase encoded by the plasmid pGP1-2. The expression of the T7 RNA

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

Strain	Genotype	Source
MC4100	*araD139 ΔlacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	12
H1443	As MC4100 but *aroB*	12
H1717	As H1443 but *fhuF*::λ*plac* Mu	12
H1771	As H1717 but *feoB7*	12
H1858	As MC4100 but *fhuF*::MudX	This work
SO67	As H1858 but *feoA*::Tn*5*	This work
SO74	As H1858 but *feoB*::Tn*5*	This work
SO78-SO73	As H1858 but *feoA*::Tn*5*	This work
H5107	As MC4100 but *feoB-lacZ*	This work
H5108	As H5107 but *fnr-250 zcj-637*::Tn*10*	This work
H2331	As H1443 but Δ*fur*	This work
H5101	As H2331 but *feoB*::Tn*5*	This work
H5102	As H2331 but *feoA*::Tn*5*	This work
H1756	As H1443 but *fur-31 fhuF*::λ*plac* Mu *zge-53*::Tn*10*	This work
RK5288	As MC4100 but *fnr-250 zcj-637*::Tn*10*	28
BR158	*aroB tsx malT tonB*	11
H5125	*tsx malT tonB feoA*::Tn*5*	This work
H1526	*tsx malT tonB feoB*::Tn*5*	This work
H5127	*aroB tsx malT tonB feoA*::Tn*5*	This work
H1528	*aroB tsx malT tonB feoB*::Tn*5*	This work
SM10	*thi thr leu fhuA lacY supE recA*::RP4-2Tc::Mu Km λ*pir*RK6	18

polymerase is under the control of the lambda p_L promoter and the gene for the heat-sensitive lambda repressor *c*I857. For localizing FeoB, the procedure as described by Staudenmaier et al. (27) was used, but without radioactive labeling, since FeoB was so strongly expressed that it could be identified by Coomassie blue staining of the gel. Because of the differential centrifugation used to separate outer and inner membranes, the cytoplasmic membrane was contaminated by outer membrane proteins.

Iron uptake experiments. Strains were grown overnight in TY medium under anaerobic conditions and inoculated in nutrient broth medium to about 2×10^8 cells per ml and incubated at 37°C under aerobic conditions and shaking or under anaerobic conditions in a filled 100-ml Erlenmeyer flask without shaking. At about 8×10^8 cells per ml, the culture was harvested, washed once in M9 medium (17) at 4°C, and kept on ice in M9 medium (0.2% glucose, 0.1 mM nitrilotriacetate) at 1×10^9 cells per ml. The transport was started after 5 min for warming up at 37°C by addition of 3 μM ferrous iron labeled with ^{55}Fe. The stock solution contained 300 μM $^{55}FeCl_3$ (12 MBq/μmol) and 100 mM ascorbate to reduce the iron. At appropriate times, samples were drawn, filtered on 0.45-μm-pore-size filters, and washed two times with 2 ml of 0.1 mM LiCl. Incorporated iron was determined by liquid scintillation counting.

Nucleotide sequence accession number. The accession number X71063 ECFEOAB was given to the *feoAB* gene sequence by the EMBL data library at Heidelberg, Germany.

RESULTS

Isolation of *feo*::Tn*5* mutants. Strain H1771 *feoB7* (12) was used to isolate *feo* complementing clones with high- and low-copy-number vectors. However, the rare complementing clones turned out to be highly unstable. For this reason, we tried to clone a transposon-inactivated *feo* gene. *feo*::Tn*5* mutants were isolated by the following procedure. Strain H1858 contained the iron-regulated reporter gene *fhuF*::Mud1X (operon fusion of *lac* to *fhuF*). On MacConkey lactose plates at high iron concentrations (40 μM iron added), this strain gives white colonies because of repression by Fur-Fe^{2+} of the *lac* fusion. When the free iron in the medium was complexed by 50 μM 2,2'-bipyridine, red colonies appeared, showing derepression of the *fhuF-lacZ* fusion. Cotransduction of *zge-53*::Tn*10 feoB7* into strain H1858 led to red colonies at 40 μM iron added. This indicated derepression of the fusion gene because of the missing iron supply caused by the defective ferrous iron transport system. This Feo phenotype was used to screen a pool of H1858 Tn*5* mutants for red colonies on MacConkey lactose medium. However, not only insertions in *feo* but also

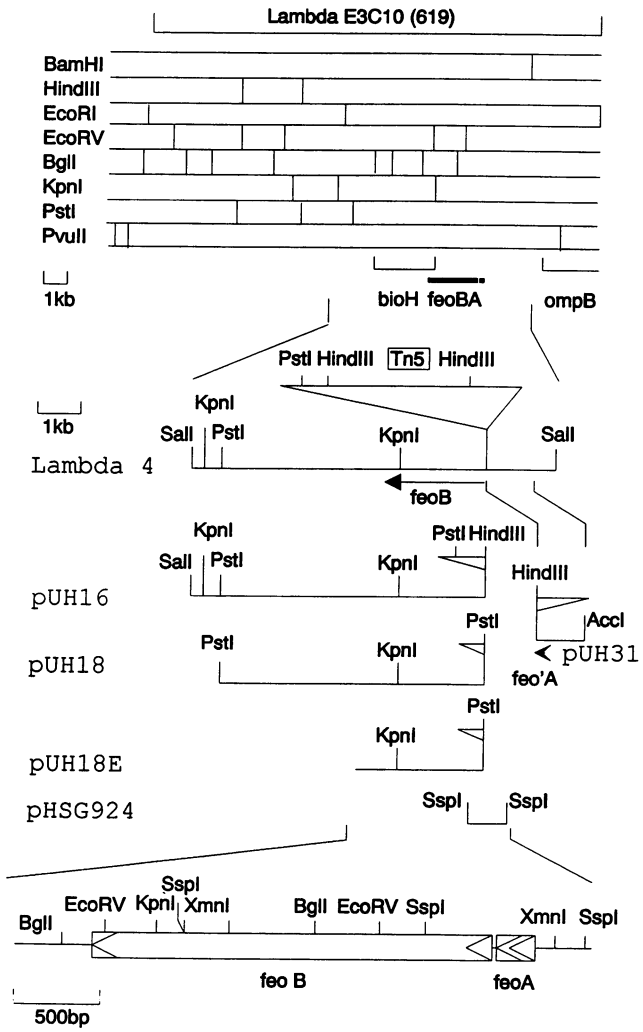

FIG. 1. In the upper part the restriction map of the *E. coli* chromosome covered by phage lambda 619 (16) is shown. *feoAB* is located at 74.9 min between *ompB* and *malA*. The restriction map of the insert of phage lambda 4 and the inserts of the derived plasmids are given. The coding region of *feoB* on phage lambda 4 is indicated by an arrow, and the C-terminally truncated *feoA* is indicated by an arrow under pUH31, which is a subclone derived from phage lambda 4 (*Hin*dIII-*Acc*I fragment). Phage lambda 4 was derived from EMBL3. Vector of pUH16 was pACYC184 (25), vector for pUH18 and pUH18E was pT7-6 and for pUH31 was pT7-5 (34), and vector for pHSG924 was pHSG575 (35).

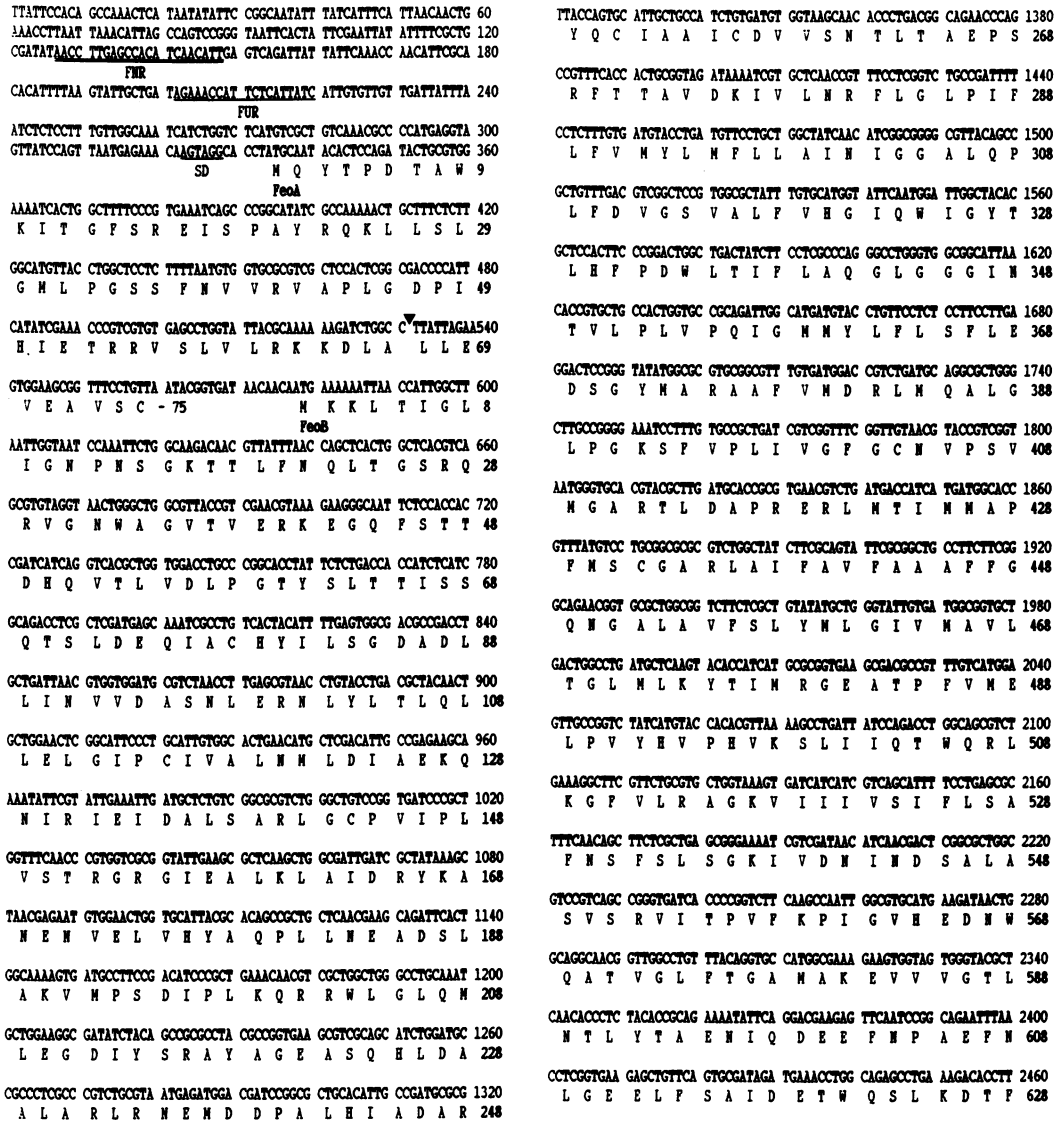

FIG. 2. Nucleotide sequence of the *feo* region and the amino acid sequence of the open reading frame FeoA and the protein FeoB. The arrowhead indicates the position of Tn*5*.

insertions in *tonB* or *fur* could have led to red colonies. For this reason red colonies were pooled, and phage P1 was grown on these cells and used to transduce strain H1756 *zge-53*::Tn*10* to neomycin resistance. *zge-53*::Tn*10* was known to be 90% cotransducible with *feoB7*. The neomycin-resistant transductants were tested for tetracycline resistance, and those which were sensitive were assumed to carry a *feo*::Tn*5* mutation. By retransduction into strain H1858, the mutant phenotype (derepression of *fhuF*) was confirmed. The mutants SO67 *feoA*::Tn*5* and SO74 *feoB*::Tn*5* were used for further studies (for the distinction between *feoA* and *feoB*, see below).

Cloning and sequencing of *feo*. In many attempts we were unable to clone the *feo* genes by complementation in strain H1771. Very few white colonies were found on MacConkey plates after introduction of different *E. coli* gene banks. The clones isolated were unstable. Therefore, the gene region was cloned into a lambda vector. Chromosomal DNA of SO67 *feoA*::Tn*5* was partially digested by *Sau*3A, and fragments of about 20 kb were ligated into the vector lambda EMBL3. The clone lambda 4 containing Tn*5* was identified by hybridization to a Tn*5* DNA probe. DNA from both sides of Tn*5* was cloned into the plasmids pUH31 and pUH16 (Fig. 1). Further subcloning into the vector pBSK and exonuclease III digestion generated an ordered set of deletions which was used to sequence the *feo* gene region (Fig. 2). The open reading frame *feoA* had been interrupted by the inserted Tn*5*. The restriction map of the phage lambda 4 insert indicated that *feo* should be on phage 619 (E3C10) from the Kohara collection (16). Hybridization with the 7.7-kb *Pst*I fragment of pUH18 confirmed the location of *feo* on this phage. This result was in contrast to the previous mapping locating *feo* near 38 min on the genetic map of *E. coli* (12). Another trial to clone the whole *feo* gene region with *Bam*HI-*Eco*RI from the phage 619 into the vector pBSK failed. Also, it was not possible to clone the *Kpn*I-*Bam*HI fragment with a shortened *feoB* gene into a high-copy-

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CAGCCTTAGC GTACTGATGA ACCCCATTGA AGCCAGCAAA GGCGACGGCG AAATGGGTAC 2520
  S  L  S  V  L  M  N  P  I  E  A  S  K  G  D  G  E  M  G  T  648

CGGGGCGATG GGCGTGATGG ATCAGAAATT CGGTAGCGCA GCAGCAGCTT ACAGCTACCT 2580
  G  A  M  G  V  M  D  Q  K  F  G  S  A  A  A  A  Y  S  Y  L  668

GATTTTCGTC CTGCTGTATG TACCATGTAT CTCGGTGATG GGGGCTATCG CCCGTGAATC 2640
  I  F  V  L  L  Y  V  P  C  I  S  V  M  G  A  I  A  R  E  S  688

AAGCCGTGGC TGGATGGGCT TCTCCATCCT GTGGGGGCTG AATATCGCTT ACTCACTGGC 2700
  S  R  G  W  M  G  F  S  I  L  W  G  L  N  I  A  Y  S  L  A  708

AACATTGTTC TATCAGGTCG CCAGCTACAG TCAGCATCCA ACTTACAGCC TGGTGTGCAT 2760
  T  L  F  Y  Q  V  A  S  Y  S  Q  H  P  T  Y  S  L  V  C  I  728

TCTGGCGGTT ATCCTGTTTA ACATCGTGGT TATCGGTCTG CTGCGCCGCG CGCGTAGCCG 2820
  L  A  V  I  L  F  N  I  V  V  I  G  L  L  R  R  A  R  S  R  748

GGTGGATATC GAACTGCTGG CAACCCGCAA GTCGGTAAGC AGTTGCTGCG CAGCCAGCAC 2880
  V  D  I  E  L  L  A  T  R  K  S  V  S  S  C  C  A  A  S  T  768

CACCGGTGAT TGCCATTAAT GGCTTCACTT ATTCAGGTGC GCGATTTGCT GGCGTTACGG 2940
  T  G  D  C  H  -                                              773

GGCCGTATGG AAGCGGCCCA GATAAGCCAG ACATTGAACA CTCCACAGCC AATGATTAAC 3000

GCCATGCTGC AACAACTGGA AAGTATGGGC AAAGCCGTGC GGATTCAGGA AGAACCTGAC 3060

GGCTGCCTCT CTGGCAGTTG TAAAAGCTGC CCGGAAGGAA AAGCCTGTCT GCGCGAGTGG 3120

TGGGCGCTGC GTTAACCTTA CTCCATCGCC TGTTTTGGAA AGCCCGGTAT GCGTCTGCAT 3180

CCGGGCTTTT TTGCGTGCGG CTTTCCATAA AAATGCAACT CTTGCAGCAC GGCGTAAGTT 3240

CCTTTGAAAG CATCTCGCAG GGATGAAAAC TCGCTAATAC ACAGGTGTGG AGTGGCGCGT 3300

AGAGTCGCGG CATTCAAACA ACAGGTGAAG GAACGCCATG AGCAAAAAG              3349
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FIG. 2—*Continued.*

number vector or in the low-copy-number vector pWKS30 (37).

The 924-bp *Ssp*I fragment containing the *feoA* gene region was cloned from phage 619 into plasmid pHSG924 (Fig. 1). The DNA fragment overlapping the Tn*5* insertion site was sequenced and showed complete agreement with the previously determined sequences at the transposon insertion site, indicating that no deletions or rearrangements had occurred.

No obvious promoter region with a −10 and −35 motif was found. However, a site with homology to Fnr binding sites was detected (127 to 148) in that region. Fnr is an activator of anaerobic energy generating systems like fumarate and nitrate reductases. In addition, further downstream a binding site for the regulatory protein Fur (Fur box 202 to 220) was detected.

An open reading frame of 75 amino acids, named FeoA, and a calculated molecular mass of 8,371 Da started at bp 334. *feoB* started at bp 578, 16 bp after the stop codon of *feoA*. *feoB* coded for a protein of 773 amino acids with a calculated molecular mass of 84,473 Da. By the method of Klein et al. (15), FeoB was predicted to be a membrane protein with eight hydrophobic transmembrane helices. Two sequences (1 to 21 and 79 to 100) showed homologies to nucleotide binding sites of ATPases (see Fig. 7). Their distance of 58 amino acid residues was in the range found in other ATPases. It was likely that ferrous iron transport was driven by ATP hydrolysis.

Two possible termination loops are indicated after the stop codon of *feoB*.

A comparison of bp 2515 to 3349 revealed identity to the sequence of *bioH* (bp 1742 to 2576 as deposited at EMBL [23]). This places *feoAB* between *bioH* and the *ompB* operon at 74.9 min on the genetic map of *E. coli*. The reasons for the wrong mapping are not clear. We were unable in some strains to cotransduce *zge-53*::Tn*10* with *aroB*, which points to rearrangements in the chromosome of the mutant studied as a possible reason for this mistake.

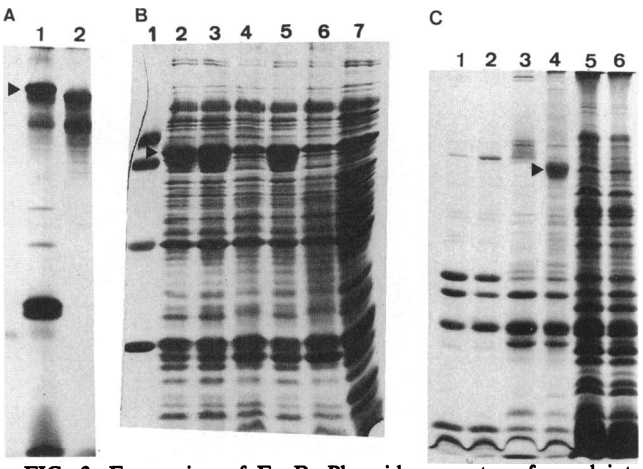

FIG. 3. Expression of FeoB. Plasmids were transformed into strain WM1576, and the proteins encoded were expressed with the help of the T7 RNA polymerase-promoter system (34). (A) Autoradiogram of rifampin-treated cells labeled with [^{35}S]methionine. Proteins of whole cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1, WM1576 (pUH18); lane 2, WM1576 (pUH20) with a truncated *feoB* gene. (B) Proteins of whole cells were separated by SDS-PAGE and stained with Coomassie blue. The position of FeoB at an apparent molecular mass of about 70 kDa is indicated by an arrow. Lane 1, molecular mass standards (phosphorylase *b*, 97 kDa; bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa); lane 2, pUH18 *feoB*; lane 3, pUH18E *feoB*; lane 4, pBSK 18-18 *feoB* mutant; lane 5, pBSK18E (insert from pUH18E, T7 promoter at 5′ end of *feoB*) *feoB*$^+$; lane 6, pBKS E18 (T7 promoter at 3′ end of *feoB*) phenotype FeoB$^+$, but no expression of *feoB* from the T7 promoter; lane 7, pBSK vector. (C) Localization of FeoB in WM1576. Shown are outer membranes of cells with pUH30 (lane 1) and pUH18 (lane 2); cytoplasmic membranes, contaminated with outer membrane proteins, of cells with pUH30 (lane 3) and pUH18 (lane 4); and soluble proteins of cells with pUH30 (lane 5) and pUH18 (lane 6). FeoB (arrow) is visible only in the cytoplasmic membrane fraction (lane 4).

Identification of the gene products. For the expression of the encoded proteins, the system of Tabor and Richardson (34) was used where the genes of interest are cloned behind the gene ϕ10 promoter of phage T7 and efficient transcription is obtained with the T7 RNA polymerase. WM1576(pUH18) was labeled with [^{35}S]methionine, and FeoB was identified as a protein with an apparent molecular mass of about 70 kDa (Fig. 3A). FeoB is highly expressed with the T7 system, and even in whole cells without radioactive labeling the protein can be identified (Fig. 3B). The protein was localized in the cytoplasmic membrane (Fig. 3C).

With the plasmid pUH31 containing the truncated open reading frame of *feoA*, a protein of about 9 kDa was found. However, since we did not observe this protein with the plasmid pHSG924 up to now, further experiments are necessary to see whether the open reading frame is translated into a protein.

Phenotype of *feo* mutants. To test the importance of *feoA* and *feoB* for the growth of cells, the mutations *feoA*::Tn*5* and *feoB*::Tn*5* were introduced into the mutant BR158 *aroB tonB*. This strain is unable to produce its own siderophore enterochelin (enterobactin) because of the *aroB* mutation, and it is unable to transport iron(III) siderophores because of

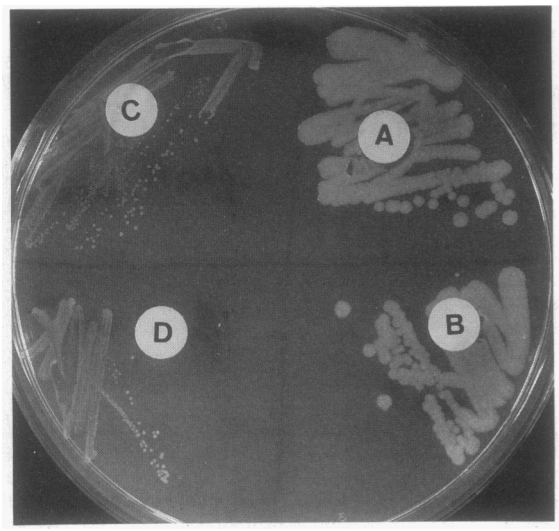

FIG. 4. Growth of H5125 *tonB feoA* (A), H5126 *tonB feoB* (B), H5127 *tonB aroB feoA* (C), and H5128 *tonB aroB feoB* (D) on TY medium plates under aerobic conditions.

Gene	
aerA (iucA)	G A T A A T G A G A A T C A T T A T T
fhuA	C T T T A T A A T A A T C A T T C T C
fepA	A T T A T T G A T A A C T A T T T G C
fhuE	T A C A A A C A A A A T T A T T C G C
fecA	T G T A A G G A A A A T A A T T C T T
tonB	G A A T A T G A T T G C T A T T T G C
fepB	G A A A A T G A G A A G C A T T A T T
fur	T A T A A T G A T A C G C A T T A T C
sodA	G A T A A T C A T T T T C A A T A T C
Consensus FUR	G A T A A T G A T A A T C A T T A T C
feo	A G A A A C C A T T C T C A T T A T C

Gene	
frdA	A A A A A T C G A T C T C G T C A A A T T T
frdA (P.vulgaris)	A A C C A T C A T T C A T A T C A A A T T T
glpA	A A T G A C G C A T G A A A T C A C G T T T
dmsA	C C C T T T G A T A C C G A A C A A T A A T
narG	A C T C T T G A T C G T T A T C A A T T C C
nirB	G A A T T T G A T T T A C A T C A A T A A G
Consensus FNR	A A A * T T G A T * * * * A T C A A * T T T
feo	A A C C T T G A G C C A C A T C A A C A T T

FIG. 5. Sequence comparisons with the Fur box and the Fnr box in front of *feo*. The sources of the Fur box sequences are given by Braun and Hantke (4), and the sources of the Fnr boxes are given by Eiglmeier et al. (9).

the *tonB* mutation. P1 transduction from strains SO67 *feoA*::Tn*5* and SO74 *feoB*::Tn*5* into strain BR158 and selection for neomycin resistance led to many unexpectedly well growing transductants. The transductants were then further characterized, and it was found that *aroB*$^+$ had been cotransduced with *feo*. No *feo aroB* transductants were detected. Cotransduction rate of both *feo* loci with *aroB*$^+$ into strain H1443 was only 13 to 18%. The reason for the good growth of the *aroB*$^+$ *tonB feo* strains was not the secretion of enterochelin into the medium, since it has been shown that iron(III)-enterochelin uptake is TonB dependent. However, a dihydroxybenzoate-promoted TonB-independent uptake of iron has been described (11). This seems to be the main source of iron for the *feo tonB* double mutants.

To construct a *feo tonB aroB* triple mutant, the double mutant *feo aroB* was chosen as a donor for the transduction into BR158. The transductants were streaked for single colonies on nutrient broth. The *tonB feo aroB* mutant formed microcolonies on this medium, while the *tonB feo aroB*$^+$ mutant was able to grow to large colonies. On TY medium, the *tonB feo aroB* strains were able to form colonies, but they were much smaller than the *tonB feo* strain colonies (Fig. 4). There was no major difference in the phenotype between the *feoA*::Tn*5* and the *feoB*::Tn*5* mutants. Growth of *feoB tonB aroB* mutants on TY and nutrient broth could be stimulated by the addition of 10 mM sodium citrate or by high concentrations of iron-loaded 2,3-dihydroxybenzoate.

Regulation of *feo* by iron. Ferrous iron uptake was shown to be repressible by iron. In addition, ferrous iron uptake was derepressed in a *fur* mutant (12). A Fur binding site was found in the upstream region of *feo* by comparison to the consensus sequence (4). Of the 19 bp, 12 were identical to the consensus sequence (Fig. 5). The nonidentical base pairs were also found in other well-established Fur binding sites, except for a C instead of a T in position 6.

The presence of a Fur binding site was also demonstrated in vivo. Strain H1717 with *fhuF*::lambda p*lac* Mu as a reporter gene is repressed by Fur on MacConkey agar with 40 μM iron added. By introducing a Fur binding site on a high-copy-number plasmid, the low level of Fur protein is titrated out, the reporter gene *fhuF* is derepressed (30), and the transformants grow as red colonies on the MacConkey agar plates with iron. Strain H1717 was transformed with the plasmid pBKS31 carrying the insert of pUH31 or with pHSG924. Transformants with the high-copy-number plasmid pBKS31 yielded red colonies, while transformants with the low-copy-number plasmid pHSG924 remained white. This, together with the known sequence, indicated that there is a Fur box on plasmid pBKS31 able to titrate the Fur receptor in strain H1717 (30).

Influence of Fnr on *feo* expression. Fnr is the transcriptional activator of anaerobic respiratory genes. Fe(II) has been shown to influence the activity of Fnr. Severe iron limitation led to a reduced activity of Fnr (21). A homology to Fnr binding sites was found in the upstream region of *feo* (Fig. 2 and 5). However, no canonical −10 region was detected in a distance of 18 to 24 bp as was described for six Fnr-activated genes by Eiglmeier et al. (9).

To gain more insight into the regulation of *feo* by Fnr, an operon fusion *feo-lacZ* was constructed. The 1.3-kb *Kpn*I-*Eco*RV fragment of *feoB* was cloned into pGP704 (18). *lacZ* from plasmid pJU8 (8) was cloned behind the *'feoB'* fragment. pGP704 is not able to replicate in a cell without the presence of the *pir* gene in *trans* and can be used for the generation of insertion mutants (18). The *'feoB'-lacZ*-containing plasmid was crossed into MC4100. Those cells in which the homology of the *'feoB'* fragment led to integration of the pGP704 derivative into the chromosome were selected with ampicillin. Although *lacY* is missing in this strain, low iron-regulated β-galactosidase activity was observed with strain H5107 *feoB-lacZ* on MacConkey agar plates. β-Galactosidase activity was tested from cells grown under anaerobic conditions in TY medium with 1% KNO_3 added. In the presence of iron, 72 U of β-galactosidase was produced in strain H5107 *feoB-lacZ*, while in strain H5108 *feoB-lacZ fnr*, 14 U were found. Inactivation of *fnr* led to a fivefold decrease of β-galactosidase activity in the presence of iron, indicating that Fnr is acting as an activator for *feo*. With 50 μM of dipyridyl, 85 U of β-galactosidase was observed in strain H5108 *feoB-lacZ fnr*, demonstrating a sixfold derepression probably mediated by Fur.

Complementation and iron transport. Plasmids pUH18, pUH31, and pHSG924 were transformed into different *feo* mutants. Strain H1771 *feoB7* and strain SO74 *feoB*::Tn*5* were complemented by pUH18, growing as white colonies

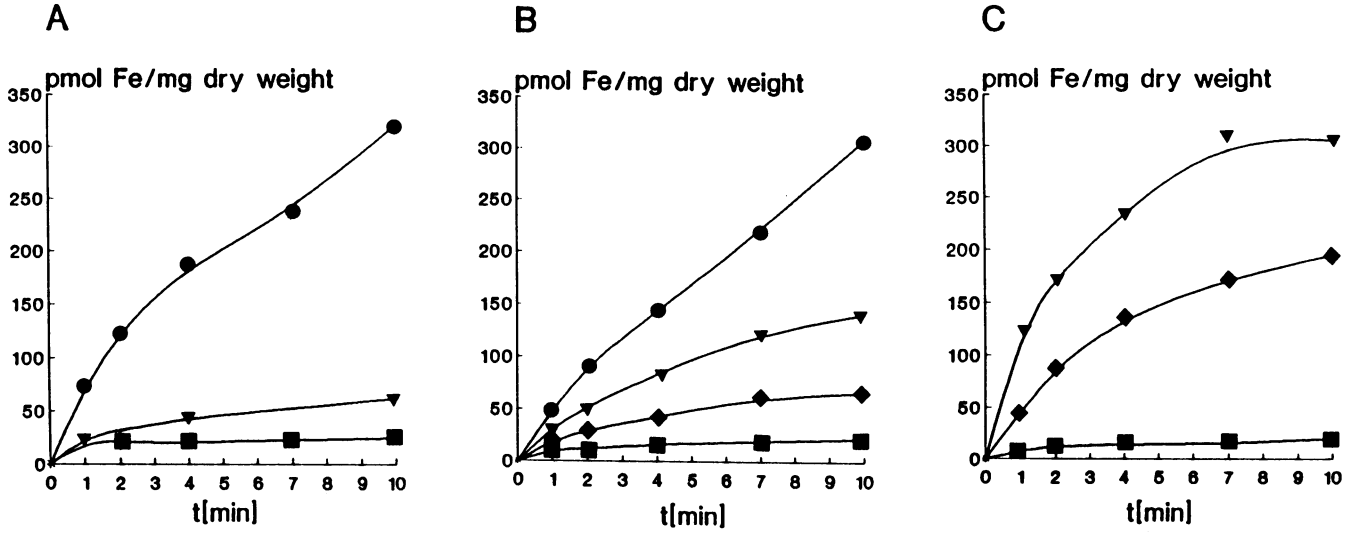

FIG. 6. Ferrous iron uptake. Cells were grown under aerobic conditions (A) or anaerobic conditions (B and C) in nutrient broth medium, and uptake of $^{55}Fe^{2+}$ was measured. (A and B) Strain H1717 (▼), strain H1771 *feoB7* (■), strain H1771 *feoB7* (pBSK18E *feoB*$^+$) (●), and strain SO67 *feoA*::Tn*5* (◆) results are shown. (C) Iron uptake by the following *fur* mutants: strain H2331 Δ*fur* (▼), strain H5101 Δ*fur feoB*::Tn*5* (■), and H5102 Δ*fur feoA*::Tn*5* (◆).

on MacConkey lactose agar plates with 40 μM iron. Plasmid pUH31 was unable to complement any *feo* mutant. Plasmid pHSG924 was able to complement the strains SO67, SO68, SO69, SO70, SO71, SO72, and SO73. However, this complementation was only partially effective, since single colonies were red to weakly red, while in more crowded regions of the plate the colonies were white. The reason may be a polar effect of the Tn*5* insertion on the expression of *feoB*.

Ferrous iron uptake was significantly lower in H1771 *feoB* than in the parent strain H1717 (Fig. 6). Complementation with pBSK18E led to a very high uptake of iron and was not very much influenced by the aerobic or anaerobic growth conditions. However, in *feoA*::Tn*5* mutants, ferrous iron uptake was only partly reduced. Since these results were highly variable, the double mutants H5102 Δ*fur feoA*::Tn*5* and H5101 Δ*fur feoB*::Tn*5* were constructed in strain H2331 Δ*fur*. The *fur* mutation was introduced to obtain a constitutive high expression of the *feo* genes and to avoid variations due to differing amounts of iron in the growth medium. The iron uptake in the *feoA* mutant was about 60% as high as in the parent strain (Fig. 6), confirming the results obtained with the *fur*$^+$ strains.

DISCUSSION

We had great difficulties in cloning the *feo* gene region. There are two possible reasons for this failure. High expression of an integral membrane protein has often been shown to be deleterious for the cells. The other, less likely, possibility is that an uncontrolled high expression of the FeoB ATPase activity leads to an energy deprivation.

This is the first ferrous iron transport system studied at the molecular level. At first glance, FeoA (75 amino acid residues) and FeoB (773 amino acid residues) seemed to be comparable to the cadmium export system (*cadAC*), in which one relatively large membrane protein, CadA (727 amino acid residues), and CadC (122 amino acid residues) catalyze the ATP-driven export of Cd^{2+} (22). This transport system is a member of a family of cation-translocating E1E2 ATPases, including K^+- and Ca^{2+}-ATPases (26). However, no sequence homologies to these and any other proteins were detected in the data base. A search for specific sites indicated that there are two domains in *feoB* with homology to nucleotide binding sites (Fig. 7) as described by Walker et al. (36). This type of structure is found in eucaryotic and bacterial F-type ATPases and in many transport-related ATPases (traffic ATPases), as has been analyzed by Mimura et al. (19). It is interesting to note that the binding protein-dependent transport systems, including the iron(III) siderophore transport systems, also belong to this superfamily (5). However, obvious homologies between FeoB and the traffic ATPases were observed only in the phosphate binding domain and not in the nucleotide binding fold, which is more similar to the domains of the *E. coli* ATPase and phosphofructokinase (36). How the transport of ferrous iron via Feo is energized has not been tested, but the observed similarities lead to the prediction that the uptake is driven by ATP.

The iron regulation of this system by Fur was observed during the first characterization of the system (12) and was confirmed by further experiments. The suggested Fur binding site deviates in position 6 from the consensus, where the highly conserved T is replaced by a C (Fig. 5). Further experiments are necessary to prove this suggestion and to see why the C is tolerated. Similarly, for the suggested Fnr box no convincing −10 region has been found, since the TATTAT in position 158 (Fig. 2) seems to have an unallowable short spacing of 9 bp to the Fnr box. There is no doubt that Fnr is activating the expression of *feo*, however. Ferrous iron uptake is certainly important for the iron supply of the cells. This was demonstrated in a *tonB* mutant which is unable to use iron(III) siderophores. However, the dependence on *feo* could be demonstrated on nutrient broth and TY medium plates only in an *aroB* background. Enterochelin, the siderophore of *E. coli*, can be secreted by the *aro*$^+$ strains, but they should not be able to use it, because of the *tonB* mutation. This contradiction can be explained by a TonB-independent 2,3-dihydroxybenzoate iron uptake system (11). The *aro*$^+$ strains are able to produce 2,3-dihydroxybenzoate as a precursor of enterochelin.

Protein	Residue	Sequences
Bovine ATPaseβ	148	A K G G K I G L F * G G A G V G K T V F I M
E.coli ATPaseβ	141	A K G G K V G L F * G G A G V G K T V N M M
E.coli ATPaseα	160	G R G Q R E L I I * G D R G T G K T A L A I
Adenylate kinase	5	L K K S K I I F V V G G P G S G K G T Q C E
RecA-protein	56	L P M G R I V E I Y G P E S S G K T T L T L
PstB	33	I A K N Q V T A F I G P S G C G K S T L L R
MalK	26	I H E G E F V V F V G P S G C G K S T L L R
HisP	29	A R A G D V I S I I G S S G S G K S T F L R
SfuC	26	V A A G S R T A I V G P S G S G K T T L L R
FecE	25	L P I G K I T A L I G P N G C G K S T L L N
FhuC	34	F P A G K V T G L I G H N G S G K S T L L K
FeoB	1	M K K L T I G L I G N P N S G K T T L F N

Bovine ATPaseβ	245	F R D Q E G Q D V L L F I D N I F R F T Q A
E.coli ATPaseβ	231	F R D * E G R D V L L F V D N I Y R Y T L A
E.coli ATPaseα	269	F R D * R G E D A L I I Y D D L S K Q A V A
Adenylate kinase	106	E R K * I G Q P T L L L Y V D A G P E T M T
Phosphofructok.	89	L K K * H G I Q G L V V I G G D G S Y Q G A
FeoB	79	H Y I L S G D A D L L I N V V D A S N L E R

FIG. 7. ATP binding site homologies to ATPases and traffic ATPases. The upper part shows the alignment to the phosphate binding domain (5, 36). The lower part shows the alignment to part of the nucleotide binding fold (36). No obvious similarities of FeoB to the traffic ATPases (19) were seen in this region.

The triple mutant forms only microcolonies on nutrient broth; however, growth could be obtained after citrate was added. Citrate may also be the reason for the slight growth observed on TY medium plates, since it is known that the ferric-citrate uptake system is induced above 0.1 mM citrate (13), and induction is observed in TY medium. The low molecular mass of the ferric iron dicitrate complex of 443 Da may allow diffusion of this complex through the outer membrane, independent of FecA, as has also been observed in *E. coli* for the cloned Sfu iron uptake system of *Serratia marcescens* (1). This iron transport system has been shown to use ferric iron, but the way that iron(III) reaches the periplasm is not known. Proteins homologous to SfuA have been detected in *Neisseria* spp., and it is tempting to speculate that in this organism the ferric iron comes from transferrin, which is bound by a TonB-dependent receptor to the cell surface (2, 6).

Ferrous iron uptake under oxygen-limited conditions in the intestine seems to be more important for *E. coli* than ferric iron transport. Recently, it was shown that *feo* mutants were less efficient in the colonization of the mouse intestine than *feo*$^+$ strains (31). Iron(III) uptake by enterochelin, citrate, or aerobactin did not influence the colonizing abilities of the strains tested (32). Similarly, it has been shown for aerobactin-defective mutants of *Shigella* spp. that they are not altered in their virulence (20), and it has been speculated that heme may be a source of iron for these intracellular pathogens (24). We think that also, in this case, ferrous iron has to be considered as an important additional source.

ACKNOWLEDGMENTS

The technical assistance of M. Döring is gratefully acknowledged. We thank I. Stojiljkovic for help in construction of the *lac* fusion and discussion, R. Koebnik for advice at the computer, and G. Weinstock, E. Bremer, and J. Kohara for sending strains and phages. We are also grateful to J. Crosa and V. Braun for critical reading of the manuscript.

The work was supported by the Deutsche Forschungsgemeinschaft (SFB323).

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