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Mutations Affecting Iron Transport in *Escherichia coli*

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A mutant of *Escherichia coli* K-12 unable to form an essential component of the enterochelin-dependent iron transport system has been isolated. This strain carries a mutation in a gene designated *fep*, mapping close to two genes, *entA* and *entD*, concerned with enterochelin synthesis. Strain AN102, which carries the *fep*$^-$ allele, accumulates large quantities of enterochelin and gives a growth response to sodium citrate. The cytochrome b_1 and total iron content, and the measurement of the uptake of $^{55}Fe^{3+}$, indicate an impairment of the enterochelin-dependent iron transport system. The growth response to sodium citrate is related to the presence, in strain AN102, of an inducible citrate-dependent iron transport system.

A relationship between compounds containing 2,3-dihydroxybenzoate and the metabolism of iron was first suggested by Ito and Neilands (7) after the isolation of 2,3-dihydroxybenzoylglycine from cultures of *Bacillus subtilis* grown under conditions of iron deficiency. 2,3-Dihydroxybenzoate was later shown to be a growth factor for multiple aromatic auxotrophs of *Escherichia coli* (3). This growth requirement could be replaced either by high concentrations of iron, or by sodium citrate, depending on the strain of *E. coli* examined (17). A study of the enzymes involved in the biosynthesis of 2,3-dihydroxybenzoate in *Aerobacter aerogenes* and *E. coli* has shown their levels to be regulated by the concentration of iron in the growth medium (18). 2,3-Dihydroxybenzoate is metabolized further in *E. coli* and *A. aerogenes* to form enterochelin, a cyclic trimer of 2,3-dihydroxy-*N*-benzoylserine (10; O'Brien and Gibson, Biochim. Biophys. Acta, *in press*). 2,3-Dihydroxy-*N*-benzoylserine has been isolated from culture supernatants of *E. coli* (2) but this compound may be a degradation product of enterochelin (10). Wang and Newton (15, 16) isolated a mutant strain of *E. coli* B/r unable to convert 2,3-dihydroxybenzoate to its serine conjugates. The mutant strain required a higher concentration of iron for maximal growth than did the wild-type strain, and measurements of $^{59}Fe^{3+}$ uptake in iron-starved cells indicated an impaired iron-transport activity.

Somerville (13) isolated mutants with deletions in the tryptophan region of the *E. coli* genome from a strain carrying a mutation in the common pathway of aromatic biosynthesis. The deletion mutants were unable to grow on a citrate-glucose-mineral salts medium supplemented with the aromatic amino acids and 4-aminobenzoate unless 2,3-dihydroxybenzoate was added. Wang and Newton (15, 16) also studied mutants carrying a deletion in the tryptophan region of the genome and demonstrated that they require a higher concentration of iron for maximal growth than does the wild-type strain. The measurement of the uptake of $^{59}Fe^{3+}$ by iron-starved cells of such deletion mutants indicated an impairment of the iron transport system that was not rectified by the addition of either citrate or 2,3-dihydroxy-*N*-benzoylserine.

The present paper describes a biochemical and genetic investigation of a mutant strain of *E. coli* K-12 lacking another component of the iron uptake system(s). The properties of this mutant are compared with those of mutants blocked in the pathway of biosynthesis of enterochelin.

MATERIALS AND METHODS

Chemicals. Chemicals used were of the highest purity obtainable and, unless otherwise specified, were not further purified. $^{55}Fe^{3+}$ was obtained as a carrier-free solution of $FeCl_3$ in 0.1 M HCl from The Radiochemical Centre, Amersham, England. Enterochelin and 2,3-dihydroxy-*N*-benzoylserine were isolated from culture supernatants as described previously (9; O'Brien and Gibson, *in press*).

Bacterial strains. All the strains used were derived from *E. coli* K-12 and are listed in Table 1.

Culture media. The medium used for growth of cells, except those used in iron-uptake experiments, was double-strength medium 56 described by Monod et al. (8). To the sterilized mineral salts base was

TABLE 1. *Strains of Escherichia coli K-12*

Strain	Relevant genetic loci[a]	Other information
AB259	*thi*⁻	Obtained from J. Pittard
AN31	*entA*⁻, *thi*⁻	Isolated after MNNG[b] treatment of AB259
AN34	*his-4*⁻, *proA*⁻, *argE*⁻, *pheA*⁻, *tyrA*⁻, *trp*⁻	Obtained from M. Huang
AN42	*entD*⁻, *his-4*⁻, *proA*⁻, *argE*⁻, *pheA*⁻, *tyrA*⁻, *trp*⁻	Isolated after MNNG treatment of AN34
AB3311	*metB*⁻	Hfr Reeves 1
AN96	*metB*⁻, *fep*⁻	Isolated after MNNG treatment of AB3311
AB1515	*leu*⁻, *proC*⁻, *purE*⁻, *trp*⁻, *thi*⁻	Obtained from J. Pittard
AN102	*leu*⁻, *proC*⁻, *trp*⁻, *thi*⁻, *fep*⁻	Derived from AB1515 by transduction with P1kc grown on AN96
AN90	*leu*⁻, *proC*⁻, *trp*⁻, *thi*⁻, *entD*⁻	Derived from AB1515 by transduction with P1kc grown on AN42
AN103	*leu*⁻, *proC*⁻, *trp*⁻, *thi*⁻, *entA*⁻	Derived from AN90 by transduction with P1kc grown on AN31
AB3248	*aroF*⁻, *aroG*⁻, *aroH*⁻, *proA*⁻, *argE*⁻, *ilv*⁻, *his-4*⁻	Obtained from J. Pittard

[a] The following symbols stand for structural genes concerned with various biosynthetic pathways: *thi*, thiamine; *ent*, enterochelin; *his*, histidine; *pro*, proline; *arg*, arginine; *phe*, phenylalanine; *tyr*, tyrosine; *trp*, tryptophan; *met*, methionine; *leu*, leucine; *pur*, purine; *ilv*, isoleucine valine; *aroF*, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase (tyr); *aroG*, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase (phe); *aroH*, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase (trp). The gene *fep* codes for an essential component of the enterochelin-dependent iron-transport system as described in this paper.

[b] *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

added a sterile solution of either glucose or succinate at a final concentration of 30 mM. Additional growth factor supplements were added, when necessary, at the following final concentrations: enterochelin, 1 μM; 2,3-dihydroxybenzoate, 10 μM; 2,3-dihydroxy-*N*-benzoylserine, 20 μM; L-histidine, 200 μM; L-proline, 1 mM; L-arginine, 700 μM; L-phenylalanine, 200 μM; L-tryptophan, 100 μM; L-tyrosine, 200 μM; L-methionine, 150 μM; L-leucine, 300 μM; L-isoleucine, 320 μM; L-valine, 360 μM; adenine, 150 μM; thiamine, 1 μM; sodium citrate, 10 mM.

Isolation of mutants. Cells were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and mutants were selected on solid media as described previously (4). The mutants sought were those unable to grow on succinate as sole carbon source but able to grow on glucose.

Selection media for transduction experiments. Strains carrying mutant alleles were selected by their growth response, on succinate media, to citrate (for *fep*⁻), to 2,3-dihydroxybenzoate (for *entA*⁻), to enterochelin or to 2,3-dihydroxy-N-benzoylserine (for *entD*⁻). The basal medium used was the medium 56 described by Monod et al. (8), except that no ferrous sulfate was added. When using a mutant strain unable to synthesize enterochelin as recipient in transduction experiments, 0.1 mM 2,2'-dipyridyl was included in the selection media to reduce background growth.

Measurement of growth. Cultures of 10 ml in 120-ml side arm flasks were incubated at 37 C in a New Brunswick Metabolyte shaker bath. Turbidity was measured at intervals in a Klett-Summerson colorimeter with a blue filter.

Transduction techniques. The technique used for transduction experiments was that described by Pittard (12).

Examination of culture supernatants for 2,3-dihydroxybenzoate and its derivatives. The total amount of 2,3-dihydroxybenzoate, free and combined, in each culture supernatant was measured by the following procedure. Portions (25 ml) of supernatant were acidified with 2.5 ml of 2.5 M H_2SO_4 and extracted with two 25-ml volumes of redistilled ethylacetate. The combined extracts were evaporated to dryness under vacuum, and the residue was shaken with 2.5 or 25 ml of ethylacetate (depending on the amount of 2,3-dihydroxybenzoate present), and the absorbance at 315 nm of the resulting solution was measured (ϵ_M 3,100) (O'Brien and Gibson, *in press*). The solution was then evaporated, the residue was taken up in a known volume of ethyl acetate, and a measured sample was applied to a 20-cm cellulose thin-layer plate which was developed and examined as previously described (10).

Estimation of cytochrome b_1. The cytochrome b_1 content was measured in a membrane-containing fraction prepared by ammonium sulfate precipitation as described by Cox et al. (Cox, Newton, Gibson, Snoswell, and Hamilton, Biochem. J., *in press*) and diluted to a concentration of about 10 mg of protein/ml. Difference spectra between $Na_2S_2O_4$-reduced and -oxygenated samples were recorded. The wavelength pair and molar extinction coefficient used to estimate cytochrome b_1 were ΔE_{560}-ΔE_{575} (ϵ_M 17,500).

Estimation of iron. Iron determinations were carried out by atomic-absorption spectroscopy.

Measurement of iron uptake. Cells used for studies of iron uptake were grown on glucose-nutrient agar slopes, inoculated directly into a low-iron medium (EGA), and incubated with shaking at 37 C. After growth into early stationary phase, a 0.1-ml portion was used to inoculate 10 ml of fresh EGA medium, and the culture was incubated as before. The cells were harvested by centrifugation, either in the mid- or late-exponential phase of growth. The EGA medium contained: 10 mM ammonium sulfate, 1 mM $MgCl_2$, 40 μM $CaCl_2$, 40 mM KH_2PO_4, and 25 mM glucose. The *p*H was adjusted to 6.9 with NaOH, and the appropriate growth factors were added. The medium was made with glass-distilled water which had been passed through a column of freshly cycled Dowex 50 (H^+). The final medium contained about 0.7 μM iron, and was supplemented, if required, with 1 μM $FeCl_3$ or 10 mM sodium citrate, or both. All glassware used was autoclaved with a 0.5% ethylenediaminetetraacetic acid (EDTA) solution, rinsed five times with glass-distilled water, and then autoclaved twice with glass-distilled de-ionized water.

The medium used for uptake measurements was as above, except that the concentration of phosphate was halved and tryptophan was omitted. The harvested cells (*see above*) were suspended in the uptake medium to give a turbidity of 200 Klett units. All subsequent incubations and transfers were carried out by using polyethylene or polypropylene apparatus. Another sample of the uptake medium, containing $^{55}FeCl_3$ (1 μM), stabilized with 10 μM sodium citrate, was shaken 0.5 to 1 hr at 37 C and membrane-filtered (filters, Millipore Corp.) before use. Equal volumes of this solution and of the cell suspension were mixed at zero time with the compounds to be tested, and the suspension was then sampled at intervals. The samples were filtered through membrane filters which were then washed twice with 2 ml of 0.9% NaCl (w/v) containing 1 mM Fe^{3+}-EDTA complex. The dried filters were placed in scintillation vials and counted with a Triton-toluene (2:1, v/v) scintillant (11) in a Packard 3320 counter.

RESULTS

Mutant strains of *E. coli* K-12 unable to synthesize enterochelin have been isolated in this laboratory (R. K. J. Luke, Ph.D. Thesis, Australian National University, Canberra, 1970). One of these, strain AN31, carries a mutation in the *entA* gene affecting one of the enzymes converting chorismate to 2,3-dihydroxybenzoate. Another of these, strain AN42, carries a mutation in the *entD* gene, affecting an enzyme after 2,3-dihydroxybenzoate in the pathway of biosynthesis of enterochelin. The genes *entA* and *entD* have been shown, by interrupted mating, to be between the *purE* and *aroG* genes on the *E. coli* genome (R. K. J. Luke, Ph.D. Thesis, Australian National University, Canberra, 1970).

During an examination of mutant strains of *E. coli* K-12 unable to grow on succinate but able to grow on glucose as sole carbon source, a mutant (strain AN96) was isolated which would grow on succinate in the presence of citrate. This mutant, when grown on glucose, contained low levels of cytochromes and excreted high levels of enterochelin into the growth medium. It therefore seemed likely that the mutation in strain AN96 may have affected some component of the iron-transport system. The mutant allele *fep*$^-$, present in strain AN96, and the two mutant alleles *entA*$^-$ and *entD*$^-$ were each mapped by cotransduction. Transductants containing each of the mutant alleles were prepared from a common parent strain to provide a set of strains suitable for comparing the effects of the various mutations.

Mapping of the fep, entA, and entD genes by cotransduction. *E. coli* strain AB1515 carries a mutation in the *purE* gene. The generalized transducing phage P1kc was grown on strains AN96, AN31, and AN42, or on derivatives of these strains, and the resulting lysates used to transduce *purE*$^+$ into strain AB1515; the transductants obtained were then tested for the presence of the *fep*$^-$, *entA*$^-$, or *entD*$^-$ alleles, respectively. Cotransduction of the *entD*$^-$ allele with the *purE*$^+$ allele occurred at a frequency of 13% (10 colonies out of 78 colonies tested) and cotransduction of the *fep*$^-$ allele with the *purE*$^+$ allele occurred at a frequency of 2% (1/54), but no cotransduction between the *entA*$^-$ allele and the *purE*$^+$ allele could be demonstrated (0/77). *E. coli* K-12 strain AB3248 carries a mutation in the *aroG* gene, and the P1kc lysates prepared on strains AN96 and AN31 were used to transduce *aroG*$^+$ into strain AB3248. The transductants obtained were then tested for the presence of the *fep*$^-$ or *entA*$^-$ alleles. No cotransduction could be demonstrated between either of the mutant alleles and the *aroG*$^+$ allele (*fep*$^-$/*aroG*$^+$, 0/126; *entA*$^-$/*aroG*$^+$, 0/77).

The *purE*$^+$, *fep*$^-$ cotransductant (AN102) and a *purE*$^+$, *entD*$^-$ cotransductant (AN90) derived from AB1515 were purified and retained for further work. The P1kc lysates prepared on strains AN31 and AN42 were used to transduce *fep*$^+$ into strain AN102. The transductants obtained were then tested for the presence of *entA*$^-$ or *entD*$^-$ alleles. Cotransduction of the *entD*$^-$ allele with the *fep*$^+$ allele occurred at a frequency of 74% (53/72), and cotransduction of the *entA*$^-$ allele with the *fep*$^+$ allele occurred at a frequency of 54% (37/68). The P1kc lysate prepared on strain AN31 was used to transduce *entD*$^+$ into strain AN90. The transductants obtained were then tested for the presence of the *entA*$^-$ allele. Cotransduction of the *entA*$^-$ allele with the *entD*$^+$

allele occurred at a frequency of 30% (33/109). An *entA*$^-$, *entD*$^+$ cotransductant (AN103) was purified and retained for further work. The frequencies of cotransduction between the various markers described above are summarized in Fig. 1.

Growth characteristics of strain AN102 (fep$^-$). Strain AN102 (*fep*$^-$) grows slowly with glucose as sole carbon source in the double-strength minimal medium 56 but was unable to grow when succinate was used as the sole source of carbon. Enterochelin (1 μM) or 2,3-dihydroxybenzoate (10 μM) failed to stimulate the growth of this strain, although at these concentrations the compounds gave maximal growth of strains AN90 (*entD*$^-$) or AN103 (*entA*$^-$), respectively. However, the presence of 10 mM citrate in the medium greatly increased the growth rate of strain AN102 (*fep*$^-$) with either glucose or succinate as sole carbon source (Fig. 2). Citrate also stimulated the growth of AN90 (*entD*$^-$) and AN103 (*entA*$^-$).

Enterochelin and related compounds in culture supernatants. Enterochelin and a number of related compounds are excreted by *E. coli* K-12 into the growth medium. Although iron deficiency results in greater excretion of the compounds, wild-type *E. coli* AB1515, even when grown in the double-strength 56 medium containing about 8 μM iron, formed the various compounds in amounts detectable by two-dimensional chromatography (Fig. 3a). Unlike strain AB1515, strain AN102 (*fep*$^-$) in the mid-exponential phase of growth mainly excreted enterochelin into the growth medium (Fig. 3c). The concentration of enterochelin excreted was about 30 μM which is about 10 times the total amount of 2,3-dihydroxybenzoate-containing compounds excreted by strain AB1515. Under similar conditions of growth, strain AN90 (*entD*$^-$) accumulated about 100 μM 2,3-dihydroxybenzoate (see Fig. 3b), whereas strain AN103 (*entA*$^-$) did not form detectable quantities of any compounds containing 2,3-dihydroxybenzoate. Since citrate promoted the growth of strain AN102 (*fep*$^-$), culture supernatants of this strain grown to mid-exponential phase in the presence of 10 mM citrate were examined. The total amount of 2,3-dihydroxybenzoate-containing compounds was less than one-tenth of the amount formed in the absence of citrate. The amount of enterochelin formed was lower than in the absence of citrate, but the relative concentration of 2,3-dihydroxybenzoate had increased (Fig. 3d).

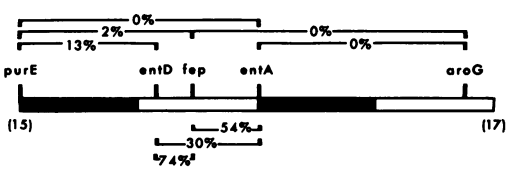

FIG. 1. *Genetic map showing the approximate positions of the fep, entD, and entA genes in relation to the purE and aroG genes. The cotransduction frequencies are shown, and the divisions are equal to about 1-min intervals, although the figures in parentheses are the positions of the purE and aroG genes according to the map of Taylor and Trotter (16).*

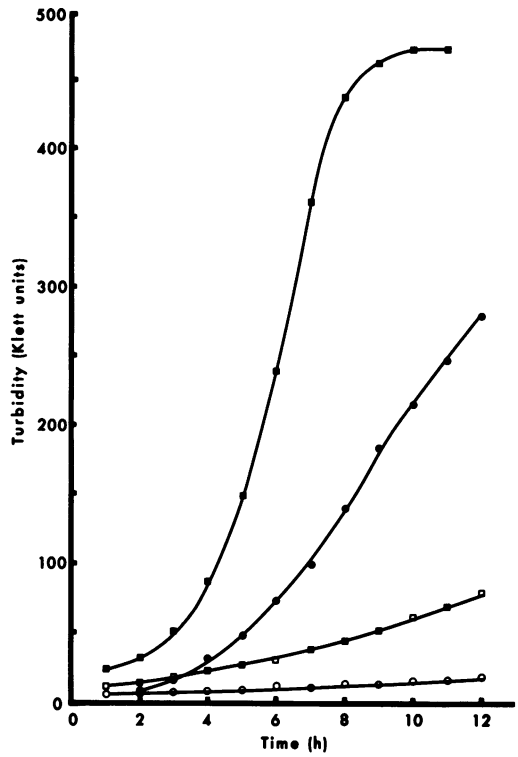

FIG. 2. *Growth of strain AN102 (fep) on media with either glucose (□, ■) or succinate (○, ●) as sole source of carbon in the presence (■, ●) or absence (□, ○) of citrate (10 mM). The conditions of growth are described in the text.*

Concentrations of total iron and cytochrome b_1. Crude cell extracts of strain AB1515, AN102 (*fep*$^-$), AN103 (*entA*$^-$), and AN90 (*entD*$^-$) were prepared and fractionated by ammonium sulfate precipitation into a "membrane-containing" fraction and a "soluble" fraction. Membrane fractions from strains AB1515, AN103 (*entA*$^-$), and AN90 (*entD*$^-$) contain the same amounts of iron (about 8.5 nmoles/mg of protein) but those from strain AN102 (*fep*$^-$) contained less than half of this amount (Table 2). These values were not affected by incubating the cell extract with 10 mM EDTA for 3 hr at 4 C before ammonium sulfate precipitation. When strain AN102 (*fep*$^-$) was

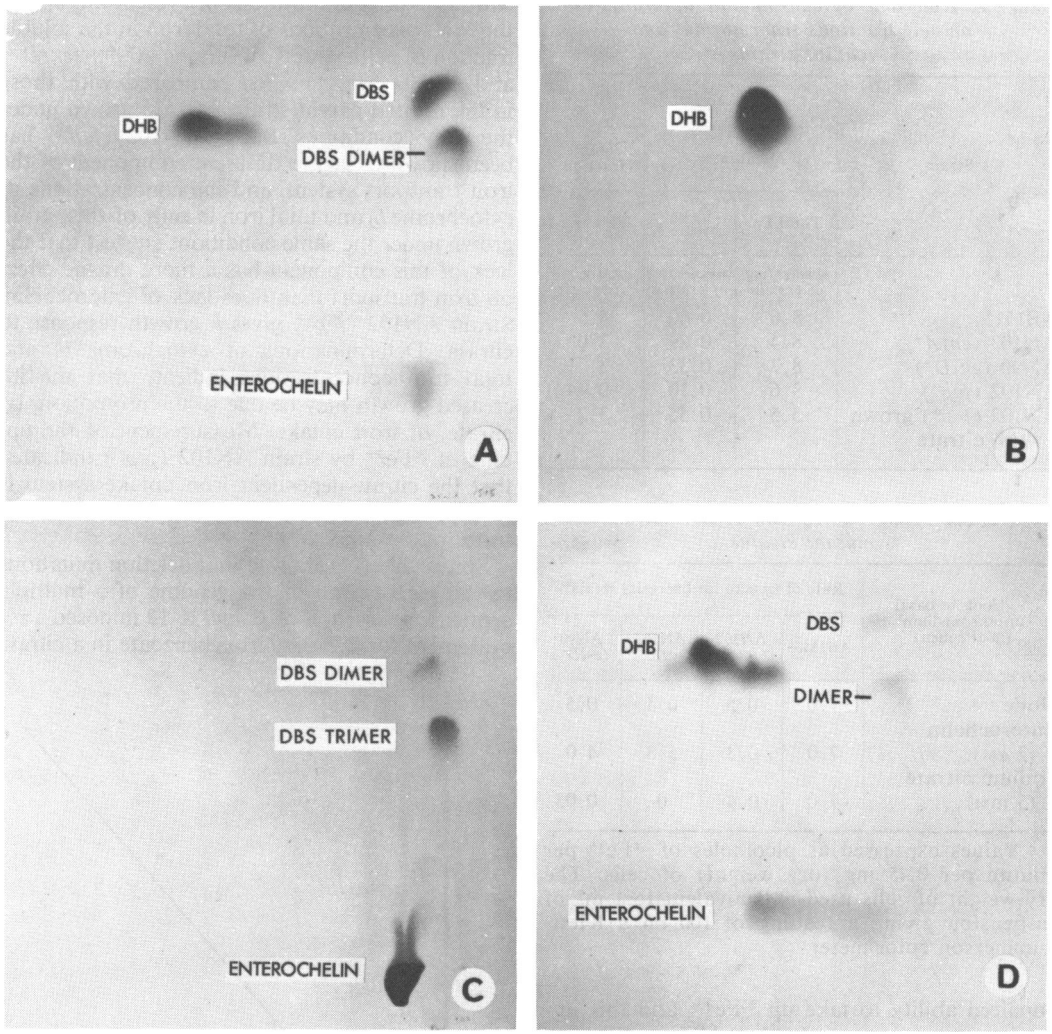

FIG. 3. *Two-dimensional chromatograms showing 2,3-dihydroxybenzoate-compounds extracted from culture supernatants. A, strain AB1515; B, strain AN90 (entD⁻); C, strain AN102 (fep⁻) grown on glucose; D, strain AN102 (fep⁻) grown on glucose plus citrate. The amounts chromatographed were equivalent to 24 ml of supernatant for A and D, and 8 ml of supernatant for B and C. Abnormal R_F values and "tailing" in D were due to chromatography twice in the first dimension because of lipid in the ethyl acetate extracts. Abbreviations: DHB, 2,3-dihydroxybenzoate; DBS, 2,3-dihydroxy-N-benzoylserine; DBS dimer and DBS trimer, dimer and linear trimer of 2,3-dihydroxy-N-benzoylserine, respectively.*

grown in the presence of citrate the level of iron in the membranes was increased but was still less than that found for the other strains.

The concentrations of iron in the soluble fractions of strains AN90 (*entD⁻*) and AN103 (*entA⁻*) are lower than in the soluble fractions of strain AB1515. The iron concentration in the soluble fraction from strain AN102 (*fep⁻*) is only 20% of that in strain AB1515 (Table 2). The concentrations of cytochrome b_1 (Table 2) also support the conclusion that strains AN90 (*entD⁻*), AN103 (*entA⁻*), and AN102 (*fep⁻*) are iron-deficient, the deficiency being most pronounced in strain AN102 (*fep⁻*). The cytochrome b_1 concentration in strain AN102 (*fep⁻*) grown in the presence of 10 mM citrate is similar to that in AB1515.

Measurements of iron uptake. Cell suspensions of strains AB1515, AN102 (*fep⁻*), AN103 (*entA⁻*), and AN90 (*entD⁻*), grown under iron-deficient conditions, were tested for their ability to take up $^{55}Fe^{3+}$. The wild-type strain AB1515 was able to take up $^{55}Fe^{3+}$ rapidly without any additions to the basal uptake medium (Table 3). Strains AN103 (*entA⁻*) and AN90 (*entD⁻*) had an

TABLE 2. *Iron and cytochrome b_1 determinations on cell fractions from normal and mutant strains*

Strain	Cell fraction		
	Membranes		Soluble fraction
	Total Fe	Cytochrome b_1	Total Fe
	nmoles/mg of protein	*nmoles/mg of protein*	*nmoles/mg of protein*
AB1515	8.6	0.60	4.5
AN103 (*entA*$^-$)	8.4	0.29	3.0
AN90 (*entD*$^-$)	8.7	0.33	3.2
AN102 (*fep*$^-$)	3.6	0.10	0.9
AN102 (*fep*$^-$) grown with citrate	5.5	0.55	3.2

TABLE 3. *Uptake of iron by normal and mutant strains*

Addition to basal uptake medium (final concn)	Rate of uptake for bacterial strain[a]			
	AB1515	AN102 (*fep*$^-$)	AN103 (*entA*$^-$)	AN90 (*entD*$^-$)
None	7.0	0.5	0.5	0.5
Enterochelin (2 μM)	7.0	0.3	5.8	4.0
Sodium citrate (5 mM)	7.0	0.4	0	0.03

[a] Values expressed as picomoles of $^{55}Fe^{3+}$ per minute per 0.45 mg (dry weight) of cells. The dry weight of cells used is equivalent to 1 ml of suspension giving a reading of 100 on a Klett-Summerson colorimeter.

impaired ability to take up $^{55}Fe^{3+}$, and this impairment was overcome by the addition of enterochelin. The ability of strain AN102 (*fep*$^-$) to take up $^{55}Fe^{3+}$ efficiently was not restored by the addition of either enterochelin or citrate (Table 3). Strain AN102 has, however, been shown to give a growth response to citrate, and cells grown in the presence of citrate have normal cytochrome b_1 levels (Table 2). The uptake of iron by strain AN102 (*fep*$^-$) was examined after growth of this strain in the presence of citrate. Such cells showed a citrate dependent uptake of $^{55}Fe^{3+}$ (Fig. 4).

DISCUSSION

The experiments reported in this paper confirm the involvement of enterochelin in the iron-transport system of *E. coli* K-12. Thus strains AN90 (*entD*$^-$) and AN103 (*entA*$^-$) have an impaired ability to take-up $^{55}Fe^{3+}$ in the absence of added enterochelin. This impairment may be correlated with the low concentrations of cytochrome b_1 and the low concentrations of total iron in the soluble fraction of cell extracts of strains AN90 (*entD*$^-$) and AN103 (*entA*$^-$) when compared with those in the normal parent strain AB1515 grown under the same conditions. Strain AN102 (*fep*$^-$) has been shown to lack a different component of the iron transport system, and the concentrations of cytochrome b_1 and total iron in cells of this strain grown under the same conditions suggest that the lack of this component has a more drastic effect on iron transport than does lack of enterochelin. Strain AN102 (*fep*$^-$) gives a growth response to citrate. Determinations of cytochrome b_1 and total iron concentrations indicate that the increased growth may be due to the promotion, by citrate, of iron uptake. Measurement of the uptake of $^{55}Fe^{3+}$ by strain AN102 (*fep*$^-$) indicated that the citrate-dependent iron uptake system is only present in cells grown in the presence of citrate.

Somerville (13) showed that deletion mutations in the *tonB* region of the genome of a multiple aromatic auxotroph of *E. coli* K-12 imposed a requirement for 2,3-dihydroxybenzoate in a citrate

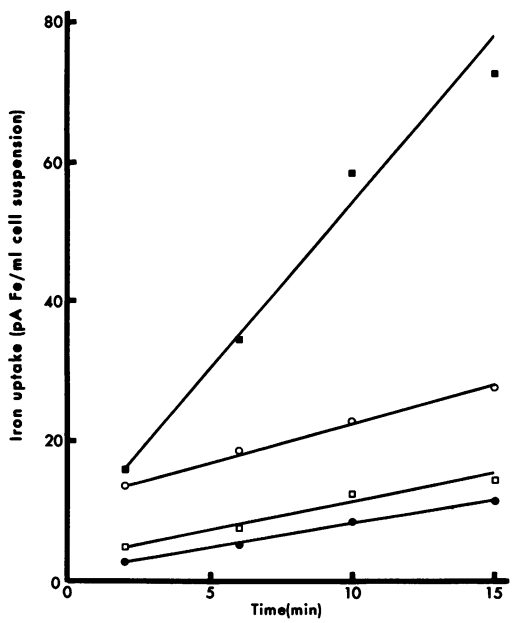

FIG. 4. *Effect of sodium citrate on the uptake of $^{55}Fe^{3+}$ by cells of strain AN102 (fep$^-$) grown with and without citrate. Symbols: ○, cells grown without citrate, no addition to the uptake medium; ●, cells grown without citrate, citrate (5 mM) added to the uptake medium; □, cells grown with citrate (10 mM), no addition to uptake medium; ■, cells grown with citrate (5 or 10 mM) added to the uptake medium. For conditions of growth of cells and details of uptake medium, see text.*

medium, presumably indicating that in such a deletion mutant the citrate-dependent iron uptake mechanism is impaired, but the enterochelin system is still operative. However, Wang and Newton (15, 16) showed that *tonB* deletion mutants able to form 2,3-dihydroxy-*N*-benzoylserine, and presumably enterochelin, had an increased requirement for iron, which would indicate that in these strains both the citrate and enterochelin systems have been affected. To explain the apparent discrepancy between these two observations, we suggest that, in the deletion mutants, the enterochelin system is still partially functional. The observations that point mutations of the *tonB* locus result in strains which show the same growth response to iron as the deletion mutants (15, 16) may indicate that mutations affecting the *tonB* locus cause alterations in the cell membrane such that two independent uptake systems might be impaired.

Evidence has now accumulated that there are a number of distinct uptake mechanisms for iron in various species of the enterobacteria. Thus, in *E. coli* K-12, the enterochelin and citrate systems are operative, whereas in *E. coli* W it is likely that the citrate system is lacking, since multiple aromatic auxotrophs from this strain show an absolute requirement for 2,3-dihydroxybenzoate both in the absence and presence of citrate (17). *A. aerogenes* NCW, when grown under iron-deficient conditions, excretes both enterochelin and an hydroxamate, aerobactin, into the culture medium (5). There is, however, no direct evidence that aerobactin is involved in iron transport.

Significant features of the chromatogram of the culture supernatant from strain AN102 (*fep*$^-$) compared with the chromatogram of the supernatant from the normal parent strain AB1515 are, as well as the high level of enterochelin, the lack of 2,3-dihydroxybenzoate and the relatively small amounts of the other 2,3-dihydroxybenzoate-containing compounds. The comparative lack of 2,3-dihydroxybenzoate would suggest that the pathway of biosynthesis from chorismate to 2,3-dihydroxybenzoate is controlled differently from the 2,3-dihydroxybenzoate to enterchelin pathway. This suggestion is consistent with the observation that higher concentrations of iron are required for the repression of the enzymes converting chorismate to 2,3-dihydroxybenzoate than are required for the repression of the enzymes converting 2,3-dihydroxybenzoate to its serine conjugates (I. G. Young, *unpublished work*; 1, 18). It may also be noted that strain AN102 (*fep*$^-$), when grown in the presence of citrate which promotes iron uptake, accumulates more 2,3-dihydroxybenzoate and less enterochelin in the medium. The low level of 2,3-dihydroxy-*N*-benzoylserine and of other 2,3-dihydroxybenzoate-containing compounds in the culture supernatant of strain AN102 (*fep*$^-$) suggests that formation of such compounds is related to the uptake of iron by the enterochelin-dependent system.

The genetic mapping by cotransduction of the *fep*$^-$, *entA*$^-$, and *entD*$^-$ alleles indicates that these mutations are in the same region of the *E. coli* genome. The cotransduction data do not appear to be consistent with the time of 2 min given between *purE* and *aroG* on the map of Taylor and Trotter (14) and would suggest that there is at least a 4-min interval between these two markers. Herbert and Guest (6) suggested that there is at least a 5-min interval between *purE* and *gal*, the latter gene being closely linked to *aroG*. At least two other genes concerned with enterochelin synthesis map in the same region of the chromosome (R. K. J. Luke, Ph.D. Thesis, Australian National University, Canberra, 1970), and the significance of this grouping of related genes remains to be determined.

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