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Enterochelin System of Iron Transport in *Escherichia coli*: Mutations Affecting Ferric-Enterochelin Esterase

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Three mutant strains of *Escherichia coli* have been isolated which are lacking ferric-enterochelin esterase activity. This enzyme catalyzes the hydrolysis of the enterochelin moiety of ferric-enterochelin to yield ultimately three molecules of *N*-2,3-dihydroxybenzoylserine. The mutants (designated *fes*$^-$) were shown to be unaffected in enterochelin biosynthesis, capable of enterochelin-mediated iron uptake, and able to utilize ferric-dihydroxybenzoylserine complexes normally. When grown under iron-deficient conditions, however, they showed an absolute requirement for added iron or citrate, a phenotype characteristic of mutants defective in some part of the enterochelin system of iron uptake. These results support the theory that iron, taken up by the cell as ferric-enterochelin is only available for general cell metabolism after hydrolysis of the ligand by enterochelin esterase. The three *fes*$^-$ strains were shown to be affected in the B component of enterochelin esterase. The *fesB* gene which is probably the structural gene coding for component B of the esterase, was shown to be located at about minute 14 on the *E. coli* chromosome together with seven other genes involved in the enterochelin system of iron transport.

Recent studies have established the existence of an iron transport system in *Escherichia coli* which utilizes the iron-binding compound enterochelin, which is a cyclic trimer of *N*-2,3-dihydroxybenzoylserine (DBS) (3, 10). This compound is also formed by *Salmonella typhimurium* and has been called enterobactin (13). Under conditions of iron starvation *E. coli* synthesizes enterochelin and excretes it into the medium (4). Enterochelin is a powerful chelator of ferric iron and probably serves to solubilize any iron which is present in an insoluble form and therefore not readily available to the cell. It has been proposed that ferric-enterochelin is taken into the cell and that due to the high stability of this complex the enterochelin molecule must be hydrolyzed before the iron can be released for general cell metabolism (3, 10).

An intracellular enzyme, enterochelin esterase, capable of catalyzing the hydrolysis of the enterochelin moiety of ferric-enterochelin to yield DBS as the ultimate product, has been found (3, 10). Evidence to date, that this enzyme is involved in iron metabolism, comes from the findings that DBS is present in high concentrations in the culture supernatant fluids of *E. coli* grown under iron-deficient conditions and that, like the enterochelin biosynthetic enzymes, enterochelin esterase is repressed by relatively low levels of iron added to the growth medium (10, 15).

Enterochelin esterase has been found to separate on gel filtration into two components, neither of which is active alone. One of these components has a molecular weight greater than 140,000 and has been designated component A (10). The other component, B, has a molecular weight of about 22,000.

Six classes of mutants (designated *entA-F*) which are unable to synthesize enterochelin and one mutant (designated *fep*$^-$) which is blocked in enterochelin-mediated iron uptake have been isolated (4, 6, 16). The six *ent* genes and the *fep* gene are clustered together on the *E. coli* chromosome at about minute 14 according to the chromosome map. All of the mutants grow poorly in iron-deficient media, but their growth can be stimulated by the addition of iron or of citrate, which facilitates the entry of iron into the cell by some process independent of the

enterochelin system (4).

This present study was undertaken to establish the physiological importance of enterochelin esterase as part of the enterochelin iron transport system. Mutants lacking enterochelin esterase were obtained and characterized, and the gene concerned was located on the *E. coli* chromosome.

MATERIALS AND METHODS

Chemicals. Chemicals of analytical reagent quality were used; they were not further purified, except for ethyl acetate which was redistilled before use.

Preparation of enterochelin. The supernatant fluid from a 1-liter stationary-phase culture of strain AN260 (*fep*$^-$) was adjusted to about *p*H 1 with concentrated H_2SO_4 and extracted twice with an equal volume of ethyl acetate. The extracts were evaporated under reduced pressure over 50 ml of 0.1 M sodium phosphate buffer (*p*H 7.0) containing 5 mM $FeSO_4$. The dark red solution of ferric-enterochelin was washed twice with an equal volume of ethyl acetate, acidified, and extracted with two equal volumes of ethyl acetate. The enterochelin solution in ethyl acetate was then washed twice with equal volumes of 0.1 M phosphate buffer (*p*H 7.0), and the concentration of enterochelin was determined spectrophotometrically from the absorbance at 315 nm (E = 9,390) (11). The ethyl acetate was removed by evaporation, and the enterochelin was dissolved in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 6.5) containing 20% (v/v) ethanol, to give a concentration of 1 mM. A 1 mM solution of ferric-enterochelin was prepared by evaporating the ethyl acetate solution over the appropriate volume of 0.1 M sodium phosphate buffer (*p*H 7.0) containing 1 mM $FeSO_4$. About 20 μmoles of ferric-enterochelin was obtained per liter of original culture supernatant fluid by this procedure.

Bacterial strains. All of the strains used (Table 1) were derived from *E. coli* K-12, were maintained on nutrient agar slopes containing glucose (30 mM) and sodium citrate (10 mM), and were stored at 4 C.

Media. The basal medium used for the growth of cells was a modification (16) of the medium 56 described by Monod et al. (8). Iron content of the medium with no iron added was found to be about 2 μM (15). Glucose and growth factors were sterilized separately and added where necessary at the following concentrations: glucose, 30 mM; sodium citrate, 10 mM; L-proline, 1 mM; L-arginine, 0.7 mM; L-phenylalanine, 0.2 mM; L-tyrosine, 0.2 mM; L-tryptophan, 0.2 mM; L-leucine, 0.3 mM; *p*-aminobenzoate, 20 μM; *p*-hydroxybenzoate, 20 μM; thiamine, 1 μM. Solid media were prepared by the inclusion of 1.5% (w/v) Difco agar. Where stated, 2,2'-dipyridyl (0.1 mM) was added to the medium before autoclaving, to accentuate the growth responses to iron on agar media. A special low-iron growth medium was used for iron uptake studies and for growth responses in liquid medium and is described below together with the iron uptake methods.

Isolation of mutants. Strain AN92 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine under the conditions described by Adelberg et al. (1). The cells were then allowed to grow for about two generations in nutrient broth, washed twice with the basal mineral salts medium, and plated onto a glucose-mineral salts agar medium containing shikimate (1 μM) but no added iron. The plates were incubated for 4 days at 37 C, and all colonies were marked. An agar overlay containing sodium citrate to give a final concentration of 10 mM was then added, and the plates were incubated a further 4 days. All colonies appearing after enrichment with sodium citrate were tested for their response to citrate on glucose-mineral salts-agar plates containing 2,2'-dipyridyl (100 μM), shikimate (50 μM), and no added iron. Those isolates showing an absolute response to citrate were selected for further study.

Growth of cells and preparation of cell extracts. Cells were grown in glucose-mineral salts medium supplemented with shikimate (0.2 mM) and iron (1 μM) in 1-liter quantities in 2-liter flasks shaken at 37 C. Cells were harvested in late logarithmic phase and washed once with 0.1 M sodium phosphate buffer (*p*H 7.0). The washed cells were resuspended in 1 ml of 0.1 M Tris-hydrochloride buffer (*p*H 8.0) for each 1 g (wet weight) of cells and disrupted in a Sorvall Ribi cell fractionator at 20,000 lb/inch^2, to give the cell extract. Extracts were used immediately after preparation, although enterochelin esterase activity was found to be fairly stable when stored at −13 C. Protein concentrations were determined with the Folin phenol reagent (5).

Estimation of ferric-enterochelin esterase activity. The reaction mixture for the determination of enterochelin esterase activity contained 0.15 μmole of ferric-enterochelin or enterochelin in 0.1 M sodium phosphate buffer (*p*H 7.0) and 0.15 ml of cell extract. A control tube containing boiled cell extract was included in each test to estimate the nonenzymatic degradation of enterochelin under the conditions of the assay. After 1 hr of incubation at 37 C, 0.1 ml of 1 M H_2SO_4 and 2 ml of ethyl acetate were added, and the mixtures were emulsified thoroughly and allowed to stand for 30 min. The amount of enterochelin hydrolyzed was determined spectrophotometrically by comparing the absorbance of the ethyl acetate solution before and after extraction with an equal volume of 0.1 M phosphate buffer (*p*H 7.0). Under these conditions the enterochelin remains in the ethyl acetate, but any hydrolysis products are extracted quantitatively into the phosphate buffer. It has been shown (10) that enzymatic hydrolysis of ferric-enterochelin under the conditions used, where enterochelin is in excess, is proportional to enzyme concentration, is linear over a 2-hr period, and yields a mixture of DBS and its dimer in the ratio of about 4:1. The enterochelin esterase activities are quoted in terms of nanomoles of enterochelin hydrolyzed per milligram of protein in 1 hr.

Preparation of components A and B of ferric-enterochelin esterase. A cell extract of strain AN260 was prepared and centrifuged at 18,000 rev/min for 20 min. The supernatant fluid had an activity of 52 nmoles of ferric-enterochelin hydrolyzed/mg of pro-

TABLE 1. *Strains of E. coli K-12 used*

Strain no.	Sex	Relevant genetic loci[a]				Source or reference
		ent	*fep*	*fes*	Other	
AN92	F⁻	+	+	+	*proA2, argE3, pheA1, tyrA4 trp-401, aroB351*	
AN156	F⁻	*A403*	+	+	As for AN92	Isolated from AN92 after treatment with NTG[b]
AN260	F⁻	+	*402*	+		
AN272	F⁻	+	+	*B411*		
AN274	F⁻	+	+	*B412*		
AN276	F⁻	+	+	*B413*		
AB1515	F⁻	+	+	+	*proC⁻, leu⁻, trp⁻, purE⁻*	
AN194	F⁻	+	+	+	*proC⁻, leu⁻, trp⁻*	P1 transductants derived from AB1515
AN261	F⁻	+	*402*	+	*proC⁻, leu⁻, trp⁻*	
AN273	F⁻	+	+	*B411*	*proC⁻, leu⁻, trp⁻*	
Hlip22	Hfr	+	+	+	*lip22*	

[a] Genetic nomenclature is that used by Taylor (14).
[b] NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

tein in 1 hr, and 5 ml of this supernatant fluid (250 mg of protein) was applied to a column of Sephadex G-150 (1.5 by 64 cm) equilibrated with 0.05 M Tris-hydrochloride buffer (*p*H 8). The protein was eluted with the same buffer at 12 ml per hr, and 3-ml fractions were collected. The peak fractions containing component A (fraction 12) and component B (fraction 25) were found by mixing column fractions and testing for enterochelin esterase activity as described previously (10).

Examination of culture supernatant fluids. Cultures were grown to late logarithmic phase in the supplemented glucose basal medium containing shikimate (0.5 mM), and supernatant fluids were examined by two-dimensional chromatography as described previously (11).

Iron uptake studies. Precautions were taken in the preparation of the media used both for iron uptake and for the growth of cells to minimize their iron content. Major media components, such as phosphates, ammonium sulfate, and glucose, with the lowest iron content available were chosen, and the water was deionized and twice glass-distilled. The glassware was cleaned and then autoclaved with 0.5% (w/v) ethylenediaminetetraacetate (EDTA), followed by thorough washing with distilled and double-distilled water, and finally autoclaved twice in the double-distilled water.

The "low-iron" growth medium contained: 20 mM sodium phosphate and 20 mM potassium phosphate (*p*H 6.9), 10 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, and 40 μM $CaCl_2$. This basic medium was autoclaved and supplemented with 1 mM sodium citrate. Glucose and other supplements were added as specified above, but shikimate was not used.

The uptake medium was made up as follows. A solution containing 30 mM NaH_2PO_4, 10 mM KH_2PO_4, 10 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, and 40 μM $CaCl_2$ (*p*H 4.5) was autoclaved and left to stand for at least 7 days to allow endogenous iron to precipitate. The medium was then filtered through a membrane filter (Millipore, HAWP, pore size 0.45 μm), adjusted to *p*H 6.9 with a 3:1 mixture of 5 M NaOH-5 M KOH, and autoclaved again. Before use this medium was diluted with an equal volume of water and supplemented with glucose and other nutrients described for the growth medium, except that tryptophan and citrate were omitted. The Na^+ to K^+ ratio of 3:1 was found previously to be essential to maintain the iron in a soluble form (H. Rosenberg, *unpublished results*).

Strains to be tested were inoculated into the low-iron growth medium from a freshly grown nutrient agar-glucose slope containing 1 mM sodium citrate. After shaking overnight at 37 C, the cells were diluted with fresh growth medium to a density of 7 to 8 Klett units and allowed to grow into early exponential phase (cell density of 50 to 60 Klett units). The suspensions were cooled to 0 C over a 15-min period, centrifuged and washed twice with cold uptake medium, and resuspended in cold uptake medium to give a turbidity of 200 Klett units. At this stage cells could be stored for up to 90 min at 0 C before use without affecting their capacity for iron uptake.

Iron uptake measurements. Solutions of 2 μM $^{55}FeCl_3$ in uptake medium, containing the compounds to be tested, were shaken at 37 C for about 45 min, membrane-filtered, and stored at 0 C before use. Directly before each assay the cell suspension and ^{55}Fe solution were warmed to 37 C. An equal volume of the ^{55}Fe solution and the cell suspension were then mixed, and ^{55}Fe uptake was measured by filtering 0.5 ml of the mixed suspension at intervals through membrane filters (Gelman metricel, pore size 0.45

μm) which were then washed twice with 2 ml of 0.9% (w/v) NaCl containing 1 μM $FeCl_3$. The thoroughly dried filters were placed in scintillation vials, 10 ml of 0.6% (w/v) butyl 1,3,4-phenylbiphenylyloxadiazole scintillator in toluene was added, and the vials were counted in a Packard 3320 counter.

Cells were routinely assayed for iron uptake under the following conditions: (i) 1 μM $^{55}FeCl_3$ with no added chelating agents; (ii) 1 μM $^{55}FeCl_3$ plus 100 μM sodium nitrilotriacetate (NTA); (iii) 1 μM $^{55}FeCl_3$ plus 100 μM NTA plus 1 μM enterochelin. Blank determinations, in which cell suspension was replaced by uptake medium, were carried out for each solution, and uptake data were corrected accordingly.

Membrane filters were soaked before use for 30 min in 0.9% NaCl (w/v) containing 40 μM ammonium Fe^{3+}-EDTA complex and then washed three times in glass distilled deionized water. All incubations and transfers were performed by using either polyethylene or polypropylene apparatus.

Genetic techniques. Transduction with the generalized transducing phage P1Kc was carried out as described by Pittard (12).

RESULTS

Isolation of mutants. It was predicted that mutants lacking enterochelin esterase activity, and therefore blocked in the release of iron from ferric-enterochelin, would be unable to grow on iron-deficient media unless citrate or iron were added. Strain AN92 was used as parent strain because it is blocked in the common pathway of aromatic biosynthesis and the amount of enterochelin formed by the cells can be regulated according to the quantity of shikimate supplied in the growth medium. Mutants showing an absolute requirement for citrate were isolated as described in Materials and Methods. To separate out those mutants affected in enterochelin synthesis, all of the mutants were tested for their growth response to enterochelin, and the enterochelin responders were discarded. Twelve independently isolated mutants were obtained which showed an absolute requirement for citrate and no response to enterochelin. Examination of culture supernatant fluids (*see below*) confirmed that enterochelin biosynthesis was unaffected in these mutants.

Growth response to iron. When grown on the glucose-mineral salts-agar medium with no added iron, the parent strain AN92 was found to require a minimum concentration of 1 μM shikimate for full growth. Under these conditions the twelve mutant strains, in contrast to AN92, showed an absolute requirement for added iron.

Occurrence of enterochelin and related compounds in culture supernatant fluids. The culture supernatant fluids of each of the mutant strains contained high levels of enterochelin and small quantities of its three hydrolysis products and were very similar to the supernatant fluid of strain AN102 (*fep*$^-$) which was described in detail previously (4). In a control experiment, it was found that, when enterochelin was incubated in the culture medium, nonenzymatic hydrolysis occurred which was sufficient to account for the levels of hydrolysis products which were found in the culture supernatant fluids. The supernatant fluid from the parent strain (AN92) grown under the same conditions showed less enterochelin and relatively more of the three hydrolytic products of enterochelin, as expected for a strain able to hydrolyze enterochelin.

Response to DBS. It was shown previously that DBS can serve as a growth factor for *ent*$^-$ strains, although it is considerably less efficient than enterochelin (9). It was predicted that mutants lacking enterochelin esterase would respond to DBS since it is the product of enterochelin esterase activity, but that mutants blocked in enterochelin-mediated iron uptake might not. Because enterochelin forms much more stable complexes with iron than does DBS (10), it was necessary to carry out this test under conditions where the cells were unable to synthesize enterochelin, in order to avoid interference with DBS-mediated iron uptake. The mutants were therefore tested for response to DBS on minimal agar medium containing no shikimate. Three of the mutants (AN272, AN274, and AN276) grew well in the presence of DBS, suggesting that they might be deficient in enterochelin esterase. The remaining nine mutants did not respond to DBS, suggesting that they were blocked in enterochelin-mediated iron uptake (*fep*$^-$).

Estimation of enterochelin esterase activity in the mutants. Cell extracts prepared from the parent (AN92) and the mutant strains were tested for enterochelin esterase activity, with ferric-enterochelin used as substrate. The three mutants that responded to DBS (strains AN272, AN274, and AN276) had no detectable activity (Table 2) and were designated *fes*$^-$. In contrast, cell extracts from the parent strain and each of the other nine mutants that did not respond to DBS possessed esterase activity. The esterase activities obtained with extracts of the parent strain (AN92) were generally lower than those of the mutants because, under the conditions used for the preparation of cell extracts, enterochelin esterase is partially repressed in the parent strain.

A number of the cell extracts of the above strains were also tested for esterase activity with enterochelin rather than ferric-entero-

TABLE 2. *Ferric-enterochelin esterase activities in cell-free extracts*

Extract prepared from	Ferric-enterochelin esterase activity[a]
AN92 (parent)	4, 9
AN260 (*fep*⁻)	35, 53
AN272 (*fes*⁻)	<0.5, <0.5
AN274 (*fes*⁻)	<0.5
AN276 (*fes*⁻)	<0.5

[a] Esterase activity (nanomoles ferric-enterochelin hydrolyzed per milligram of protein in 1 hr) was determined as described in Materials and Methods.

chelin as substrate. Extracts possessing esterase activity hydrolyzed only about 30% as much enterochelin as ferric-enterochelin in 1 hr, suggesting that ferric-enterochelin is the preferred substrate. The *fes*⁻ strains were unable to hydrolyze either enterochelin or ferric-enterochelin. Because crude cell extracts contain quantities of iron and other metals, it is possible that the observed enterochelin hydrolysis represents the hydrolysis of ferric-enterochelin, formed by chelation of endogenous iron. We propose to use the trivial name ferric-enterochelin esterase throughout this paper because ferric-enterochelin is generally used as the substrate.

Enzymatic complementation tests between cell extracts from fes⁻ strains and the A and B components of ferric-enterochelin esterase. To determine whether the mutation in the three *fes*⁻ mutants had affected either the A or B component of ferric-enterochelin esterase, or both, enzymatic complementation tests were carried out. Components A and B were separated by gel filtration of an extract of strain AN260, as described in Materials and Methods. The preparations of components A and B had little esterase activity unless mixed (Table 3), indicating that a good separation of the two components had been achieved. Cell extracts from each of the three *fes*⁻ mutants gave good activity when mixed with component B, whereas complementation with component A gave no activity. These results indicate that each of the *fes*⁻ mutants possessed a normal esterase component A, the *fes*⁻ mutations resulting in an inactive component B. The gene carrying the mutations affecting the component B protein was designated *fesB* and is probably the structural gene coding for this protein.

Iron uptake studies. The enterochelin-mediated iron uptake of the three *fesB*⁻ mutants was compared with that of the parent strain (AN92) and of strain AN260. The results of previous tests suggested that strain AN260 was blocked in enterochelin-mediated iron uptake. The strains were grown under standardized iron deprivation conditions, in which the cells were unable to synthesize enterochelin due to the absence of shikimate. All strains showed a slow rate of iron uptake in the absence of any added chelating agents (Fig. 1). In each case addition of NTA (100 μM) resulted in a marked inhibition of this non-enterochelin-mediated iron uptake. Under these conditions addition of 1 μM enterochelin plus 100 μM NTA resulted in characteristic differences in iron uptake behavior between the parent strain, the *fesB*⁻ strains, and strain AN260 (Fig. 1).

Enterochelin greatly stimulated iron uptake in the parent strain, whereas strain AN260 was completely defective in enterochelin-mediated iron uptake and was therefore designated *fep*⁻. The *fesB*⁻ mutants (AN272, AN274, and AN276) showed initial rates which were comparable with that of the parent strain, but iron uptake virtually ceased after 10 min. In the parent strain uptake continued for at least 1 hr under these conditions. The *fesB*⁻ mutants were therefore not blocked in enterochelin-mediated iron uptake. The reduced amount of iron taken up by these mutants is consistent

TABLE 3. *Complementation tests between ferric-enterochelin esterase components A and B and cell extracts from fes⁻ strains*

Cell extract	Addition of enterochelin esterase component A or component B[a]	Enterochelin hydrolyzed (nmoles in 1 hr)[b]
AN260 (*fes*⁺)	None	192
None	A	≤8
None	B	14
None	A + B	91
AN272 (*fes*⁻)	A	≤8
AN272 (*fes*⁻)	B	74
AN274 (*fes*⁻)	A	≤8
AN274 (*fes*⁻)	B	64
AN276 (*fes*⁻)	A	≤8
AN276 (*fes*⁻)	B	62

[a] Components A and B of enterochelin esterase were obtained by chromatography of an extract of AN260 (*fes*⁺) on Sephadex G-150 as described in Materials and Methods.

[b] The assay for enterochelin esterase was carried out as described in Materials and Methods except that 0.075 ml of cell extract and 0.075 ml of components A and B were used.

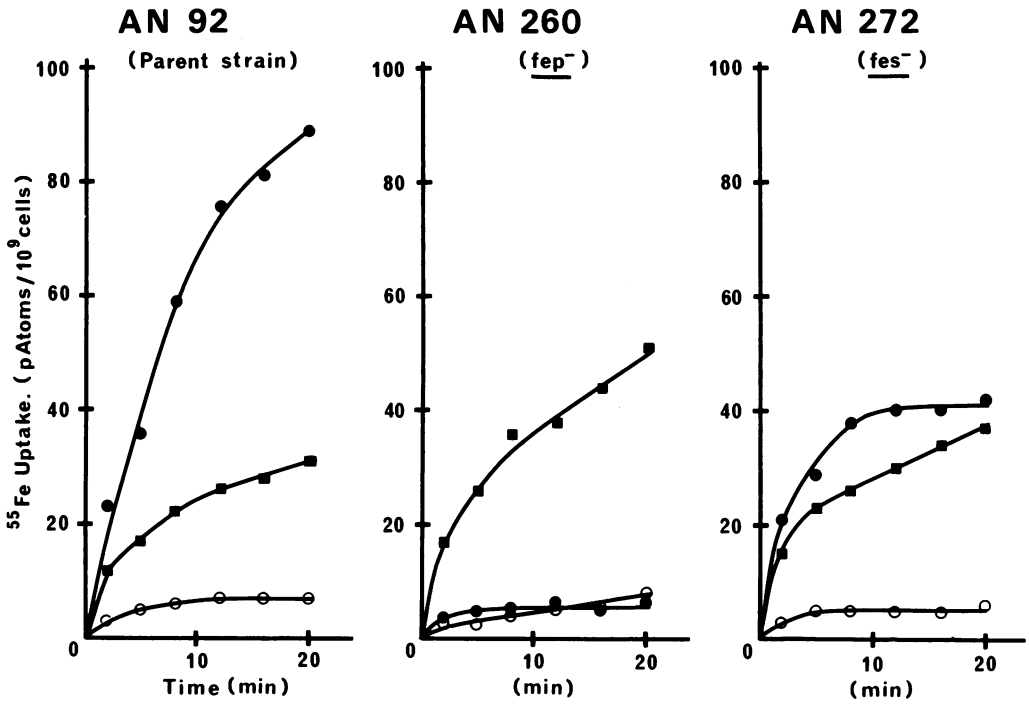

FIG. 1. *Iron uptakes by cells of AN92 (parent), AN260 (fep⁻), and AN272 (fes⁻) in the presence of 1 μM $^{55}Fe^{3+}$. Symbols: ■, no added chelating agents; ○, 100 μM sodium nitrilotriacetate (NTA); ●, 100 μM NTA plus 1 μM enterochelin. Each point represents the mean of several experiments.*

with the saturation of a pool for ferric-enterochelin, resulting from a blockage in the release of iron from this complex. At the point of saturation the intracellular concentration of ferric-enterochelin is about 50 times that in the medium, indicating the operation of an active transport system. This calculation is based on 7.5×10^{-13} ml as the volume of the cell water in an *E. coli* cell (7) and assumes uniform distribution of ferric-enterochelin throughout the cell.

Growth responses in liquid medium. In the experiments reported above, the *fesB*⁻ mutants were shown to be unaffected in enterochelin biosynthesis and excretion, capable of enterochelin-mediated iron transport, and able to utilize ferric-DBS complexes normally. Thus, the only defect in these mutants is their inability to hydrolyze ferric-enterochelin. It was therefore of interest to compare the effects of mutations in the *fesB* and *fep* genes on growth under iron-deficient conditions in order to quantitate the physiological consequences of the loss of enterochelin esterase activity.

An isogenic set of transductants (AN194, wild type; AN261, *fep*⁻; AN273, *fesB*⁻) was therefore prepared and their growth was compared in the low-iron growth medium used for iron uptake studies. Strain AN194 grew normally in the low-iron growth medium, and its growth was not further stimulated by iron or citrate. Strains AN261 (*fep*⁻) and AN273 (*fesB*⁻) barely grew at all in the low-iron medium, and the growth of both strains was greatly stimulated by the addition of iron or citrate (Table 4). It is clear that the loss of ferric-enterochelin esterase activity results in the inability of the mutant strain to grow under iron-deficient conditions where enterochelin-mediated iron transport is essential for growth. This indicates that ferric-enterochelin esterase is an essential part of the enterochelin system of iron transport.

Genetic analysis of the fesB⁻ mutants. The six *ent* genes and the *fep* gene have previously been shown to be clustered on the *E. coli* chromosome at minute 14 and to be cotransducible with the *purE* and *lip* genes at low frequency (4, 6, 16). The mutations affecting ferric-enterochelin esterase in the three *fesB*⁻ strains were also found to be cotransducible with *purE* and *lip* at low frequency and with *entA* at about 40% (Table 5). A cell-free extract of a *purE*⁺, *fesB*⁻ transductant from each of the crosses was prepared, and it was found that in each case the transductants lacked ferric-

enterochelin esterase activity. The above results indicate that the *fesB* gene is clustered together with the *fep* gene and the six *ent* genes, at about minute 14 on the *E. coli* chromosome.

DISCUSSION

A scheme summarizing the enterochelin-dependent system for iron transport in *E. coli* is given in Fig. 2. The present findings provide strong evidence that iron transported into the cell via this system remains unavailable for general cell metabolism until the enterochelin ligand has been hydrolyzed by ferric-enterochelin esterase. The resultant ferric-DBS complexes are markedly less stable than the ferric-enterochelin complex (10), and consequently iron may be more readily removed from the ligand for general metabolism, possibly via a reduction step to the ferrous state. Further support for this proposal is found in the recent demonstration (R. J. Porra, L. Langman, I. G. Young, and F. Gibson, Arch. Biochem. Biophys., *in press*) that ferrochelatase is unable to utilize iron from the enterochelin complex as a substrate for heme synthesis, whereas iron from the DBS complex is readily utilized.

In the presence of excess ferric-enterochelin, hydrolysis by the esterase yields predominantly DBS but also appreciable amounts of the DBS-dimer (10). It remains to be established whether iron from this ferric-DBS dimer complex is available for cell metabolism.

Compacted cells of mutants lacking the esterase have a distinct pink coloration which was shown to be due to ferric-enterochelin. In contrast, cells of the parent strain and mutants defective in enterochelin-mediated iron transport (*fep*$^-$) are white in color and do not accumulate ferric-enterochelin.

Bryce and Brot (2) have recently described the isolation, from crude cell extracts of *E. coli*, of a fraction possessing enterochelin esterase activity. This enzyme hydrolyzed enterochelin in the absence of iron but was inhibited by the addition of iron. The relationship between this enzyme and the A and B components of ferric-enterochelin esterase remains obscure, but the present studies have shown that mutants lacking the B component are unable to hydrolyze enterochelin either in the presence or absence of iron.

Eight genes involved in the enterochelin system of iron transport have now been shown to be located at about minute 14 on the *E. coli* chromosome. These comprise the six *ent* genes involved in enterochelin biosynthesis, the *fep* gene involved in enterochelin-mediated iron uptake, and the *fesB* gene coding for the ferric-enterochelin esterase B component. All eight genes are cotransducible with *purE* at low frequency (1–4%) and with *lip* at frequencies of from 2 to 25%. The six *ent* genes are cotransducible with the *fep* gene at frequencies ranging

TABLE 4. *Growth responses of fep$^-$ and fes$^-$ mutants in "low-iron" growth media*[a]

Supplement	Mean generation time (hr)		
	AN194	AN261 (*fep*$^-$)	AN273 (*fes*$^-$)
A. None	1.3	>7[b]	>7
Citrate			
0.1 mM	1.3	2.2	2.2
1 mM	1.3	1.4	1.1
5 mM	1.2	1.3	1.1
B. None	1.7	>7	>7
Iron			
5 μM	1.6	3.3	2.5
10 μM	1.7	2.7	2.3
50 μM	1.7	2.7	1.8

[a] Growth responses in "low-iron" growth medium (see Materials and Methods) with glucose as carbon source were measured in two separate experiments designated A and B. Cultures were shaken at 37 C in 10-ml volumes in 125-ml side-arm flasks.

[b] The *fep*$^-$ and *fes*$^-$ strains grew extremely slowly without iron or citrate, and generally grew less than two generations over a 24-hr period.

TABLE 5. *Transduction data for the fesB gene*

P1 grown on strain	Recipient strain	Marker selected	Transductants carrying unselected marker	
			No.	%
AN272 (*purE*$^+$,*fesB411*)	AB1515 (*purE*$^-$,*fes*$^+$)	*purE*$^+$	*fesB*$^-$ 1/112	1
AN274 (*purE*$^+$,*fesB412*)	AB1515 (*purE*$^-$,*fes*$^+$)	*purE*$^+$	*fesB*$^-$ 1/120	1
AN276 (*purE*$^+$,*fesB413*)	AB1515 (*purE*$^-$,*fes*$^+$)	*purE*$^+$	*fesB*$^-$ 4/111	4
AN272 (*lip*$^+$,*fesB411*)	Hfr lip22(*lip22*,*fes*$^+$)	*lip*$^+$	*fesB*$^-$ 3/132	2
AN274 (*lip*$^+$,*fesB412*)	Hfr lip22(*lip22*, *fes*$^+$)	*lip*$^+$	*fesB*$^-$ 2/114	2
AN276 (*lip*$^+$,*fesB413*)	Hfr lip22(*lip22*,*fes*$^+$)	*lip*$^+$	*fesB*$^-$ 5/120	4
AN156 (*fes*$^+$,*entA403*)	AN272 (*fesB411*,*ent*$^+$)	*fes*$^+$	*entA*$^-$ 18/39	46

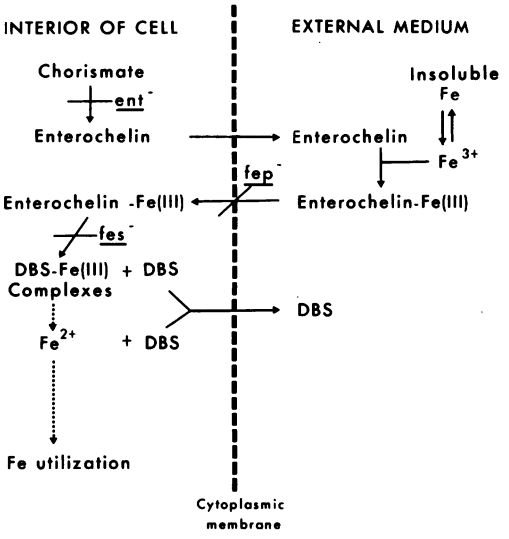

FIG. 2. *Postulated scheme depicting the operation of the enterochelin system of iron transport in E. coli.*

from 40 to 95% (6, 16), and the *fesB* gene is cotransducible with the *entA* gene at about 46%. The cotransduction frequencies suggest that these genes may be clustered together in a segment of the *E. coli* chromosome of about 0.2 to 0.5 min in length. As well as the *fesA* gene there may be other genes not yet identified coding for additional proteins of the enterochelin system. The possibility exists that all of the genes for the enterochelin system of iron transport are located at about minute 14 and may constitute one or more operons whose transcription is regulated according to the intracellular level of iron.

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