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Exogenous Induction of the Iron Dicitrate Transport System of *Escherichia coli* K-12

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Streptonigrin was used to select mutants impaired in the citrate-dependent iron transport system of *Escherichia coli* K-12. Mutants in *fecA* and *fecB* could not transport iron via citrate. *fecA-lac* and *fecB-lac* operon fusions were constructed with the aid of phage Mu dl(Ap *lac*). Strains deficient in ferric dicitrate transport which were mutated in *fecB* were as inducible as transport-active strains. They expressed the FecA outer membrane protein and β-galactosidase of the *fecB-lac* operon fusions. In contrast, all *fecA*::*lac* mutants and *fecA* mutants induced with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine did not respond to ferric dicitrate supplied in the growth medium. *tonB fecB* mutants which were lacking all *tonB*-related functions were not inducible. We conclude that binding of iron in the presence of citrate to the outer membrane receptor protein is required for induction of the transport system. In addition, the *tonB* gene has to be active. However, iron and citrate must not be transported into the cytoplasm for the induction process. These data support our previous conclusion of an exogenous induction mechanism. Mutants in *fur* expressed the transport system nearly constitutively. In wild-type cells limiting the iron concentration in the medium enhanced the expression of the transport system. Thus, the citrate-dependent iron transport system shares regulatory devices with the other iron transport systems in *E. coli* and, in addition, requires ferric dicitrate for induction.

Escherichia coli K-12 can transport iron(III) by various high-affinity systems with the use of complexing agents called siderophores. Transport systems via enterochelin, ferrichrome, or coprogen require outer membrane receptor proteins designated FepA, FhuA (TonA), and FhuE (14), respectively, and additional functions defined genetically and tentatively assigned to the cytoplasmic membrane (reviewed in references 5, 6, 22, and 27). All iron transport systems depend on the function specified by the *tonB* gene and to a lesser and variable extent on that determined by the *exbB* locus (16). The transport systems are regulated by the iron supply and by the *fur* locus (1, 2, 12, 13). At iron-limiting growth conditions, the outer membrane proteins and enterochelin (29) are synthesized in high amounts, and the activities of the transport systems are increased. *fur* mutants express these functions constitutively (1, 2, 12).

Frost and Rosenberg reported an iron transport system in *E. coli* K-12 with a K_m of 0.2 μM Fe^{3+} and a V_{max} of 66 patoms of Fe^{3+} per mg (dry weight) (8). It was induced by 0.1 mM citrate in the growth medium. Mutations which abolished citrate-dependent iron transport were mapped at 7 min on the *E. coli* chromosome (33). They fell into two groups, mutations which were cotransducible by phage P1 with the *argF* gene and mutations which were not cotransducible. The mutants close to *argF* were later shown to lack an outer membrane protein that was induced by citrate with the same kinetics as the transport system (11, 32). Binding of Fe^{3+} in the presence of citrate to outer membranes was dependent on the content of the FecA protein (32). *E. coli* W and *Salmonella typhimurium* contain no citrate-dependent iron transport systems (8) and no citrate-inducible outer membrane proteins (32). These data have suggested that the FecA protein is a constituent of the ferric dicitrate transport system. Mutants outside *fecA* were termed *fecB* (32). With a Tn*10* transposon inserted close to the *fec* region, *fecA* and *fecB* could be cotransduced with P1, indicating that both genes are closely linked (21).

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Induction of the iron transport system required iron and citrate (21). Citrate does not serve as a carbon or energy source in this organism (9, 25, 28), and it is not transported under aerobic growth conditions. Under conditions in which 10 to 100 times more iron and citrate were in the cell than is required for induction, the transport system was not induced (21). For maintaining the induced transport system, the same threshold concentration of citrate (0.1 mM) as for the initial induction was required. Fluorocitrate and phosphocitrate were strong inducers but transported iron very poorly (21). Transport-defective *fecB* mutants expressed the FecA protein in reponse to citrate in the medium as *fecB*$^+$ strains. It seemed that ferric dicitrate did not have to enter the cytoplasm to induce the transport system. There were inducers which were not taken up or did not support iron transport or both; transport-deficient strains could be induced; intracellular citrate and iron were inactive, and there was no autocatalysis between the transport and induction system. We therefore adopted the term exogenous induction (21).

In former studies, we relied on the expression of the FecA protein in transport-negative (*fecB*) mutants as sole indicator of a functioning induction system (21, 32). The requirement of FecA for induction could not be studied. In this paper, we inserted phage Mu dl(Ap *lac*) into the *fecB* gene such that expression of β-galactosidase came under the control of citrate induction. This allowed us to study expression of the ferric dicitrate transport system quantitatively, and to reveal the function of FecA in the induction process.

MATERIALS AND METHODS

Bacterial strains and media. The *E. coli* K-12 strains used are listed in Table 1. The phages T1, T5, P1, and λNK370 were from our stock. Media containing nutrient broth (NB), tryptone-yeast extract (TY), and M9 salts-glucose were as described previously (4). The antibiotics were used at the following concentrations (micrograms per milliliter): streptonigrin, 5; ampicillin, 25; chloramphenicol, 50; streptomycin, 100; and tetracycline, 15. P1 transduction and conjuga-

TABLE 1. List of *E. coli* strains used

Strain	Genotype	Source or reference
K-12 Ymel	Wild type	This institute
AB2847	*aroB malT tsx thi*	(4)
H455	As AB2847, but *pro lac*	(12)
H1080	As AB2847, but *zhe*::Tn*10*	This study
H1290	As H455, but *cys fur-74*	(12)
H1368	As AB2847, but *zag*::Tn*10*	(21)
H1389	As H455, but *exbB*::Tn*10*	(16)
H1443	*aroB araD lac thi rpsL*	(16)
H799	As H1443, but *fecB*::Mu dl(Ap*lac*)	This study
H1452	As AB2847, but *tonB zch*::Tn*10*	This study
WA27	As AB2847, but *fecB*	(32)
WA380	As AB2847, but *fecA*	(32)
BR158	As AB2847, but *tonB*	(4)
JC10240	HrPO45 *thr recA56 ilv srl*::Tn*10 rel spc*	M. Achtmann
N541	*proAB lac his argF argI met* λ P1 *argF* Cm13	(36)
1A10	*proAB lac his argF argI met* λ P1 *argF* Cm0	(36)
PCO950	HfrR4 *thr argI argF serB purA fhuA relA spoT*	B. Bachmann
ZI311	As H1443, but *fecA recA srl*::Tn*10*	This study
ZI325	As H799, but *fecA zag*::Tn*10*, donor ZI323	This study
ZI341	As H799, but *exbB*::Tn*10*	This study
ZI342	As H799, but *tonB zch*::Tn*10*	This study
ZI345	As H1443, but *zag*::Tn*10*, donor H1368	This study
ZI367	As AB2847, but *lac*::Tn*10*	This study
ZI370	As H1443, but *fecB recA srl*::Tn*10*	This study
ZI379	As ZI367, but *fecA*::Mu dl(Ap *lac*)	This study
ZI395	As H799, but *fhuA*	This study
ZI396	As H1443, but *exbB*	This study
ZI406	As H1290, but *fecB*::Mu dl(Ap *lac*)	This study
ZI407	As PCO950, but *aroB*, donor H1080 *zhe*::Tn*10*	This study
ZI418	As H1443, but *fecB*::Mu dl(Ap *lac*)	This study
ZI419	As H799, but *tonB*	This study

tion experiments were carried out as described by Miller (26). Mutagenesis with phage Mu dl(Ap *lac*) was performed as described previously (2, 7, 9, 12). Genetic markers were transferred with the use of the transposon Tn*10* inserted either within or near these genes and selection for resistance to tetracycline. Growth on ferric dicitrate was tested on agar plates containing nutrient broth, 0.2 mM 2,2'-dipyridyl, and 1 mM citrate.

The *recA* strains ZI370 and ZI311 were obtained by transducing *srl*::Tn*10* of JC10240 into H1443. UV sensitivity of the tetracycline-resistant colonies was tested on TY plates (26).

Isolation of streptonigrin-resistant mutants impaired in ferric dicitrate uptake. Cells were grown in 5 ml of TY medium to a density of 3×10^8 cells per ml. They were washed twice with 4 ml of 0.1 M sodium citrate (pH 5.5) and resuspended in 4 ml of the citrate buffer. To each of two halves, 0.1 and 0.05 ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (1 mg/ml in citrate buffer) were added, and the mixtures were shaken for 30 min at 37°C. Cells were centrifuged, washed once in M9 medium that contained 10 mM citrate, and then incubated in this medium for 3 h at 37°C.

Mutagenized cells (5×10^8 cells in 0.5 ml) obtained by treatment with MNNG or by infection with Mu dl(Ap *lac*) were incubated for 2 h at 30°C in 5 ml of NB medium supplemented with dipyridyl and citrate. Dilutions were plated on M9 agar that contained 10 mM citrate and 5 μg of streptonigrin per ml. After incubation for 4 days at 30°C, small colonies were investigated further.

Alternatively, Mu dl(Ap *lac*)-infected cells (4×10^7 cells per ml) were incubated for 3 h at 30°C in M9-citrate-dipyridyl medium that contained 25 μg of ampicillin per ml. Then, 0.1 mg of cycloserine per ml was added and the culture incubated further for 5 h. Cells were centrifuged and shaken overnight in 5 ml of NB-ampicillin medium. The enrichment of ferric dicitrate transport mutants by treatment with cycloserine was repeated four times by a method similar to that described previously (2). The survivors were plated on TY-ampicillin plates and incubated for 48 h. The colonies were replica plated on NB-citrate-dipyridyl plates. Colonies that could not grow on the latter medium were investigated further.

Isolation of *lac*::Tn*10* mutants. *E. coli* Ymel was infected with λNK370::Tn*10*. The selection plates contained TY medium and the appropriate antibiotic. A total of 5,000 colonies were pooled, and various dilutions were plated on MacConkey-lactose agar. White colonies were picked and used to construct strain ZI367 by P1 transduction (Table 1).

Isolation of P1 *argF* Cm lysogens. The procedure for isolation of P1 *argF* chloramphenicol (Cm) lysogens was that used for lysogenization with Mu dl(Ap *lac*).

Standard procedures. β-galactosidase was determined as described by Miller (26). Protein concentration was measured with Fluram (31). Isolation of outer membranes and gel electrophoresis were performed as published previously (12, 24). Isolation of phage-resistant mutants and the determination of phage and albomycin sensitivity by cross-streak tests followed the procedure described previously (4).

Chemicals. Streptonigrin was kindly supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Ferrichrome and albomycin were donated by H.-P. Fiedler of this institute. All the other chemicals were of the highest purity commercially available.

RESULTS

Isolation of mutants defective in ferric dicitrate transport. Mutants in *aroB* unable to synthesize enterochelin were used in this study so that citrate-dependent iron transport was the only high-affinity system unless other siderophores were added. Selection for ferric dicitrate transport mutants is difficult (32, 33). We therefore tried streptonigrin since iron enhances its bactericidal activity (35). Transport-deficient strains may better resist the antibiotic owing to their reduced iron content. To test this assumption, we measured the survivors in citrate-containing cultures of transport-active and transport-inactive strains during treatment with streptonigrin. Strain AB2847 *fec*$^+$ was rapidly killed (Fig. 1). The transport-deficient strains WA380 *fecA*, WA27 *fecB*, H1452 *tonB* survived to a large extent. The mutation in *exbB* of H1389 protected cells much less. Streptonigrin was therefore included in the plates used for selection of iron transport mutants after mutagenesis with MNNG or with Mu dl(Ap *lac*). The mutants obtained are listed in Table 2. The pattern of the outer membrane proteins related to iron, Cir through Fiu, was defined according to the method outlined in the legend to Fig. 2. Only those mutants were listed which were related to ferric dicitrate transport. Since β-galactosidase

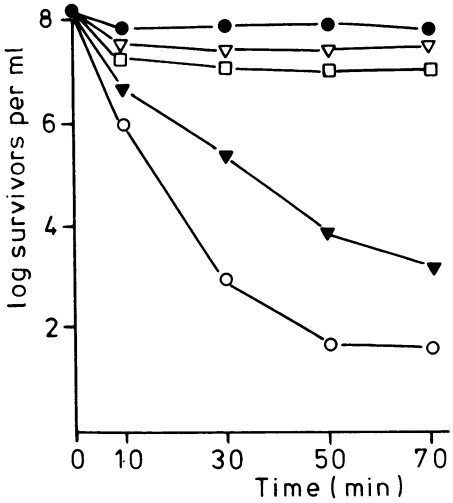

FIG. 1. Sensitivity of *E. coli* mutants to streptonigrin (5 μg/ml) in M9 medium supplemented with 10 mM citrate. At the times indicated, survivors were determined by plating on TY agar. Symbols: ●, strain WA380 *fecA aroB*; △, WA27 *fecB aroB*; H1452 *tonB aroB*; ▲, H1389 *exbB aroB*; ○, AB2847 *aroB*.

was only expressed in one of six *fecB*::Mu dl(Ap *lac*) mutants, a further selection procedure was applied by using MacConkey-lactose agar. All colonies with *lac* fusions in the correct orientation to iron-related genes (1–3, 12, 34) disclosed pink to red colors on such plates. *lac* fusions which were derepressible at low iron concentrations could be screened on MacConkey-lactose plates supplemented with 0.1 mM ferric ammonium sulfate on which a zone of low iron concentration was created by a filter paper strip impregnated with 20 mM 2,2'-dipyridyl (14). *lac* fusions to iron-regulated genes were red close to the paper strip and white further apart. The gradient in iron concentration allowed identification of mutants with altered regulatory properties. By this technique, two iron-regulated *fecB*::Mu dl(Ap *lac*) but not *fecA*::Mu dl(Ap *lac*) were found. Therefore, three additional selections with streptonigrin were carried out starting with the *lac* strains H455, ZI367, and H1443. Of 67 *tonB*$^+$ *exbB*$^+$ mutants unable to grow on ferric dicitrate as sole iron source, 11 were lacking the FecA protein. Transduction of *fec*$^+$ together with *zag*::Tn*10* from ZI345 *lac*$^+$ yielded ampicillin-sensitive *fec*$^+$ recipients indicating that Mu was inserted in the *fec* region. None of the mutants expressed β-galactosidase.

Expression of β-galactosidase in *fecB*::Mu dl(Ap *lac*) mutants under different conditions. Expression of β-galactosidase of strain H799 *fecB*::Mu dl(Ap *lac*) in nutrient broth that provides a limited concentration of iron (4) was slightly elevated and was further increased when the available iron was reduced by addition of dipyridyl (Table 3). Citrate induced synthesis of β-galactosidase strongly. Iron limitation further increased β-galactosidase activity. Addition of an excess of iron reduced expression of the enzyme in M9 medium in the presence and absence of citrate. Fluorocitrate was nearly as effective an inducer as was citrate.

Total withdrawal of iron by addition of deferri-ferrichrome to ZI395, a *fhuA* derivative unable to transport ferrichrome, strongly reduced induction of β-galactosidase in all media tested. This result demonstrates the requirement of iron for the citrate-mediated induction. The experiments were done under the same conditions employed for the withdrawal of iron by dipyridyl. The increase in the absorbance of the cultures was the same in the presence of both iron ligands. Lack of induction of the transport system with deferri-ferrichrome was therefore not caused by growth inhibition.

The *fecA* derivative ZI325 could not be induced (Table 3). Six additional MNNG-induced mutants were tested and showed the same phenotype. FecA seems to be required for induction.

Mutants in *tonB* are ferric dicitrate transport negative. To corroborate the finding obtained with *fecB* mutants that transport is not required for induction, we isolated the *tonB* mutants. Growth of the *tonB* donor BR158 was not stimulated by ferric dicitrate on NBD plates. The *tonB* recipient ZI419 *fecB*::Mu dl(Ap *lac*) disclosed no induction of β-galactosidase by ferric dicitrate (Table 3). Five spontaneous T1-resistant but T5-sensitive mutants of H799 (*tonB*) showed the same phenotype (not listed). However, transduction of *tonB* together with *zch*::Tn*10* (70% cotransduction) from H1452 into H799 resulted in ZI342 which induced β-galactosidase in response to citrate and dipyridyl (Table 3). Lower concentrations of citrate, 0.3 mM instead of 1 mM, yielded less β-galactosidase activity in the presence and absence of dipyridyl which was not observed in the *tonB*$^+$ strain H799 (Table 3). Six additional *tonB* mutants had the same phenotype (not listed). The latter *tonB* mutants were only partially T1 resistant in cross-streak tests and the *tonB* donor strain H1452 was not completely transport deficient since it disclosed a weak but clearly discernable growth promotion by ferric dicitrate on NBD plates. A result comparable to that of ZI342 was obtained with the *exbB* mutant ZI341 which showed a weak growth promotion and an induction depending on the citrate concentration. Induction of H799 *tonB*$^+$ *exbB*$^+$ was high at 0.3 mM citrate.

Mutants in *fur* expressed constitutively all iron transport systems tested. Examination of the iron dicitrate system was of interest since its induction required not only iron limitation like the other systems but also citrate. *fecB*::Mu dl(Ap *lac*) was transduced from H799 into H1290 *fur lac*. The resulting strain ZI406 *fecB*::Mu dl(Ap *lac*) *fur* contained a high β-galactosidase activity which was increased two- to three fold by citrate and slightly more by further adding dipyridyl (Table 3). This result shows that the citrate-dependent iron transport system is also under control of the *fur* locus.

Transduction experiments to determine the order of the *fec* loci. The cotransduction frequency of the markers *fec*$^+$ and *zag*::Tn*10* from strain ZI345 into the various *fec* mutants was

TABLE 2. Streptonigrin-resistant mutants of H1443[a]

No. of mutants induced by:	Membrane type	Phage T1 sensitivity	Albomycin sensitivity	Cotransduction with *zag*::Tn*10*	Mutant type
MNNG					
24	I	S	S	−	Not defined
2	II	S	S	+	*fecA*
15	III	S	S	+	*fecB*
6	IV	R	R	−	*tonB*
1	IV	S	R	−	*exbB*
Mu dl(Ap *lac*)					
17	I	S	S	−	Not defined
6	III	S	S	+	*fecB*
2	IV	R	R	−	*tonB*
1	IV	S	R	−	*exbB*

[a] S, Sensitive; R, resistant. Not defined, these mutants are not of the type listed. *zag*::Tn*10* is cotransducible with *fecA* and *fecB*.

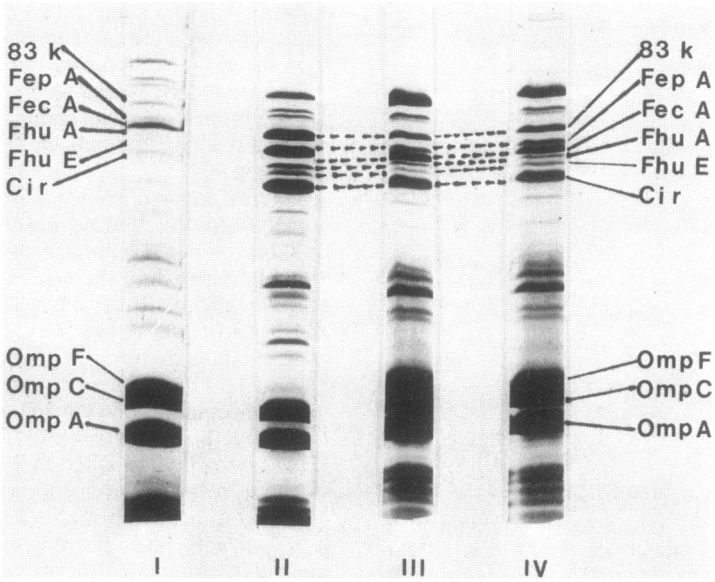

FIG. 2. Protein pattern of outer membranes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane I, strain H1443 *aroB*; lane II, ZI379 *fecA aroB*; lane III, H799 *fecB aroB*; lane IV, ZI396 *exbB aroB*. Cells were grown in TY medium that contains sufficient citrate to induce the iron transport system (see lane I, in which the iron-related proteins are suppressed except FecA). Owing to the lack of FecA in II, the other iron-related proteins are enhanced. Iron limitation in *fecB* (lane III) and *exbB* (lane IV) mutants leads to strong synthesis of FecA and of the other iron-related proteins.

determined. Selection was first for acquisition of tetracycline resistance and then for growth on iron dicitrate as sole iron source by using 100 tetracycline-resistant colonies in each experiment. Cotransduction of *argF* with *zag*::Tn*10* into strain PCO950 was determined to evaluate the cotransduction frequencies obtained between *fecA argF*$^+$ of the donor WA380 into the recipient ZI407 *fec*$^+$ *argF argI* (Table 4).

The *fecA*$^+$ transductants of the second experiment showed two phenotypes. A total of 82% grew on iron citrate plates as well as the donor strain, 18% grew slowly. Inspection of their outer membrane protein patterns revealed a fully induced FecA protein in the former and no induced FecA protein in the latter strains. This result suggests that WA380 is a *fecA* mutant, with an additional mutation in a locus that regulates *fec* expression. A number of such mutants were isolated and mapped in the *fec* region. They will be described elsewhere.

Additional transduction experiments not listed in Table 4 demonstrated that *fecB* cannot be cotransduced with *argF*, and *fecA*, *fecB*, *zag*::Tn*10* are not cotransducible with *proA,B* or *lac*.

Attempts to complement *fec* loci. Complementation experiments were performed to determine the number of loci involved in iron-dicitrate transport and to test *cis-trans* effects. Seven strains containing different F′ plasmids bearing chromosomal markers of the *lac* region were conjugated with the strains ZI311 and ZI370. Moreover, strains WA380 *fecA* and WA27 *fecB* were lysogenized with two derivatives of phage P1 which contain an 11-kilobase-pair fragment of the chromosome between two IS1 insertion elements in the *argF* region (36). Neither the F′ plasmids nor the P1 derivatives could complement *fec* mutants.

DISCUSSION

Streptonigrin turned out to be a suitable antibiotic to select for mutants deficient in ferric dicitrate transport. Since previously there was no positive selection, the isolation of such mutants was laborious (32, 33). Streptonigrin is subject to autoxidation which in turn yields reactive oxygen radicals and peroxide (17, 18, 35). The formation of the latter is frequently catalyzed by iron. A lower iron content reduced the activity of streptonigrin so that a lower amount of reactive oxygen derivatives were formed, and consequently, less DNA damage occurred. We have shown previously that streptonigrin can successfully be used for selection of mutants deficient in aerobactin synthesis and ferric-aerobactin transport (3) so that this antibiotic may generally be applicable to study iron metabolism.

This paper provides further evidence that iron transport via citrate is not required for induction of the transport system since transport-deficient mutants in *fecB* retained inducibility. Transport is defined here as the ability of cells to concentrate iron in an energy-dependent process such that iron is retained by the cells when they are washed with salt solutions on membrane filters. Since, at pH 7, iron is never present in any quantity as free ferric ion but is always ligated by some molecule, the actual transport mechanism is complex.

The other interesting finding is the apparent requirement of the FecA outer membrane protein for induction. Iron, provided it is complexed to citrate, binds to the FecA protein (32). The question arises as to how much further ferric dicitrate has to be translocated to act as the inducer. Presumably, iron and citrate have to act as a complex. In the presence of a 20-fold excess of citrate over iron, the complex consists mainly of ferric dicitrate (30). In this respect, it is important that induction is *tonB* dependent. The tight *tonB* mutants, for which BR158 is an example, were inactive in ferric dicitrate transport; in the *tonB fecB*::Mu dl(Ap *lac*) derivative, β-galactosidase was not inducible. We have reported previously (21) that, in strain BR158, the FecA protein was also not inducible. In the *fecB*::Mu dl(Ap *lac*) mutant ZI342 which has received the *tonB* mutation from H1452, β-galactosidase could be induced provided a suffi-

TABLE 3. Specific activity of β-galactosidase in mutants at different culture conditions[a]

Strain	Culture conditions[a]	β-Galactosidase activity
H799 *fecB* Mu dl(Ap *lac*)	NB	20
	NB/Dip	50
	NB/Cit	250
	NB/0.3 mM Cit	235
	NB/Cit/Dip	300
	NB/0.3 mM Cit/Dip	290
	M9/$FeSO_4$	10
	M9/Dip	30
	M9/$FeSO_4$/Cit	90
	M9/Cit	250
	M9/Cit/Dip	265
	NB/Fluorocit	230
ZI395 *fhuA fecB*::Mu dl(Ap *lac*)	NB	22
	NB/Cit/Def	50
	M9/Cit/Def	30
	NB/Fluorocit/Def	35
ZI325 *fecA fecB*::Mu dl(Ap *lac*)	NB	4
	NB/Cit	7
	NB/Cit/Dip	6
ZI342 *tonB fecB*::Mu dl(Ap *lac*)	NB	20
	NB/Cit	190
	NB/0.3 mM Cit	100
	NB/Cit/Dip	230
	NB/0.3 mM Cit/Dip	115
ZI419 *tonB-158 fecB*::Mu dl(Ap *lac*)	NB	25
	NB/Cit	30
	NB/Cit/Dip	30
ZI341 *exbB fecB*::Mu dl(Ap *lac*)	NB	25
	NB/Cit	210
	NB/0.3 mM Cit	170
	NB/Cit/Dip	275
	NB/0.3 mM Cit/Dip	190
ZI406 *fur fecB*::Mu dl(Ap *lac*)	NB	120
	NB/Cit	270
	NB/Cit/Dip	295

[a] Cells were pregrown overnight in tryptone-yeast extract medium. They were transferred into either nutrient broth (NB), or M9 medium supplemented with 1 mM citrate (Cit), 0.1 mM 2,2'-dipyridyl (Dip), 0.2 mM $FeSO_4$, 1 mM fluorocitrate (Fluorocit), or 50 μM deferri-ferrichrome (Def). Dipyridyl or deferri-ferrichrome was added after 2 h growth in the media. β-Galactosidase activity was measured after additional growth for 3 and 5 h. The values given are the average of two determinations. They varied by 10% in absolute terms but within one experiment even the small differences obtained under the various culture conditions and among the different strains were reproducibly found.

ciently high concentration of ferric dicitrate was supplied. Since H1452 showed a weak growth promotion by ferric dicitrate, the mutation in *tonB* did not entirely inactivate the transport system. Owing to the *fecB* insertion mutation, the *fecB tonB* double mutant was completely transport inactive, showing that $tonB^+$ was active in the induction process independent of transport or release of iron into the cytoplasmic membrane or the cytoplasm. Also, partially T1-sensitive *tonB* mutants not listed in this paper showed the same phenotype. If transport from the outer membrane to the cytoplasmic membrane is *tonB*-dependent, as has been argued for several reasons (5, 6, 10, 15, 22), the ferric dicitrate complex would not stay at the outer membrane receptor for induction. Rather, the ferric dicitrate complex would enter the periplasm and act from there by, for example, binding to a protein that spans the cytoplasmic membrane. It could also be transferred to a periplasmic binding protein that translocates the complex to a cytoplasmic membrane protein involved in induction. The same protein could also interact with the transport system in the cytoplasmic membrane. Dual functions are known for certain periplasmic binding proteins that channel sugars to the chemotactic as well as to the transport system (19, 23). Search for a periplasmic binding protein for $^{55}Fe^{3+}$-labeled ferric dicitrate, for altered protein patterns in the mutants, and in response to induction did not disclose one (data not shown).

The notion that the FecA protein is required for induction rests on 11 *fecA*::Mu dl(Ap *lac*) mutants and on 6 *fecA fecB*::Mu dl(Ap *lac*) double mutants which expressed no β-galactosidase activity. It is unlikely that all of the Mu insertions in *fecA* have the wrong orientation, and that all of the *fecA* mutants are in fact regulatory mutants. Rather, the mapping data suggest that *fecA* and *fecB* are not controlled by the same promoter. The transposon *zag*::Tn*10* between both loci would have to inhibit expression of either of them.

If FecA is required for induction, one has to ask whether it is also necessary for transport because lack of synthesis of the transport system would show apparent lack of activity.

TABLE 4. Cotransduction frequency of markers in the *fec* region[a]

Recipient	Unselected marker	Cotransduction frequency (%)
WA27 *fecB*	*fecB*$^+$	63
WA380 *fecA*	*fecA*$^+$	50
H799 *fecB*::Mu dl(Ap *lac*)	*fecB*$^+$	11
ZI379 *fecA*::Mu dl(Ap *lac*)	*fecA*$^+$	5
PC0950 *argI argF*	*argF*$^+$	14
ZI407 *aroB argI argF*	*fec*	8

[a] In the first five experiments, strain ZI345 *fec*$^+$ *zag*::Tn*10* was used as donor on which phage P1 was grown. Tetracycline resistance was selected. In the last experiment, strain WA380 *fecA* was the donor, and growth on arginine was selected.

This question was studied with *fur* mutants which somewhat express the ferric dicitrate transport system in M9 medium without citrate (12). *fecA* mutants of *fur* strains were transport inactive, showing that FecA performs a dual role, one for induction and one for transport (data not shown).

Previously, it has been shown that the minimal concentration of citrate for induction of the transport system was reduced from 100 μM in *fur*$^+$ strains to 10 μM in *fur* derivatives. This finding could now be supported by quantitative measurements of β-galactosidase. The low concentration of citrate in NB medium yielded in the *fur* strain a sixfold-higher enzyme activity than in *fur*$^+$ strains (Table 3). Addition of citrate led to no higher β-galactosidase activity than the level reached in *fur*$^+$ strains. We consider the *fur* protein as a repressor when iron is bound to it (1, 12, 13). The repressor was inactivated when iron dissociated at low iron concentrations. Iron was certainly also required for the action of citrate since its removal by deferri-ferrichrome prevented induction of β-galactosidase in the fusion strain. We have shown previously that the citrate-dependent transport system could not be induced when iron was trapped either in ferrichrome or in enterochelin (21). The mutants used in these experiments were specifically inactive in ferrichrome and enterochelin transport.

The transduction experiments prove that the order of genes is *proAB*, *argF*, *fecA*, *fecB*, *lac*. None of the F′ plasmids bearing various fragments of this region contained the *fec* locus. The P1 *argF* Cm phages were unable to complement *fec* mutants so that *fec* is not located between the two IS1 elements of this region. The region between *proA,B* and *lac* is notoriously difficult to study genetically. It contains an accumulation of IS elements. Identical IS elements can cause inversions and deletions which lead to changes in the order and the distances of genes.

Only *E. coli* K-12 contains *argF* and *argI* which encode the same enzyme. *argF* is lacking in *E. coli* W and *S. typhimurium* (20). Both strains are also devoid of the citrate-dependent iron transport system (8). These data suggest that *E. coli* K-12 received the *argF fec* region from other organisms, possibly by plasmids from which it was transposed into the K-12 chromosome. In fact, transposition of the *argF* region has been observed (36).

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