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Genetic Control of Hydroxamate-Mediated Iron Uptake in *Escherichia coli*

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Cells of *Escherichia coli* can derive iron from a variety of chelators (siderophores) in addition to enterochelin, the catechol derivative excreted by many enteric bacteria. The genetic control of hydroxamate siderophore utilization was investigated in mutants of *E. coli* K-12 selected for resistance to lethal agents which adsorb to the *tonA* protein of the outer membrane (albomycin, colicin M, and phages T5 and φ80). Many of the mutants were unable to utilize hydroxamate siderophores as an iron source. This phenotype was termed Fhu, for ferric hydroxamate uptake. Mutants carrying lesions in the *tonA* region of the chromosome were studied and fell into several types. Members of one class had lost some or all of the *tonA* receptor protein's functions in that they were resistant to the lethal agents and unable to utilize ferrichrome and its analogs, although able to respond to the hydroxamate rhodotorulic acid (FhuA phenotype). Other mutants were unable to utilize any of the hydroxamate siderophores tested and were resistant to albomycin, although many were sensitive to the other lethal agents (FhuB phenotype). Members of these classes lacked ferrichrome-mediated iron uptake. Strains carrying transposon Tn*10* insertions in *fhuA* (previously termed *tonA*) lacked the 78,000-molecular-weight outer membrane protein previously described; insertions in *fhuB* retained this protein and had no detectable change in outer membrane composition. Three-point transduction crosses revealed the gene order to be *pan-fhuA-fhuB-metD* in the min 3.5 region of the chromosome map. Complementation analyses with F′ merodiploid strains showed that *fhuA* and *fhuB* comprise separate transcription units which are both required for utilization of ferrichrome. Response of diploid strains to rhodotorulic acid suggested the existence of a third gene, *fhuC*, required for utilization of this siderophore, but not ferrichrome. It is suggested that *fhuB* encodes a transport component in the cytoplasmic membrane that is necessary for the uptake of all hydroxamate siderophores following their receptor-mediated passage across the outer membrane.

The outer membrane of enteric bacteria contains a number of proteins which are involved in both transport of specific nutrients and attachment of phages and colicins (12). Several of these proteins have been implicated in the uptake of ferric ions chelated to a variety of siderophores (1). Enterochelin is the catechol siderophore produced and excreted by many enteric bacteria (18). Its uptake requires the function of a specific outer membrane receptor protein, which is also the receptor for colicins B and D (16, 17), and the *tonB* product (8). Additional mutants exist which may define another component responsible for the subsequent uptake of ferric enterochelin across the cytoplasmic membrane (5, 21).

Neilands' group has shown that *Salmonella typhimurium* can derive iron not only from enterochelin, but also from a variety of hydroxamate siderophores of diverse structure, including the fungal products ferrichrome and rhodotorulic acid (14). The *tonB* function is required for all chelate-mediated iron uptake at a step subsequent to binding to receptor (8, 17). Mutants of *S. typhimurium* resistant to the toxic ferrichrome analog albomycin were altered in their responses to some or all hydroxamate siderophores, whereas they responded normally to enterochelin (14). At least 10 phenotypic classes were distinguished from their response to a variety of hydroxamate siderophores. The genetic lesion in many of these mutants was linked to *pan* on the chromosome map.

The ferrichrome receptor has been identified in *Escherichia coli* as the product of the *tonA* gene and serves as receptor for colicin M and phages T1, T5, and φ80 (3, 6, 19). An analogous locus in *S. typhimurium*, linked to *pan*, was

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defined by certain albomycin-resistant mutants that were also resistant to phage ES18 (13). These mutants were unable to respond to ferrichrome and its analogs, but could utilize rhodotorulic acid and other hydroxamates. These mutants lacked an outer membrane protein of 78,000 molecular weight, just as in the analogous mutants of *E. coli* (2). This suggested that there would be separate transport systems for various hydroxamate siderophores, as well as several common components. Braun et al. (1) described albomycin-resistant mutants which retained the TonA protein and sensitivity to the lethal agents employing this protein. These mutants were defective in the transport of ferrichrome and carried lesions in the *tonA* region of the chromosome map, near *pan*.

In an attempt to better define the steps of hydroxamate-mediated iron uptake, we obtained a collection of *E. coli* K-12 mutants by selection for resistance to individual lethal ligands of the TonA protein (albomycin, colicin M, or phages T5 or ϕ80*vir*). This paper describes some mutants in which the genetic lesion was linked to *pan*, i.e., in the *tonA* region. Many of these mutations affected hydroxamate siderophore utilization and transport. Several mutations define a second genetic locus necessary for hydroxamate-mediated iron uptake; this does not appear to affect the outer membrane receptor protein and may be involved in transport across the cytoplasmic membrane. The existence of a third gene responsible for transport of rhodotorulic acid, but not of ferrichrome, is inferred from the results of the complementation analysis.

We propose the use of the mnemonic *fhu* for those genetic loci involved in the uptake of ferric hydroxamates. This designation is somewhat preferable to *sid* (14) in that it allows distinction from the transport systems for other nonhydroxamate siderophores which possess separate uptake systems. The term *tonA* is therefore replaced by the physiologically meaningful term *fhuA*. Other cistrons are designated *fhuB*, etc.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* K-12 strains used in this study are listed in Table 1. Mutants were first isolated in strain RK4617, which was a *pan-6* derivative of strain MX364, a suppressor-free strain supplied by M. Oeschger. The *pan-6* allele had been introduced by cotransduction with *metD* (utilization of D-methionine as methionine source), which had been obtained by selection for resistance to methionine sulfoximine and α-methylmethionine (11). The *entA* allele was introduced from strain AN248 (supplied by P. Reeves) by cotransduction with a Tn*10* transposon inserted near *entA*. The *pan-6* allele in Ktu51 was introduced by transduction of strain AB2847 (*aroB*) from a derivative of RK4617 which carried a Tn*10* transposon inserted between *pan* and *fhuA* (*tonA*).

The colicin M- and B-producing strains and the bacteriophages T5, ϕ80*vir*, and P1*kc* were from the laboratory collection. Phage λNK370 (*b*221 *c*I857 *c*I171::Tn*10* 0261[UGA]) and its host NK5336 (Su^+ *uga*) were from N. Kleckner via C. Beck.

Media and growth factor assays. The minimal salts medium A (7) and M63 (15) and M9 were used without addition of iron. The rich medium was L broth (15). The minimal media were supplemented with glucose or succinate (0.5%) and required amino acids (100 μg/ml) and vitamins (2 μg/ml). Hydroxamate siderophores were added at ca. 2 μM, 2,3-dihydroxybenzoic acid (DHB) was added at 20 μM, and citrate was added at 10 mM.

The ability of siderophores to support growth was tested on M63 agar plates with succinate as a carbon source and the appropriate siderophore incorporated in the medium. On this medium, even without addition of non-utilizable iron chelators such as dipyridyl or nitrilotriacetic acid, growth was essentially totally dependent on addition of a siderophore. Responses to siderophores by the mutants were also tested by placing filter paper disks on plates of M63-succinate medium seeded with an overlay of the mutant strain in M63-soft agar. Onto the disk was pipetted 5 μl of siderophore solution (ca. 1 mM). Either colony size or diameter of the growth response zone was scored after 18 and 42 h of incubation at 37°C.

Albomycin and the siderophores employed here were gifts of H. Zähner.

Electrophoresis of membrane proteins. The separation of the cytoplasmic and outer membranes by sucrose density gradient centrifugation and the separation of the constituent proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis has been described previously (1). Gels were stained with Coomassie brilliant blue.

Phage adsorption. Cells were grown in L broth into logarithmic phase and then washed twice with M9 salts and suspended in M9-glucose to a density of 1.3×10^9/ml, as described previously (10). About 3×10^7 plaque-forming units of T5 or ϕ80*vir* were added. After incubation at 37°C, adsorption was terminated by 500-fold dilution into cold M9 salts. The cells were pelleted, and the phages remaining in the supernatant fluid were titrated on strain AB2847. Rates of adsorption were measured by terminating adsorption at several time intervals. The effect of ferrichrome on adsorption was tested by adding ferrichrome to the indicated final concentration with the phage and allowing adsorption for 15 min.

Ferrichrome-mediated iron uptake. The measurement of ferrichrome-mediated uptake of ^{55}Fe by iron-starved cells was performed exactly as previously described (4).

Genetic methods. Phage P1-mediated transductions were carried out by using standard conditions (15). Selection for tetracycline resistance was on L plates containing tetracycline (20 μg/ml) after transduction for 1 h. Selection for pan^+ or trp^+ was on minimal selective plates of medium A with excess citrate (10 mM). Recombinants were purified by one cycle of single-colony isolation on selective medium and then were tested for siderophore utilization by

TABLE 1. *List of strains employed*

Strain	Genotype[a]	Source
W1485F⁻	Wild-type *E. coli* K-12; W1485 cured of F	
MX364	F⁻ *arg his trp*(Am) *lacZ*(Am) *tsx*	M. Oeschger
RK4616	F⁻ *metB his trp*(Am) *lacZ*(Am) *tsx pan-6*	Two transductions
RK4617	F⁻ *metB his trp*(Am) *lacZ*(Am) *tsx pan-6 entA zbd*::Tn*10*[b]	Cotransduction with Tn*10* inserted near *entA*
AB2847	F⁻ *thi aroB tsx malT*	(1)
KTu51	F⁻ *thi aroB tsx malT pan-6 zad*::Tn*10*	Transduction from RK4616 carrying Tn*10* near *pan*
RK4371	F⁻ *ilv metB metD proA his pan-6 entA* Δ*lac rpsL fhuA468*::Tn*10*	This study
RK4373	HfrH *thi aroE pan-6 fhuA468*::Tn*10*	This study
KL16-99	Hfr *thi recA*	K. B. Low
RK1005	F⁻ *thr leu arg proA thyA rpsL recA*	
RK3911	As RK4617, but *fhuB461*	Albomycin resistant
RK3912	As RK4617, but *fhuB462*	Albomycin resistant
RK3913	As RK4617, but *fhuB463*	Albomycin resistant
RK3914	As RK4617, but *fhuAC464*	φ80 resistant
RK3915	As RK4617, but *fhuAC465*	T5 resistant
RK3916	As RK4617, but *fhuAC466*	Colicin M resistant
RK3917	As RK4617, but *fhuA467*	Albomycin resistant
RK3918	As RK4617, but *fhuA468*::Tn*10*	Albomycin resistant
RK3920	As RK4617, but *fhuA470*	Colicin M resistant
RK3922	As RK4617, but *fhuA472*	Colicin M resistant
RK3923	As RK4617, but *fhuA473*	Albomycin resistant
RK3924	As RK4617, but *fhuA474*	Colicin M resistant
RK3927	As RK4617, but *cmt-96*	Colicin M resistant
RK3928	As RK4617, but *fhuB477*::Tn*10*	Albomycin resistant
RK3929	As RK4617, but *fhuB478*::Tn*10*	Albomycin resistant
The series RK3931 to 3949	As KTu51, but *pan*⁺ *fhu* carrying the *fhu* alleles in the same order as above	*pan*⁺ Tet-s transductants of KTu51 from *pan*⁺ transductants of RK3911 through RK3929, respectively
The series RK3951 to 3969	As RK4371, but *metD*⁺ *fhu his*⁺ *recA*, carrying the *fhu* alleles in the same order as above	*metD*⁺ transductants from strains RK3931 to 3949, respectively, then *his*⁺ *recA* from KL16-99
The series RK3971 to 3989	As RK4373, but *pan*⁺ *fhu* Tet[s]	*pan*⁺ transductants from strains RK3931 to 3949, respectively

[a] Genotype symbols and allele numbers are assigned by Bachmann, except for the proposed use of *fhu*.

[b] The designation *fhu*::Tn*10* is used to indicate that the mutation is the result of the insertion of transposon Tn*10* in the indicated gene. The designation *zbd*::Tn*10* indicates that the transposon Tn*10* is located near the *entA* locus near min 14, and was used for introduction of the *entA* allele by cotransduction with selection for tetracycline resistance.

radial streaking and for response to the lethal agents by cross-streaking on square L agar plates. The cross-streaks contained either a strip of phage or a strip of a colicin-producing strain that had been killed with $CHCl_3$ and then overlaid with L agar.

Construction of merodiploid strains. The *fhu* mutant alleles were transferred into strain RK4371 by cotransduction with *metD*⁺ and into strain RK4373 by cotransduction with *pan*⁺. One recombinant from each cross was chosen which had retained adjacent selective markers (*pan* and *proA*), but had lost the *fhuA*::Tn*10* insertion, and exhibited the expected phage and colicin resistance patterns. Each of the *fhu* derivatives of RK4371 was made *recA* by conjugal mating with strain KL16-99 and selection for His⁺ Str[r]. Inheritance of the *recA* allele was scored by sensitivity to UV irradiation. Episomes (F′) were obtained after the mating of each *fhu* derivative of RK4373 (HfrH) with strain RK3958, selecting for Pan⁺ Pro⁺ offspring. Episome-bearing transcipients appeared at a frequency of about 10^{-5}/donor cell. All strains were tested for Rec⁻ character and for ability to transfer Pro⁺ and Pan⁺ at high frequency. The *fhu*-carrying episomes were then transferred into strain RK1005 by selection for Leu⁺ Pro⁺ transcipients.

These F′-carrying derivatives of RK1005 were used to transfer the episome to each *fhu recA* recipient by placing a drop of each donor strain onto a lawn of each recipient strain on medium selective for Pro⁺ Pan⁺ offspring. Each transcipient strain was purified and tested for growth response to ferrichrome and rhodotorulic acid on M63-succinate medium. Diploid strains were always maintained on medium selective for retention of the episome. As expected, growth on non-selective medium resulted in loss of the episome and return to the Pro⁻ Pan⁻ Fhu⁻ character of the haploid recipient strain. By this method, there were two strains, each carrying the same pair of *fhu* alleles but on different genomes. The growth responses of both members of each pair were essentially the same and have been combined for presentation in Table 5.

RESULTS

Isolation of mutants. Several hundred independent mutants were obtained either spontaneously or after ethyl methanesulfonate treatment by selection for resistance to albomycin or

colicin M or phage T5 or φ80*vir*. The parental strain carried an *entA* mutation blocking biosynthesis of the enterochelin precursor DHB. The mutants exhibited considerable variety in their responses to colicin B and the other lethal agents and in their utilization of ferrichrome, ferricrosin, rhodotorulic acid, ferrioxamine B, DHB, or citrate. The genetic linkage to *pan* and *trp* was determined by P1 transduction from wild-type strain W1485F⁻. Of the 62 mutants tested, the lesion in 37 was linked to *pan-6*, that in 15 was linked to *trp*, and the remainder was unlinked to either. All except one of the strains with mutation near *trp* were also resistant to colicin B and sensitive to phage T5 and are hence altered in *tonB*. None of the mutations near *pan* conferred resistance to colicin B. Most of the mutants selected for resistance to phage T5 were of identical phenotype, in contrast to the variety seen with the other selective agents. Fifteen mutants with lesions near *pan* and with differing phenotypes were studied further (Table 2). These do not represent a random sampling of the mutants obtained.

Transposon Tn*10*-induced mutations were isolated by K. Hantke following simultaneous selection for resistance to albomycin and tetracycline in strain W1485F⁻ infected with the transposon-carrying phage λNK370. Phage P1 lysates of these strains were used to transduce strain AB2847 to tetracycline resistance. Those cases in which all (of 50) Tet^r transductants became albomycin resistant were considered to result from insertion of the Tn*10* element into a gene conferring albomycin sensitivity. Three of the insertions linked to *pan* were chosen for further study. Also useful for strain construction was the isolation of a Tn*10* insertion midway between *pan* and *fhuA*, but not in a gene of identifiable function. This was obtained by transduction from a collection of random Tn*10* insertions with simultaneous selection for inheritance of Pan$^+$ and Tet^r. The location of the transposon was verified by its cotransduction frequencies with adjoining markers.

Further biochemical and genetic characterizations were carried out after transfer of the mutations into strain KTu51 (*aroB pan*) by cotransduction with *pan*. This strain was found to suppress the effect of several of the mutant alleles. The entry of the mutant allele in these cases was verified by transfer of the appropriate mutation back out into a suppressor-free strain. Although these suppressible alleles are not described further in this paper, it is interesting that suppression did not fully restore wild-type phenotype. The suppressed strains were sensitive to phages T5 and φ80, but were somewhat resistant to albomycin and colicin M, although much less so than when the same allele was present in a suppressor-free strain.

All of the albomycin-resistant strains described here were deficient in utilization of ferrichrome and ferricrocin; however, there was no other consistent correlation of their properties (Table 2). Two general classes were apparent. First, the FhuA phenotype was characterized by resistance to all of the lethal agents and at least

TABLE 2. *Properties of mutant strains with lesions linked to pan*[a]

Strain	Allele	Selection	Alb	ColM	φ80	T5	Cit	DHB	Fc	RA	FoxB
RK3911	*fhuB461*	Alb-21	R	S	S	S	+	+	−	−	−
RK3912	*fhuB462*	Alb-26	R	S	S	S	+	±	−	−	−
RK3913	*fhuB463*	Alb-65	R	S	S	S	+	+	−	−	−
RK3914	*fhuAC464*	φ80-54	R	R	R	R	+	±	−	s	s
RK3915	*fhuAC465*	T5-64	R	R	R	R	+	+	−	s	s
RK3916	*fhuAC466*	M-1	R	R	R	R	+	+	−	s	s
RK3917	*fhuA467*	Alb-24	R	R	R	R	+	+	−	+	±
RK3918	*fhuA468*::Tn*10*	Alb-Tn	R	R	R	R	+	+	−	±	±
RK3920	*fhuA470*	M-98	R	R	R	R	+	+	−	+	+
RK3922	*fhuA472*	M-123	R	P	S	R	+	+	−	±	s
RK3023	*fhuA473*	Alb-133	R	P	S	R	+	+	−	±	s
RK3924	*fhuA474*	M-2	R	R	S	R	+	+	−	+	+
RK3927	*cmt-96*	M-96	S	S	S	R	+	+	+	+	+
RK3928	*fhuB477*::Tn*10*	Alb-Tn	R	S	S	S	+	+	−	−	−
RK3929	*fhuB478*::Tn*10*	Alb-Tn	R	S	S	S	+	+	−	−	−

[a] This table lists the properties of the derivatives of strain RK4617 carrying the listed *fhu* alleles, all of which exhibit ca. 50% cotransduction with *pan-6*. The selective agents employed were albomycin (Alb), phage φ80, phage T5, and colicin M (M). The selective agent is followed by the isolation number. Response to the lethal agents is designated by R, resistant; S, sensitive; and P, partial. Response to siderophores is designated by +, equivalent to wild type; ±, decreased growth; s, slight, limited growth after 3 days; −, no detectable growth over the very slight growth in the absence of added siderophore. The siderophores are Cit, citrate; DHB; Fc, ferrichrome, also ferricrocin; RA, rhodotorulic acid; FoxB, ferrioxamine B.

partial utilization of rhodotorulic acid. The second FhuB phenotype was sensitivity to all the lethal agents except albomycin and inability to utilize any of the hydroxamate siderophores tested. All mutants responded normally to citrate and all except two were normal in response to DHB. In addition, there were mutants which were only partially resistant to certain lethal agents or partly depressed in response to certain siderophores.

Ferrichrome uptake. The ability of iron-starved cells of the mutant strains to carry out ferrichrome-mediated iron uptake was determined (Fig. 1). All of the strains unable to utilize ferrichrome in growth promotion tests exhibited negligible ferrichrome-mediated iron uptake. No difference in relative binding of ferrichrome by energy-poisoned cells was detected. We were unable to demonstrate rhodotorulic acid-promoted iron uptake in assay systems containing nitrilotriacetic acid.

Membrane proteins. The outer and cytoplasmic membranes of the parental strain KTu51 and four Tn*10* insertion mutants were separated by sucrose gradient centrifugation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Staining of the gels with Coomassie brilliant blue revealed the absence of the 78,000-molecular-weight polypeptide, previously identified as the *tonA* (*fhuA*) product, from the membrane of the *fhuA468*::Tn*10* strain. This protein band was present in normal amounts in the membranes of the strains carrying the *fhuB477*::Tn*10* and *fhuB478*::Tn*10* insertions. No other changes were detectable in the proteins of the outer or cytoplasmic membranes in any of the strains.

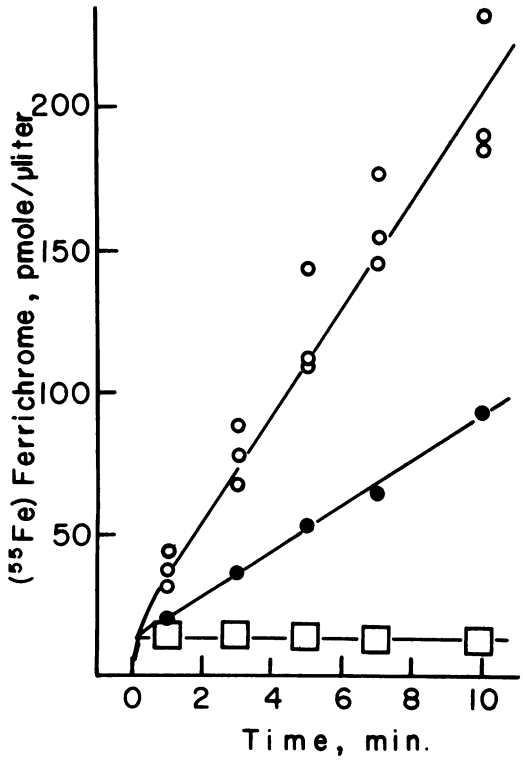

FIG. 1. *Ferrichrome-mediated iron uptake in fhu mutants. Iron-starved cells of strain KTu51 and fhu transductants of it were exposed to ^{55}Fe-ferrichrome complex and then filtered and washed at the indicated times. Symbols: ○, KTu51; ●, RK3947 (cmt-96); all other strains of the series RK3931 to 3949 gave uptake activities included within the squares.*

Phage adsorption and the effect of ferrichrome. Hantke and Braun (9) showed that, whereas ferrichrome competitively blocked phage T5 binding to isolated outer membranes or energy-poisoned or *tonB* mutant cells, it only partially inhibited T5 adsorption to wild-type cells in the presence of glucose. To study the interactions between binding sites on the receptor protein, we determined the rates of adsorption of T5 and φ80*vir* to several of the FhuA and FhuB strains and determined the effect of ferrichrome on phage adsorption (Table 3). Both phage T5 and phage φ80 adsorbed to the FhuB strains at the same rate as to the parental strain. As expected, there was negligible adsorption to the FhuA strains carrying the *fhuA467* and *fhuA468*::Tn*10* alleles. Ferrichrome at 1 μM blocked completely the adsorption of φ80. However, even at 100 μM ferrichrome, there was only a 30 to 50% inhibition of T5 adsorption to the parental strain. The same limited extent of maximal inhibition of T5 adsorption by ferrichrome was also observed in the four FhuB mutant strains tested. Lower concentrations of ferrichrome blocked adsorption more effectively in the FhuB strains than in the parental strain, probably because the mutant cells were unable to deplete the medium of the low concentrations of ferrichrome.

Strain RK3944, carrying the *fhuA474* allele, was sensitive to T5, although resistant to φ80 and the other lethal agents. Adsorption of T5 by this strain was somewhat reduced relative to the parental strain, yet there was no detectable adsorption of φ80. The adsorption of T5 was totally insensitive to the presence of ferrichrome.

Zones of killing on plates revealed that the FhuB strains were as sensitive to colicin M as the parental strain. Cross-streaking tests showed that ferrichrome protected against colicin M killing even more effectively in the FhuB mutants. Neither rhodotorulic acid nor ferrioxamine B afforded protection against colicin M or

phage T5 or φ80.

Genetic mapping of *fhuA* and *fhuB*. The order of *fhuA* and *fhuB* with respect to the nearby locus, *pan-6*, was determined by phage P1-mediated three-point transductional crosses. The donor strains were RK3938 (*fhuA468*::Tn*10*) and 3948 (*fhuB477*::Tn*10*), which were used to donate tetracycline resistance to several strains carrying *fhuA* or *fhuB* mutations. The FhuA phenotype was scored by the response of purified recombinants to colicin M and phages T5 and φ80; the FhuB phenotype was determined from the utilization of rhodotorulic acid as iron source. All recombinants were able to utilize citrate and unable to utilize ferrichrome as iron sources. The distribution of recombinants observed and the identity of the minority class of recombinants were consistent only with the gene order *pan-fhuA-fhuB* (Table 4). The cotransduction frequency of *pan* and *fhuA* was 0.50 and that of *fhuA* and *fhuB* was in the range of 0.91 to 0.96. Insufficient numbers of recombinants were tested to determine more precisely the linkage between these two loci.

In crosses of the reciprocal orientation, strains carrying the *fhuA*::Tn*10* and *fhuB*::Tn*10* transposon insertions were transduced to the utilization of ferrichrome and rhodotorulic acid, re-

TABLE 3. *Adsorption of phages T5 and φ80 and inhibition by ferrichrome*

Strain	*fhu* allele	Rate of adsorption (ml × min^{-1} × 10^{-10})	% Inhibition of adsorption by ferrichrome				
			0.01 μM	0.1 μM	1 μM	10 μM	100 μM
T5 Adsorption							
KTu51	*fhu*$^+$	1.3	—[a]	2	28	30	35
RK3948	*fhuB477*::Tn*10*	1.2	9	31	30	35	—
RK3949	*fhuB478*::Tn*10*	0.8	9	32	28	—	—
RK3931	*fhuB461*	1.1	8	32	35	—	—
RK3932	*fhuB462*	0.7	1	31	30	—	—
RK3937	*fhuA467*	<0.01	—	—	—	—	—
RK3944	*fhuA474*	0.4	—	0	0	0	0
RK4947	*cmt-1*	1.2	6	5	21	28	30
φ80 Adsorption							
KTu51	*fhu*$^+$	1.6	0	0	100	—	—
RK3948	*fhuB477*::Tn*10*	1.4	0	75	—	—	—
RK3949	*fhuB478*::Tn*10*	1.4	0	70	—	—	—
RK3931	*fhuB461*	1.2	0	91	100	—	—
RK3932	*fhuB462*	1.1	0	63	94	—	—
RK3944	*fhuA474*	<0.01	—	—	—	—	—

[a] —, Not determined.

TABLE 4. *Three-point transduction mapping of fhuA and fhuB*[a]

Recombinant genotype			No. of recombinants of genotype				
			P1(RK3928; *fhuB477*::Tn10 *pan*) × :			P1(RK3918; *fhuA468*::Tn*10* *pan*) × :	
pan	*fhuA*	*fhuB*	RK3937 (*fhuA467*)	RK3936 (*fhuA466*)	RK3944 (*fhuA474*)	RK3931 (*fhuB461*)	RK3932 (*fhuB462*)
D	D	D	91	21	59		
R	D	D	82	19	42		
R	R	D	14	8	7		
D	R	D	1	0	0		
D	D	D				80	54
R	D	D				93	45
R	D	R				4	2
D	D	R				3	5

pan — 0.50 — *fhuA* — 0.91, 0.96 — *fhuB*

[a] Selection was made for inheritance of tetracycline resistance resulting from the insertion of the transposon Tn*10* inserted in *fhuB* or *fhuA*, respectively. D, donor allele; R, recipient allele.

spectively, with P1 lysates grown on the other *fhu* mutants. The yield of *fhu*$^+$ recombinants was markedly lower (0.1 to 5%) than the number of *pan*$^+$ recombinants, as expected for recombination events between closely linked markers. The number of *fhu*$^+$ recombinants obtained was only two to three times greater than the number of revertants resulting from precise excision of the transposon with restoration of the interrupted function. Thus, although not quantitatively significant, the inheritance of the outside *pan* marker was consistent with the proposed gene order found above. In addition, crosses between mutations presumably within the same cistron (i.e., of the same phenotypic class) yielded no detectable recombinants above the level of reversion.

Complementation analysis. To demonstrate that *fhuA* and *fhuB* constitute two separate transcriptional units, stable merodiploid strains combining the mutant alleles were constructed. The mutant alleles had been introduced into both parental strains by cotransduction with *pan* or *metD*. The recipient strains were also *recA*, to maintain the episome, and *entA*, to allow scoring the growth response to exogenous siderophores. The F′ episomes, derived from HfrH *fhu* strains, were selected and maintained by selection for the *pan*$^+$ and *proA*$^+$ markers flanking the *fhu* loci. The diploid strains were tested for their ability to utilize ferrichrome and rhodotorulic acid as iron sources (Table 5). In all cases, the strains were able to utilize citrate and were dependent on addition of an exogenous siderophore for growth on M63-succinate minimal medium.

All of the mutant alleles described here were recessive to the wild-type allele with respect to the response to both siderophores. The three *fhuB* alleles tested (*fhuB461*, *462*, and *463*) complemented and were complemented by all of the FhuA-type mutants to allow utilization of ferrichrome. There was little, if any, complementation within the FhuA or within the FhuB classes. The *fhuAC464* and *fhuAC465* alleles did not complement any of the *fhuA* mutants, but did allow growth on ferrichrome in combination with any of the *fhuB* alleles. These results are indicative of the existence of two discrete cistrons necessary for ferrichrome utilization.

The response of the merodiploid strains to rhodotorulic acid stood in contrast to the results with ferrichrome. In all diploid strains which combined two mutant alleles which individually blocked the utilization of rhodotorulic acid, that diploid strain was also unable to respond to this siderophore. For example, the diploid of genotype *fhuAC464/fhuB462* responded normally to ferrichrome, but was completely unable to utilize rhodotorulic acid, by growth promotion tests on plates. In all cases, the ability to utilize rhodotorulic acid was dominant over lack of response.

TABLE 5. *Response of merodiploid strains to ferrichrome and rhodotorulic acid*[a]

Response to rhodotorulic acid: Allele	I	+	*fhuB461*	*fhuB462*	*fhuB463*	*fhuAC464*	*fhuAC465*	*fhuAC466*	*fhuA467*	*fhuA468*	*fhuA470*	*fhuA471*	*fhuA472*	*fhuA473*	*fhuA474*	*cmt-96*
Response to ferrichrome: −		+	−	−	−	−	−	−	−	−	−	−	−	−	−	+
+	+		+	±	+	±	±	+	±	+	+	+			±	+
fhuB461	−	+		−	−	+	+	+	+	+	+	+	+	+	+	+
fhuB462	−	±	−		−	+	+	+	+	+	+	+	+	+	+	+
fhuB463	−	+	−	−		+	+	+	+	+	+	+	+	+	+	+
fhuAC464	s	+	−	−	s		−	−	−	−	−	−	−	−	−	+
fhuAC465	s	+	s	−	s	−		−	−	−	−	−	−	−	−	+
fhuAC466	s	+	s	−	s	s	−		−	−	−	−	−	−	−	+
fhuA467	±	±	±	±	±	±	±	+		−	−	−	−	−	−	+
fhuA468	±	+	±	s	+	±	±	+	+		−	−	−	−	−	+
fhuA470	±	±	±	±	+	±	±	±	±	±		−	−	−	−	+
fhuA471	+	+	+	±	+	+	+	+	+	+	±		−	−	−	+
fhuA472	±		±	s	+	±	±	+	±	±					−	+
fhuA473	±		±	s	+	±	±	+	±	±	±				s	
fhuA474	+	+	+	±	+	±	±	+	±	+	+	+	+			+
cmt-96	+	+	+	+	+	±	+	+	+	+	+	+	+		+	

[a] Growth responses to ferrichrome and rhodotorulic acid on M63-succinate plates: +, equivalent colony size as *fhu*$^+$ *recA* strain; ±, lesser growth; s, slight response; −, no detectable growth after 4 days. The homozygous diploid strains all gave the same growth responses to both siderophores as the haploid strains. The data shown are the combination of both diploids with the same alleles in opposite location, i.e., whether on the F′ or the chromosome.

These results suggested the presence of only a single complementation group for rhodotorulic acid uptake.

DISCUSSION

The *fhuA* (*tonA*) protein in the outer membrane is strongly implicated in ferrichrome-mediated iron uptake. Mutants lacking this protein are unable to utilize or transport ferrichrome, and this siderophore is able to protect cells against colicin M and phages T1 and ϕ80 by competitively blocking the adsorption of these lethal agents (6, 9, 19). There is, however, no evidence that this protein is involved in the uptake of hydroxamate siderophores structurally unrelated to ferrichrome. Neither rhodotorulic acid nor ferrioxamine B, both of which can serve as iron sources, protected cells against the lethal agents which bind to the *fhuA* (*tonA*) protein. Mutants of *S. typhimurium* that were resistant to phage ES18 and lacked the analogous outer membrane protein were unable to utilize ferrichrome and its analogs, but were fully capable of employing rhodotorulic acid as iron source (13). Nevertheless, selection for resistance to albomycin or, with lower frequency, to colicin M or phage ϕ80 yielded mutations mapping in the vicinity of *fhuA* which were defective in their response to a number of other hydroxamate siderophores. This raised the possibility of the presence of a cluster of genes responsible for uptake of various hydroxamate siderophores. It is interesting that a locus responsible for utilization of citrate as iron source is in this general region of the genetic map (20).

Our results, in agreement with those of Luckey and Neilands for *S. typhimurium* (13), show that the *fhuA* (*tonA*) locus encodes the outer membrane receptor and is required for the utilization of ferrichrome and its analogs, but not for rhodotorulic acid. The existence of a second locus, *fhuB*, closely linked to *fhuA* and required for uptake of all hydroxamate siderophores tested, is strongly indicated. Since mutations in *fhuB* did not cause detectable alteration in the outer membrane protein profile, it is possible that this product is in the cytoplasmic membrane. The fact that transposon Tn*10* insertions in either *fhuA* or *fhuB* did not affect function of the other indicates that the two loci comprise separate transcription units.

The complementation analysis verified the existence of two transcription units (*fhuA* and *fhuB*) required for utilization of ferrichrome. However, there appeared to be only a single transcription unit involved in rhodotorulic acid utilization. This unit, however, included mutations in both *fhuA* and *fhuB*. If these mutations were deletions covering both *fhuA* and *fhuB*, then they should not have complemented each other to allow growth on ferrichrome. The simplest model to explain the complementation results postulates the existence of a third gene, *fhuC*, lying between *fhuA* and *fhuB* and transcribed from the *fhuB* promoter. The proposed *fhuC* product is required for uptake of rhodotorulic acid, and possibly other hydroxamate siderophores, but not for the uptake of ferrichrome or albomycin. This could possibly be an outer membrane protein, analogous to the *fhuA* protein.

According to this model, some of the *fhuA* mutations are deletions extending into *fhuC*, thereby explaining the inability of *fhuAC464* and *fhuAC465* to utilize rhodotorulic acid and their lack of receptor function. That the deletion in these mutants does not extend into *fhuB* is shown by the ability of these mutations to complement *fhuB* mutants for growth on ferrichrome. If some of the *fhuB* mutations are polar on expression of *fhuC*, this would explain the inability of those *fhuB* mutants to complement the *fhuAC* mutants for growth on rhodotorulic acid and their ability to utilize ferrichrome. Revertants of *fhuAC* mutants regaining Fhu$^+$ function were not obtained. Our choosing mutants with atypical properties, which are now explained as the result of intergenic deletions, can explain the relatively high proportion of putative deletion mutations among the population examined. Evidence for the existence of the *fhuC* gene is quite indirect and was based on the complementation patterns observed. Future efforts will be directed towards identifying the change in membrane proteins in these mutants, as well as the isolation of mutants altered only in *fhuC*. These mutants would not be selected for by the procedures used in this paper.

It must also be noted that the involvement of the *fhuB* product in the transport of ferric hydroxamate complexes has not been proven. Its possible role in the reduction or release of iron from the complex has not been excluded if there were an obligate coupling between iron accumulation and its metabolism.

From the inability of ferrichrome to protect completely against T5 adsorption, Hantke and Braun (9) suggested the presence of separate binding domains on the *fhuA* protein: one for ferrichrome, another for T5. From the competition results, it is likely that the ferrichrome-binding site is also used for the attachment of colicin M and phage ϕ80. The *fhuA474* mutation appears to affect the binding site for colicin M-ϕ80-ferrichrome, but not the site for T5 binding. Another explanation for its phenotype is that

the mutant receptor can no longer interact with the *tonB* product to allow entry of the *tonB*-dependent substrates, whereas adsorption of T5 is *tonB* independent. The fact that ferrichrome was totally unable to block T5 adsorption in this mutant favors the model of the loss of the ferrichrome-colicin M-ϕ80 receptor site.

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