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Genetic and Biochemical Characterization of the *Escherichia coli* K-12 *fhuB* Mutation

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The *fhuB* region of *Escherichia coli* K-12 was subcloned from pLC4-44 into pP *lac* to obtain pCPN1. Deletions of this recombinant plasmid were made, and a 1.4-kilobase *Pst*I fragment was further subcloned into the vector plasmid pKK177-2 to obtain pCPN12. The response of *tonA* and *tonB* strains and *fhuB* strains containing the plasmids to 15 hydroxamate siderophores were assayed. Results showed that *tonA* strains were deficient only in the utilization of ferrichrome-type siderophores, whereas *fhuB* strains were deficient in the utilization of all hydroxamate-type siderophores. The response of the plasmid-containing *fhuB* strains to the siderophores showed that the *fhuB* gene resides on a 1.4-kilobase *Pst*I fragment of DNA. The proteins synthesized by these plasmids were examined in maxicells of strain CSR603. Plasmid pCPN1 expressed five proteins of molecular weights 78,000, 40,000, 30,000, 24,000, and 13,700. By the use of deletions of pCPN1, the approximate order of the genes for these proteins was determined. Plasmid pCPN12 expressed no proteins other than the β-lactamase proteins in maxicell strain CSR603. However, in maxicell strain BN660, a *lon* mutant, it expressed a 20,000-molecular-weight protein. Inner membrane vesicles made from *tonB* and *fhuB* strains were able to transport [^{55}Fe]ferrichrome and [^{55}Fe]rhodotorulate at rates similar to those obtained in vesicles from *tonB*$^+$ and *fhuB*$^+$ strains.

Although *Escherichia coli* K-12, devoid of the ColV plasmid, is unable to synthesize hydroxamate-type siderophores, this bacterium can use a number of these iron chelators as a source of iron. Luckey et al. (23) used the antibiotic albomycin (2), a structural analog of ferrichrome (9), to demonstrate that the use of hydroxamate-type siderophores in *Salmonella typhimurium* requires the presence of a cluster of genes mapping near the *panC* locus. It was then shown in *E. coli* that the *tonA* gene, which is located adjacent to *panC*, codes for the outer membrane receptor for the prototypical hydroxamate siderophore, ferrichrome (36). Also in *E. coli*, Kadner et al. (16) reported evidence for additional functions for hydroxamate-mediated iron uptake and defined the gene sequence as *pan-tonA-fhuB-metD*. The *tonA* mutation is characterized by resistance to specific lethal agents with retention of ability to transport certain hydroxamate siderophores but not those of the ferrichrome type, which are widely distributed in fungal species. In the *fhuB* mutation, sensitivity to the lethal agents is retained, with the exception of albomycin, whereas the capacity to use hydroxamate siderophores as a class is lost.

Takeda et al. (33) searched the Clarke and Carbon (5) collection for plasmids complementing the *ponB* mutation, which maps close to the *pan* locus at minute 3.5 on the *E. coli* chromosome. Three plasmids, pLC19-19, pLC4-43, and pLC4-44 were observed to complement both *ponB* and *tonA* mutations.

Plastow et al. (29) used pLC19-19 to demonstrate that the *tonA* protein synthesized in vitro is ~2,000 M_r larger than the mature membrane protein. They also found pLC19-19 to code for a 30,000-molecular-weight (30K) outer membrane protein.

In this paper we examine the *tonA-fhuB* region of *E. coli* by using a subclone of pLC4-44. pLC4-44 was used in these studies because it carries more chromosomal material to the right of *tonA* than does pLC19-19. We concentrated on the *fhuB* region because we wanted to select for the loss of hydroxamate utilization. Furthermore, we showed that the *fhuB* gene product is required for the utilization of all hydroxamate-type siderophores, making it an interesting protein for the focus of our study. We present data concerning the expression of the *tonA-fhuB* region in maxicells of *E. coli* and also show that inner membrane vesicles from *fhuB* cells are capable of ferrichrome transport.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used, all derivatives of *E. coli* K-12, are listed in Table 1. Strain BN3300 was made from strain RW193 by selection for albomycin resistance. Strain BN3306 was prepared from RW193 by mating with strain Hfr3000YA139 to obtain *pan leu*$^+$, followed by UV mutagenesis and selection for albomycin resistance. BN3300 was found to revert, whereas BN3306 did not. Thus, BN3300 is a point mutation and BN3306 is most likely a deletion. To confirm that BN3300 and BN3306 were indeed *fhuB* mutations, they were mapped by P1 transduction to be near the *panC* and *tonA* loci, and the albomycin-resistant mutation in BN3300 was moved to F'104, which is carried by strain KLF4, by homogenotization (26) and was found to complement the *tonA* mutation in strain AN193. BN3307 was prepared from BN3306 by selecting for *thy*$^-$ with trimethoprim (26), mating with KL16-99, selecting for *thy*$^+$, and screening for sensitivity to UV light to obtain *recA*. Strain BN660 was obtained by mating strain PAM660 with strain JC10240, selecting for tetracycline resistance, and screening for sensitivity to UV light.

The vector plasmids used were pP *lac*, which is a 2.0-kilobase (kb) multicopy derivative of pGL101 (4, 34) carrying the ampicillin resistance gene of pBR322 and the *lac* UV5 operator and promoter, and pKK177-3, a 2.9-kb multicopy plasmid carrying the ampicillin resistance gene of pBR322 and the *tacI* promoter (8) (kindly donated by J. Brosius). This plasmid contains an M13 linker with sites for *Eco*RI,

TABLE 1. Characteristics and sources of *E. coli* K-12 strains used in this work

Strain	Genotype	Relevant phenotype	Origin or reference
RW193	F⁻ *proC leu trp thi lacY rpsL galK ara entA mtl xyl azi tsx supE44*	Cannot synthesize enterobactin	(35)
AN193	Same as RW193	Lacks outer membrane ferrichrome receptor	I. G. Young
BN1060	Same as AN193 but *recA*		Lab stocks
RWB7	Same as RW193 but *tonB*	Deficient in all high-affinity iron uptake and B_{12} uptake	Lab stocks
BN3300	Same as RW193 but *fhuB*	Deficient in all iron hydroxamate uptake	This study
BN3306	Same as RW193 but *panB leu*⁺ *fhuB*	Deficient in all iron hydroxamate uptake	This study
BN3307	Same as BN3306 but *recA*		This study
CSR603	F⁻ *uvrA6 recA1 phr-1 thr-1 leuB6 thi-1 rpsL31 nalA lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 supE44 tsx-33*	Killed by UV light	(31)
PAM660	F⁻ *thr-1 leuB6 proA2 lon22 his-4 met-24 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL-31 tsx-33*	Protease deficient	B. Bachmann
JC10240	HfrP.O.45 of HfrKL16 *thr-300 recA56 srl-300*::Tn*10*, relA1	*recA* near Tn*10*	(7)
Hfr3000YA139	HfrP.O.1 *pan-6, thi-1 relA1 spoT1*		(6)
BN660	Same as *PAM660* but *met*⁺ *recA srl-300*::Tn*10*	Maxicell strain with a *lon* mutation	This study
KL16-99	HfrP.O.45 *recA1 relA1 thi-1 deoB13*		(22)
KLF4	F', P.O.1 of Hfr Hayes episome: F'104 chromosomal markers: *thr-1 leuB6 proA2 his-4 recA13 argE3 thi-1 ara-14 lacY1 galK2 xyl-7 met-1 rpsL-31 tsx-33 supE44*	F'104 plasmid for homogenotization	(22)

*Sma*I, *Bam*HI, *Sal*I, *Pst*I, and *Hin*dIII distal to the *tac* promoter. Recombination ColE1 plasmids pLC4-44 and pLC19-19 carry the *tonA* region of *E. coli* K-12 (29, 33).

Restriction enzymes were purchased from Bethesda Research Laboratories (BRL) or New England Biolabs. All digestions were performed in buffers recommended by BRL or New England Biolabs at 37°C for 2 to 12 h. A 0.2-μg amount of plasmid pP *lac* and 1 μg of pLC4-44 were digested with *Hin*dIII, and the enzyme was subsequently inactivated by phenol extraction. The DNA was then precipitated with ethanol and redissolved in ligase buffer (BRL). The two fractions were mixed and ligated with 2 U of T4 DNA ligase (BRL) for 24 h at 20°C. *BAL*31 nuclease digestions were performed in buffer recommended by BRL at 22°C for 5 to 60 min, and the reaction was stopped by the addition of 20 mM EDTA.

Plasmid DNA, after amplification by chloramphenicol, was purified by using either the boiling technique (13) or lysis with Triton-EDTA (17), followed by cesium chloride-ethidium bromide equilibrium density gradient centrifugation.

Insertions of Tn*5* in the recombinant plasmids were isolated from plasmid-containing strains of BN3307 after infection with λ *c*I857 *rex*::Tn*5029*(am) *P*am80 *b*221. Plasmids containing a Tn*5* insert were identified by their ability to confer both kanamycin and ampicillin resistance after retransformation into strain BN3307. The approximate positions of the Tn*5* insertions were localized to within 100 base pairs by restriction mapping with *Hin*dIII, *Hin*cII, and *Pst*I (14).

Transformation of *E. coli* strains. Plasmids were transformed into strain BN3307 by using the calcium chloride-rubidium chloride procedure (18, 25).

Growth factor assays. The ability of siderophores to support growth was examined by placing filter paper disks on nutrient agar plates seeded with an overlay of the strain in soft nutrient agar containing 0.1 mM deferriferrichrome A. Each disk was impregnated with 10 μl of ca. 50 μM siderophore solution. The diameter of the growth response zone was scored after 12 and 24 h of incubation at 37°C.

Labeling and identification of plasmid protein products. pP *lac* and pKK177-2 derivatives in strains CSR603 and BN660 were used to label plasmid-coded proteins by the maxicell system described by Sancar et al. (31), modified to include a cycloserine step (32). Labeled whole cells were lysed and run on a 10 to 20% linear gradient of sodium dodecyl sulfate polyacrylamide as described by Ames (1), Lugtenberg et al. (24), and Hancock et al. (11). Fixed and dried gels were exposed at −70°C with Kodak X-Omat AR film. Molecular weight standards were lysozyme, 14,300; β-lactoglobin, 18,500; bovine erythrocyte carbonic anhydrase, 29,000; ovalbumin, 45,000; bovine albumin, 66,000; rabbit muscle phosphorylase *b*, 97,000; and *E. coli* β-galactosidase, 116,000 (Sigma Chemical Co.).

Transport in vesicles. *E. coli* K-12 strains RW193, BN3300,

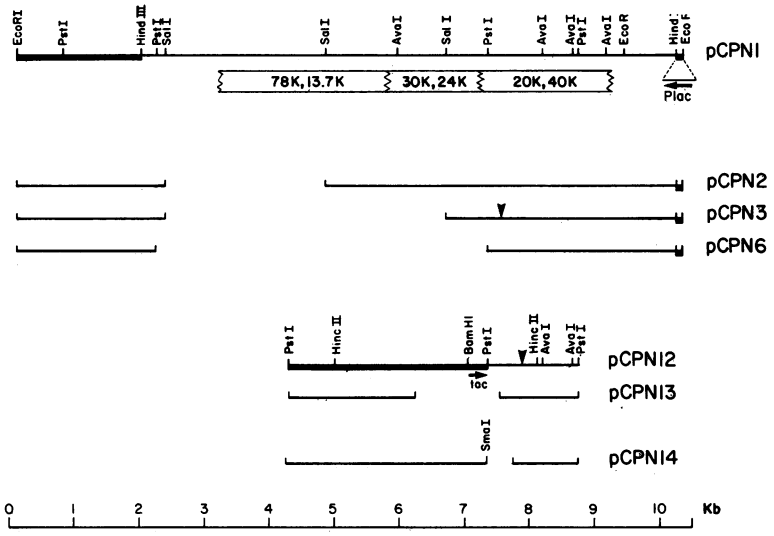

FIG. 1. Restriction maps of pCPN1 and pCPN12 and deletions of these two plasmids. Caret indicates sites of Tn*5* insertions. Proteins coded by the plasmids are diagrammed under pCPN1. Jagged lines indicate that the exact boundaries of the proteins are not known. Commas between the different polypeptides indicate that their order in the gene sequence is also unknown. Thick lines represent vector sequences.

and RWB7 were grown as described by Negrin and Neilands (27). Cells were converted to vesicles by the methods of Kaback (15) and Ramos and Kaback (30) with modifications introduced by Negrin and Neilands (27). Transport assays were performed according to Negrin and Neilands (27), except the concentration of the labeled siderophore was 1.0 μM. All of the transport experiments were repeated from two to six times.

Chemicals. [^{55}Fe]ferrichrome and [^{55}Fe]rhodotorulic acid were obtained by methods previously described (19). The specific activities of these substances were 157 and 248 mCi/mmol, respectively. All chemicals were of reagent grade. Albomycin and the various siderophores were from laboratory stocks.

RESULTS

Construction of plasmids. To identify the *fhuB* gene product, we first compared two-dimensional polyacrylamide gel electrophoresis of proteins from *fhuB* and wild-type strains. There appeared to be no differences in these gels, so we used plasmid vectors containing the *E. coli lac* UV5 and *tacI* promoters as a device to amplify the *fhuB* gene product. Because pLC4-44 carried more DNA to the right of *tonA* than did pLC19-19, the former plasmid was used for subcloning of the *tonA* region. Digestion of pLC4-44 DNA with *Hin*dIII generated an 8.25-kb fragment which was ligated into the *Hin*dIII site of pP *lac* to obtain pCPN1 (Fig. 1). pCPN2 and pCPN3 were obtained by *Sal*I digestion of pCPN1, followed by ligation, and pCPN6 was obtained by digestion of pCPN3 with *Pst*I, followed by ligation. These recombinant plasmids were all found to complement the *fhuB* mutation in both BN3300 and BN3307 (Table 2). To obtain a smaller insert containing the *fhuB* gene, we isolated the single 1.4-kb *Pst*I fragment of pCPN6 and ligated it into the *Pst*I site of pKK177-3 to yield pCPN12, which was found to complement the *fhuB* mutations in both BN3300 and BN3307.

To determine whether the 1.4-kb insert of pCPN12 could be made smaller and still complement *fhuB* mutants, pCPN12 was converted to its linear form by cleavage with *Sal*I at a site in the linker region distal to the *tac* promoter. This product was then treated with nuclease *BAL*31 to decrease the size of the insert. The products were recircularized with T4 DNA ligase and used to transform strain BN3307. A total of 50 transformants containing modified pCPN12 were selected and screened by restriction enzyme mapping of plasmid DNA for loss of the *Sal*I site. Twelve clones were identified. All but two had lost less than 50 base pairs of the insert; one, pCPN13, had lost all of the *tac* promoter and 200 base pairs of the insert, and pCPN14 still had an intact *tac* promoter and had lost 400 base pairs of the insert. Neither pCPN13 nor pCPN14 complemented the *fhuB* mutants (Table 2).

Tn*5* insertions. Tn*5* transpositions into pCPN3 and pCPN12 were isolated by infecting plasmid-containing cells with λ::Tn*5* and subsequently screening for plasmids which conferred resistance to both kanamycin and ampicillin. One Tn*5* insert was obtained in pCPN3, and one was obtained in pCPN12. The sites of the insertions, located by restriction mapping of plasmid DNA with *Hin*dIII, *Hin*cII, and *Pst*I, are indicated on Fig. 1.

Plasmids carrying *tonA* and *fhuB*. The ability of 15 siderophores (structures in Luckey et al. [23], Llinas and Neilands [21], and Linke et al. [20]) to support the growth of strains RW193, AN193, RWB7, BN3307, and BN1060 carrying the different plasmids on nutrient broth-deferriferrichrome A plates was examined (Table 2). Plasmids pCPN1, 2, 3, 6, and 12 were found to confer FhuB$^+$ phenotype to strain BN3307, whereas the vector plasmids and pCPN13 and 14 did not. In addition, the plasmid pCPN12 with the 1.4-kb insert in either orientation was found to complement the *fhuB* mutation, indicating that the *fhuB* gene is contained within the 1.4-kb *Pst*I fragment. pCPN3 carrying the Tn*5* (pCPN3K17) insert was able to complement the *fhuB* mutation in BN3307, whereas pCPN12 carrying the Tn*5* insert (pCPN3K26) was not. pCPN1 was able to complement the *tonA* mutation in BN1060; however, as indicated in Table 2, pCPN2 was unable to complement the *tonA* mutation. Strain BN1060 carrying pCPN1 was sensitive to phages T5 and φ80, but

BN1060 carrying pCPN2 was resistant to these phages, indicating that *tonA* is not programmed in its entirety on plasmid pCPN2.

Proteins encoded by the recombinant plasmids. To identify the protein products encoded by the plasmids shown in Fig. 1, [^{35}S]methionine- and [^{35}S]cysteine-labeled samples from CSR603 strains carrying the various plasmids were run on 10 to 20% sodium dodecyl sulfate polyacrylamide gels. In addition to the β-lactamase proteins, pCPN1 coded for five proteins of apparent molecular weight 78,000 (*tonA*), 40,000, 30,000, 24,000, and 13,700 (Fig. 2). Although there are several other bands at ca. 13,700, the lowest band appears to be stronger, and the larger bands may be precursors. pCPN2 is a *Sal*I deletion of pCPN1 and codes for polypeptides of molecular weights 40,000, 30,000, and 24,000, which indicates that the *tonA* protein and the 13.7K protein are located at least partially in the 2.45-kb *Sal*I fragment. The 40K protein was the only detectable product of pCPN3 and pCPN6, indicating that the 30K and 20K proteins are encoded wholly or in part by the 1.85-kb *Sal*I fragment of pCPN2. These proteins are shown on the restriction map of pCPN1. pCPN3K17, which is pCPN3 with a Tn*5* insert, no longer expresses the 40K protein; because pCPN3K17 can complement an *fhuB* mutation, and thus expresses *fhuB* function, this indicates that this protein cannot be the *fhuB* gene product.

Only the β-lactamase proteins of molecular weights 29,500 and 28,300 are expressed by pCPN12 and pCPN14 in

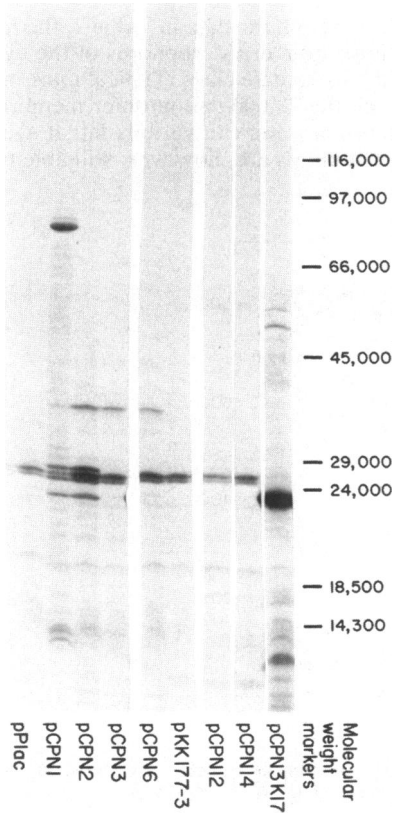

FIG. 2. Autoradiograph of a polyacrylamide gel electrophoretic separation of [^{35}S]methionine- and [^{35}S]cysteine-labeled polypeptides produced in maxicells of strain CSR603 containing, with the exception of pCPN13, the plasmids shown in Fig. 1.

TABLE 2. Response of different strains to siderophores

Strain	Reaction with the following siderophore:[a]																
	Ferrichrome	Ferrichrome C	Ferricrocin	Ferricrysin	Ferrirhodin	Sake colorant A	Sake colorant B	Albomycin	Desferal	Norcardamin	Rhodotorulic acid	Dimerum acid	Coprogen B	Schizokinen	Arthrobactin
RW193	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AN193	−	−	−	−	−	−	−	−	−	±	+	+	+	+	±
BN3307	−	−	−	−	−	−	−	−	−	±	−	−	−	−	±
RWB7	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
BN3307(pP *lac*)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
BN3307(pCPN1)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BN3307(pCPN2)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BN3307(pCPN3)	+	+	+	+	+	+	+	+	±	±	+	+	+	+	±
BN3307(pCPN6)	−	−	−	−	−	−	−	−	±	±	+	+	+	+	±
BN3307(pKK177-3)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
BN3307(pCPN12)	+	+	+	+	+	+	+	+	±	±	+	+	+	+	±
BN3307(pCPN13)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
BN3307(pCPN14)	+	+	+	+	+	+	+	+	±	±	+	+	+	+	±
BN3307(pCPN3K17)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
BN3307(pCPN12K6)	+	+	+	+	+	+	+	+	±	±	+	+	+	+	±
BN1060(pCPN1)	+	+	+	+	+	+	+	+	±	±	+	+	+	+	±
BN1060(pCPN2)	±	±	±	±	±	±	±	−	±	±	+	+	+	+	±

[a] +, Good growth with a large halo; ±, a small halo; −, no growth.

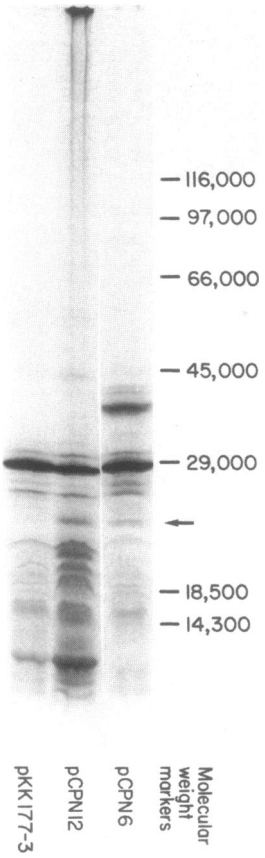

FIG. 3. Autoradiograph of a polyacrylamide gel electrophoretic separation of [^{35}S]methionine- and [^{35}S]cysteine-labeled polypeptides produced in maxicells of strain BN660 containing plasmids pKK1773, pCPN12, and pCPN6. The arrow points to the 20K putative *fhuB* protein.

CSR603, which led us to suspect that the *fhuB* gene product might be a small protein which is degraded by proteases in the cell. To attempt to overcome this problem, we used a *lon* mutant, BN660, as a maxicell strain. *lon* mutants have a number of variations, including reduced degradation of abnormal and normal proteins (39, 40). Figure 3A is an autoradiograph of [^{35}S]methionine- and [^{35}S]cysteine-labeled polypeptides synthesized by plasmids in UV-irradiated whole cells of strain BN660. A 20K protein is expressed by plasmids pCPN12 and pCPN6. This is not expressed by the vector plasmid pKK177-3. The 20K protein is also expressed by pCPN1, pCPN2, and pCPN3, abut not by pP *lac* in maxicells from strain BN660 (data not shown). This protein is indicated on Fig. 1 as being coded by the 1.4-kb *Pst*I fragment.

Transport in vesicles. In an attempt to define the function of the *fhuB* gene product, we studied the transport of [^{55}Fe]ferrichrome and [^{55}Fe]rhodotorulic acid in inner membrane vesicles made from strains RW193, BN3300, and RWB7. Vesicles made from strains BN3300 and RWB7 were able to transport both [^{55}Fe]ferrichrome and [^{55}Fe]rhodotorulic acid at rates similar to vesicles prepared from strain RW193 (Fig. 4). The values found were 10.02, 12.12, and 9.52 pmol of ferrichrome iron transported per mg of vesicle protein per min for RW193, BN3300, and RWB7, respectively. The initial rate for ferric rhodotorulate in both RW193 and BN3300 was 50 pmol/mg of vesicle protein per min. Similar rates were obtained for both siderophores in strain BN3306.

DISCUSSION

As can be seen from the data in Table 2, the *tonA* function enables the utilization of siderophores of the cyclohexapeptide ferric trihydroxamate class. Typical *tonA* mutants, such as AN193, lack the 78,000-dalton outer membrane receptor for the ferrichromes and the various lethal agents listed in Table 1. Such mutants are, however, still able to respond to

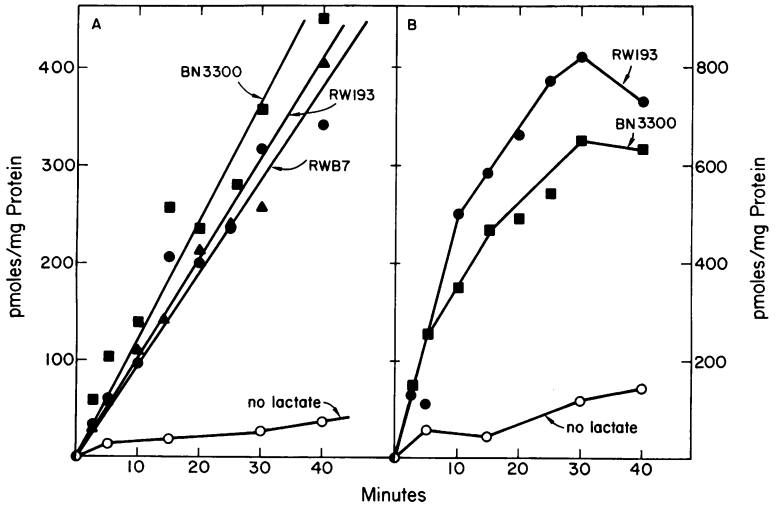

FIG. 4. [^{55}Fe]ferrichrome uptake (A) and [^{55}Fe]rhodotorulic acid uptake (B) in inner membrane vesicles. Symbols: ●, RW193 (*tonB*$^+$ *fhuB*$^+$); ■, BN3300 (*tonB*$^+$ *fhuB*); ▲, RWB7 (*tonB fhuB*$^+$); ○, no lactate added to the vesicles (all strains). An sample of stock vesicle suspension containing 0.8 to 1.5 mg of protein was made in 9 ml of buffer and incubated at 37°C for 15 min on a gyratory shaker set at 200 rpm. D-(−)-lactate was then added to give a final concentration of 20 mM, followed immediately by addition of ^{55}Fe-labeled siderophore to a final concentration of 1.0 μM. At various times 1.0-ml samples were withdrawn, diluted with 5 ml of 0.1 M lithium chloride, and passed through 0.45-μm membrane filters (HA; Millipore Corp.) which had been presoaked for 1 h in 1 ml of the appropriate siderophore. The filters were washed with 10 ml of lithium chloride solution, dried, and counted.

a variety of other ferric hydroxamate siderophores, such as rhodotorulic acid. The mnemonic *fhu*, for ferric hydroxamate utilization, is thus not particularly accurate. We propose to use the designations *tonA* and *fhuB* for the specific genotypes given in Table 1 until a systematic nomenclature, based on more complete knowledge, can be evolved.

Three siderophores have thus far been shown to require outer membrane receptors. In addition to the *tonA* protein, an 81,000-dalton outer membrane receptor serves as the common binding site for ferric enterobactin and colicin B (10, 28). In ColV-bearing strains of *E. coli*, the receptor for ferric aerobactin has been identified as a 74,000-dalton protein also binding cloacin (3). A requirement for additional genetic functions for use of siderophore-complexed iron can be easily imagined. However, a search for a shockable component in ferrichrome transport in *S. typhimurium* gave negative results (M. Luckey, Ph.D. dissertation, University of California, Berkeley, 1976). Based on experiments with vesicles, there is some indication for the presence of a discrete transport system for ferrichrome in the inner membrane of *E. coli* (27). Release of iron from the siderophores will require a reductase and possibly also a modification system (12).

Our data do not disclose the role of the *fhuB* gene product in this metabolic hierarchy. We have cloned the function and restricted it to a 1.4-kb fragment which expresses a 20K protein in maxicells that carry the *lon* mutation (Fig. 3). Therefore, this 20K protein may be the *fhuB* gene product. Since it is expressed only in maxicells carrying *lon*, it appears to be unstable and suffers proteolytic digestion. It is possible, however, that the 20K protein could be a degradation product, since *lon* mutants are devoid of only one protease. This protein is also expressed in pCPN6 (Fig. 3), and in pCPN1, 2, and 3. Hence it does not appear to be a truncated polypeptide made by the smaller insert in pCPN12.

To determine whether the plasmids carrying the Tn*5* insert expressed the 20K protein, we examined their expression in BN660 (data not shown). These gels were difficult to interpret because of over expression of the proteins from the Tn*5* insert and because of high background in the BN660 strain. Strain BN660 carries the *recA* mutation and not *uvrA* and *phr-1* and thus must be exposed to more UV light than strain CSR603 to kill the cells. Even so, there are always some surviving BN660 cells which contribute to the high background. CSR603 with the *lon* mutation may help to alleviate this problem.

In addition to digesting pCPN12 with *BAL*31 nuclease from the side near the *tac* promoter, we also tried to digest from the other side of the insert, first with *Hin*dIII and then with *BAL*31 nuclease. However, we were unable to obtain any fragments smaller than the 1.4-kb insert which still had *fhuB* function. This observation, taken together with the data from the Tn*5* insertions in both pCPN3 and pCPN12, indicate that the minimum size for the *fhuB* gene is ca. 1.25 kb.

It may appear that the same region, the left side of the insert of pCPN3, encodes both the 20K and 40K proteins. The 40K protein is missing in pCPN3K17, and pCPN12 encodes a 20K protein. There is possibly enough DNA in the insert in pCPN12 to code for both a 20K and a 40K protein. Furthermore, the Tn*5* insert in pCPN3K17 could be polar and eliminate both proteins.

In inner membrane vesicles made from *fhuB* strains, the rate of transport of [^{55}Fe]ferrichrome and [^{55}Fe]rhodotorulic acid was similar to that observed in vesicles from an *fhuB*$^+$ strain. These data indicate that the *fhuB* gene product is not required for transport of hydroxamate-type siderophores across the inner membrane of *E. coli*. Vesicles prepared from a *tonB* strain were also able to transport the iron of both siderophores. Weaver and Konisky (37) obtained similar results in spheroplasts made from *tonB* mutants. Furthermore, the rates we measured for ferrichrome transport in vesicles were very close to those obtained in spheroplasts, i.e., 10.0 pmol/min per mg of protein in vesicles and 10.3 pmol/min per mg of protein in spheroplasts. However, Wookey et al. (38) observed that although *tonA* mutants took up ferrichrome after exposure to pronase, *tonB* and *fhuB* mutants did not, suggesting that these functions are required for transport of ferrichrome across the inner membrane. Our results do not support this interpretation. In previous work, Negrin and Neilands (27) ruled out the possibility of ferrichrome binding to the inner membrane, with the iron released by reduction. Tritiated ferrichrome was transported into inner membrane vesicles at the same rate as [^{55}Fe]ferrichrome. Furthermore, given that the void space of vesicles is about 2.2 μl/mg of protein (15) and assuming an average vesicle content of more than 400 pmol of ferrichrome per mg of protein at saturation (Fig. 4), the internal concentration of ferrichrome may reach 210 μM. Since the external substrate level is 1.0 μM, this is a concentration factor of 200. This exceeds the concentration which could be attributed to simple binding.

In addition to the putative *fhuB* protein and the 78K *tonA* protein, we found four other proteins coded by the 8.25-kb insert in pCPN1. There are two to four proteins encoded in the region between the *tonA* and *fhuB* genes (Fig. 2 and 3). In view of the location of their genes, it seems possible that these proteins are related in some way to hydroxamate utilization in *E. coli*.

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