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An Aspartate Deletion Mutation Defines a Binding Site of the Multifunctional FhuA Outer Membrane Receptor of *Escherichia coli* K-12

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The FhuA protein of the outer membrane serves as a receptor for phages T5, T1, and ϕ80, for colicin M, for the antibiotic albomycin, and for ferrichrome and related siderophores. To identify protein regions important for the multiple FhuA activities, *fhuA* genes of spontaneous chromosomal mutants which expressed wild-type amounts of the FhuA protein were sequenced. A mutant which was partially T5 sensitive but impaired in all other functions was missing aspartate residue 348 of the mature protein as a result of a three-base deletion. This aspartate residue is part of the hydrophilic sequence Asp-Asp-Glu-Lys. Replacement by site-specific mutagenesis of each of the Asp residues by Tyr, of Glu by Val, and of Lys by Met reduced FhuA activity but less than the Asp deletion did. Ferrichrome inhibited binding of phage ϕ80 and of colicin M to these mutants in an allele-specific manner. A completely resistant derivative of the Asp deletion mutant contained, in addition, a leucine-to-proline substitution at position 106 and eight changed bases, converting at positions 576 to 578 an Arg-Pro-Leu sequence to Ala-Arg-Cys. The latter mutations and the Leu-to-Pro replacement alone did not alter sensitivity to the phages but reduced sensitivity to colicin M and albomycin 10- to 1,000-fold. The proline replacements probably disturb FhuA conformation and, in concert with the Asp deletion, inactivate FhuA completely. It is concluded that the Asp deletion site defines a region of FhuA which directly participates in binding of all FhuA ligands. Growth promotion studies on iron-limited media revealed that certain siderophores of the hydroxamate type, such as butylferrichrome, ferrichrysin, and ferrirubin, are taken up not only via FhuA but also via the FhuE outer membrane receptor protein.

The multifunctional FhuA (formerly designated TonA) receptor protein in the outer membrane of *Escherichia coli* is required for the uptake of the iron carrier ferrichrome, the structurally analogous antibiotic albomycin, and colicin M and for infection by phages T1, ϕ80, and T5 (5–7). Transport of ferrichrome, albomycin, and colicin M through the outer membrane and infection by T1 and ϕ80 depend in addition on the TonB protein, which itself is influenced by the ExbBD proteins (11) and alternatively by the TolQR proteins in *exbBD* mutants (4, 5). Point mutations in TonB which suppress point mutations in the TonB box of FhuA (27), and the physical stabilization of the TonB protein by FhuA (5, 14), suggest a direct binding of FhuA to TonB. It is thought that the TonB protein assumes two conformations of which the energized state activates FhuA so that surface-bound ferrichrome, albomycin, and colicin M are released into the periplasm (5, 7), while the same conformation triggers release of DNA from the heads of phages T1 and ϕ80 (16). To understand this multifactorial process, the arrangement of the FhuA protein in the outer membrane and its active sites must be determined. Small C-terminal deletions in FhuA reduced the stability against cellular proteases, insertion into the outer membrane, and activity toward the FhuA ligands (13, 18, 28). Insertion of 6-bp linkers into *Hpa*II and *Cfo*I sites of the *fhuA* gene reduced FhuA activity to various extents for all ligands with the exception of a Ser-Ser insertion after residue 321 of the mature protein, which selectively abolished uptake of ferrichrome while leaving phage and colicin receptor functions intact (9). A Glu-Leu insertion after residue 82 lowered most strongly the TonB-dependent functions while causing only a 10-fold reduction in T5 sensitivity (9). These findings, as well as the fact that FhuA contains neither obvious hydrophobic transmembrane helices nor a porinlike structure, are not sufficient to propose a model for FhuA topology in the outer membrane.

Previously, we isolated a spontaneous chromosomal mutant on tryptone-yeast extract (TY) agar plates seeded with 10^9 cells of *E. coli* B9 *fepA cir aroB* and 10^8 T1 phages (7). The mutation was mapped by P1 transduction at min 4 of the *E. coli* K-12 linkage map. The mutant, designated B9/41, produced wild-type amounts of the FhuA protein with an unaltered electrophoretic mobility on sodium dodecyl sulfate (SDS)-polyacrylamide gels. It displayed 1% T5 plating efficiency compared with the B9 strain and was resistant to T1, ϕ80, colicin M, and albomycin. Ferrichrome did not serve as an iron donor. A spontaneous derivative of B9/41, designated 41/2, isolated on plates seeded with T5, was completely resistant to all agents examined even though it expressed normal amounts of the FhuA protein. This mutant was used to isolate a spontaneous T5 host range mutant which exhibited a plating efficiency as high as on B9 (7).

To characterize the mutations thought to be located in the *fhuA* gene of both strains, chromosomal DNA was isolated and amplified by the polymerase chain reaction (PCR) (1, 23). A deletion of an aspartate residue (Asp-348) found in mutant B9/41 resulted in the phenotype previously described. Mutations at two additional sites were required to abolish completely all FhuA-related activities of mutant 41/2.

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TABLE 1. *E. coli* K-12 strains and plasmids used

Strain or plasmid	Genotype and phenotype	Reference or source
Strains		
AB2847	*aroB malT thi tsx*	7
UL3	AB2847 *fhuA recA* Tn*10*	28
B9/41	AB2847 *cir fepA fhuA*	7
41/2	AB2847 *cir fepA fhuA*	7
MS172	F⁻ *araD139 lacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR aroB thi fhuE*::λ*plac* Mu53	26
H1020	*lac*(Am) *trp*(Am) *his metB tsx zad*::Tn*10*	K. Hantke
HK97	MS172 *fhuA*	This study
Plasmids		
pHK16	pSU18 *fhuA* (41/2 after PCR)	This study
pHK44	pSU18 *fhuA* (B9/41 after PCR)	This study
pHK581	pMa5-8 *fhuA* wild type	This study
pHK582	pMc5-8 *fhuA* wild type	This study
pHK762	pT7-6 *fhuA* (B9/41 after PCR)	This study
pHKD348	pMc5-8 *fhuA* (ΔD348)	This study
pHK348	pMc5-8 *fhuA* (D348Y)	This study
pHK349	pMc5-8 *fhuA* (D349Y)	This study
pHK350	pMc5-8 *fhuA* (E350V)	This study
pHK351	pMc5-8 *fhuA* (K351M)	This study
pHK471	pMc5-8 *fhuA* (R471L)	This study
pHK472	pMc5-8 *fhuA* (D472Y)	This study
pHK100	pT7-6 *fhuA1,2,3,4,5*	This study
pHK101	pT7-6 *fhuA1*	This study
pHK102	pT7-6 *fhuA3*	This study
pHK103	pMc5-8 *fhuA4*	This study
pHK104	pT7-6 *fhuA5*	This study
pHK105	pT7-6 *fhuA1,3*	This study
pHK106	pT7-6 *fhuA1,4*	This study
pHK107	pT7-6 *fhuA1,5*	This study
pHK108	pT7-6 *fhuA1,3,4*	This study
pHK109	pT7-6 *fhuA1,4,5*	This study
pHK110	pT7-6 *fhuA3,4*	This study
pHK111	pT7-6 *fhuA4,5*	This study
pHK112	pMc5-8 *fhuA3,4,5*	This study
pT7-6		30
pSU18		31
pHSG576		H. Schöffler

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *E. coli* K-12 strains and plasmids used are listed in Table 1. They were grown in TY medium or in nutrient broth (NB) as described previously (6, 20). To reduce the available iron in NB medium, 2,2'-dipyridyl (0.2 mM) was added to make NBD medium. If required, the media contained antibiotics in order to maintain the plasmids (ampicillin, 30 μg/ml; chloramphenicol, 25 μg/ml; tetracycline, 15 μg/ml). *E. coli* HK97 *fhuA fhuE* was obtained by phage P1 transduction of *zad*::Tn*10* of strain H1020 into strain 41/2, selecting for tetracycline resistance and FhuA⁻ phenotype. From the resulting HK9 strain, the mutated *fhuA* gene was cotransduced with *zad*::Tn*10* into strain MS172 *fhuE*.

Recombinant DNA techniques. Isolation of chromosomal DNA (1, 29) and plasmids (24, 33), use of restriction enzymes, ligation, agarose gel electrophoresis, recovery of DNA fragments from agarose, and transformation (24) were done by standard techniques. DNA was sequenced by the enzymatic dideoxy method (25), using [^{35}S]ATP for labeling.

Chromosomal DNA was amplified by PCR, starting from either isolated DNA or single colonies on TY agar (15). Primer PHS19 (5'-GCAGCGGTGGCATGCGTATCTTGCC GG-3') annealed upstream of *fhuA* at bp 112 to 138 (10) and contained an *Sph*I restriction site; primer PHS20 (5'-AAGCGTGCGCCCGGGCACACGAAAGGA-3') annealed downstream of *fhuA* at bp 2911 to 2885 and contained a *Sma*I site. The reaction mixture was composed of 1 μg of DNA in 10 μl of water, 1 μg of each of primers PHS19 and PHS20 in 1 μl, the four deoxynucleotides each at 0.2 mM in a total of 1 μl, 10 μl of PCR buffer (0.1 M Tris hydrochloride, 0.5 M KCl, 25 mM $MgCl_2$, 0.01% gelatin, pH 8.3), and 2.5 U of *Taq* polymerase (0.6 μl), adjusted to 0.1 ml with distilled water. The reaction mixture was heated for 1 min at 94°C, 2 min at 54°C, and 4 min at 72°C in a Bio-Med Thermocycler 60 (Bio-Med, Theres, Germany). The reactions were stopped after 20, 30, and 40 cycles and examined by agarose gel electrophoresis to ascertain whether the expected *fhuA* DNA fragment of 2.8 kb had been formed. The purest sample (single band) was obtained after 20 cycles; the highest yield was obtained after 30 cycles. The 2.8-kb fragment was recovered from the agarose gel by extraction with phenol, cut with *Sph*I and *Sma*I, cloned into the high-copy-number plasmid pSU18 (31), cleaved with *Sph*I-*Sma*I, and dephosphorylated. The pSU18 derivative carrying the *fhuA* gene of strain B9/41 was designated pHK44; that of strain 41/2 was designated pHK16. For construction of the other plasmids used, see Fig. 2.

Site-directed mutagenesis. Single-nucleotide replacements were introduced by using pMa5-8 and pMc5-8 phasmid vectors as previously described for the construction of mutants in the TonB box of *fhuA* (27). The oligonucleotides used for mutagenesis were as follows (replaced nucleotides are underlined): MutA, 5'-CGTCGTTTATGATGAG; MutB, 5'-CGTTGATTATGAGAAG; MutC, 5'-GTTGATGATGTGAAGCTG; Mut2, 5'-CGTCGTTGATGATGAGATGCTGCAAAAC; MutD, 5'-GATAAACGTTATGACAAAC; and MutF, 5'-CCGATAAACTTGATGACAAAC.

Growth promotion assay. Since the *E. coli aroB* mutants used do not synthesize their own siderophore enterochelin (enterobactin), they are unable to grow on NBD agar plates. If they use a siderophore supplied on a paper disk, a growth zone develops where the siderophore diffuses from the disk into the surrounding NBD agar. The diameter of the growth zones is related in a logarithmic manner to the concentration of the siderophore. The diameter of the growth zones includes the diameter of the paper disk (6 mm). Since the concentration of the siderophore in the diffusion zone is unknown, we present the amounts of the siderophores placed onto the disks (10 μl of 1, 0.3, 0.1, 0.03, and 0.01 mM solutions). The plates were usually inspected after incubation overnight at 37°C. This is a very sensitive and reliable assay. The siderophores were of the following sources: albomycin (from H.-P. Fiedler, Department of Microbiology/Biotechnology, Universität Tübingen) and ferrichrome, ferricrocin, ferrichrysin, enantioferrichrome, propylferrichrome, butylferrichrome, tetraglycylferrichrome, ferrichrome A, ferrichrome C, *O*-(phenyl)-amino ferricrocin, ferrirhodin, hexahydroferrirhodin, ferrirubin, and coprogen (from G. Winkelmann, Department of Microbiology/Biotechnology, Universität Tübingen). The structures of these compounds have been described elsewhere (32).

Determination of sensitivity to phages T5, T1, and φ80 and to albomycin and colicin M. Tenfold dilutions of phage, albomycin, and colicin M solutions (4 μl) were dropped onto TY agar plates overlaid with TY top agar which contained 10^8 cells of the strain to be tested. After incubation overnight at 37°C, the highest dilution at which a clear (turbid) zone of

TABLE 2. Phenotypes of *fhuA* mutants close to the Asp-348 site

Strain	Amino acid replacement[a]	Sensitivity to[b]:					Growth on ferrichrome[c]
		T1	T5	φ80	ColM	Albo	
UL3/pHK582	VDDEKL[d]	6	6	7	5	4	35
UL3/pHK348	VYDEKL	6	6	7	4	3	30
UL3/pHK349	VDYEKL	6	6	7	5	3	30
UL3/pHK350	VDDVKL	6	6	7	3	3	30
UL3/pHK351	VDDEML	6	6	7	5	3	30
UL3/pHKD348	V-DEKL		5		2	0	26

[a] The replaced amino acids are indicated in boldface.
[b] The last of 10-fold dilutions which resulted in turbid zones of growth inhibition are listed. For example, 6 means that the phage stock solution could be diluted 10^6 times. ColM, colicin M; Albo, albomycin.
[c] Expressed as the diameter (in millimeters) of the growth stimulation zone. Ten microliters of a 1 mM solution of ferrichrome was placed on the paper disk.
[d] Wild type.

growth inhibition occurred was recorded. The number of phage plaques in the lysis zone was counted.

Inhibition of ligand binding by ferrichrome. Cells to be tested were grown in TY medium. In the logarithmic growth phase, 0.1 ml of the culture was suspended in 3 ml of TY top agar (10 g of agar per liter), which contained 30 μl of 10 mM ferrichrome. The suspension was poured over TY agar plates (16 g of agar per liter; 25 ml), which were incubated at room temperature for 1 h before 4 μl of 10-fold dilution series of the phages T1, T5, and φ80, colicin M, and albomycin were dropped onto the plates.

SDS-polyacrylamide gel electrophoresis (PAGE). Cells were grown to a density of 10^9/ml, sedimented by centrifugation, and suspended in 25 μl of buffer containing 4% SDS, 20 mM Tris hydrochloride, and 0.2 mM EDTA (pH 8.0). These samples were heated for 5 min in a boiling water bath and then diluted with the same volume of sample buffer consisting of 0.2 ml of 0.625 M Tris hydrochloride, 0.4 ml of 10% SDS, 0.4 ml of 50% glycine, 0.4 ml of 0.01% bromophenol blue, and 0.1 ml of β-mercaptoethanol. Ten microliters of each sample was applied to the polyacrylamide gel. The stacking gel was composed of 3% acrylamide, 0.08% bisacrylamide, 0.02% *N,N,N',N'*-tetramethylethylenediamine, 0.1% SDS, and 0.125 M Tris hydrochloride (pH 6.8); the running gel (0.75 mm thick; 12 to 14 cm long) consisted of 11% acrylamide, 0.08% bisacrylamide, 0.2% SDS, and 0.375 M Tris hydrochloride (pH 8.8). During polymerization, the running gel was layered over with isopropanol. Electrophoresis was run in a buffer consisting of Tris hydrochloride (3.1 g), SDS (1 g), and glycine (14.4 g), dissolved in water to 1,000 ml (pH 8.8) at a constant current of 30 mA until bromophenol blue had reached the end of the gel. The gel was stained for 40 min with Serva blue (Serva, Heidelberg, Germany) dissolved in 45% methanol–10% acetic acid–45% water (vol/vol/vol) and destained by repeated washing with 20% methanol–7.5% acetic acid–72.5% water (vol/vol/vol).

RESULTS

Characterization of mutant B9/41. The DNA of strain B9/41 was isolated and amplified by PCR. The resulting plasmid, pHK44, contained the *Eco*RI, *Hin*dIII, *Sal*I, *Sau*I, and *Mlu*I restriction fragments deduced from the nucleotide sequence. Sequencing of the *fhuA* gene revealed a mutation at positions 1681 to 1683 (numbering according to the published *fhuA* sequence [10]), in which three nucleotides had been deleted, leading to the loss of an aspartate residue. This mutation was located on the *fhuA* *Pfl*MI-*Sal*I fragment. Replacement of the wild-type *Pfl*MI-*Sal*I fragment on plasmid pHK582 by the mutant fragment resulted in *E. coli* UL3 transformants which displayed the phenotype of B9/41 (Table 2). The residual sensitivity to colicin M, which has not been noted previously (7), comes from the higher colicin activity used in these experiments compared with that used previously. In addition, cross-streak tests were performed previously, whereas in this study the solutions were dropped onto the agar plates. The drop test is a more sensitive assay than the cross-streak test.

Strain UL3, used to characterize the functions of the cloned mutated *fhuA* genes, did not express a FhuA protein (28). SDS-PAGE of B9/41 cells showed the FhuA protein (7). Transformants of UL3 carrying wild-type *fhuA* on plasmid pHK582, and mutant *fhuA* on pHKD348, which determines FhuA with the Asp deletion, synthesized increased amounts of the FhuA protein due to the multicopy plasmid (see Fig. 3, lanes 5 and 6).

Specific mutagenesis at the Asp site. The deleted Asp residue is located in a highly hydrophilic region (Asp-Asp-Glu-Lys) which may form a loop at the cell surface or, although less likely, in the periplasm. Additional mutants in this region (Table 2) were constructed by site-specific mutagenesis. They showed a 10- to 100-fold-lower sensitivity to colicin M and albomycin but remained fully sensitive to phages T5, T1, and φ80. Growth on ferrichrome was also reduced. Strain UL3 transformed with the mutant *fhuA* genes produced as much FhuA protein as did UL3 carrying wild-type *fhuA* on the same plasmid (Fig. 1; compare lane 5 with lanes 7 to 10; the somewhat weaker wild-type FhuA band comes from the better iron supply during growth of UL3/pHK582 compared with the other strains). The same FhuA activities were obtained when the mutated *fhuA* genes were excised from the high-copy-number plasmid pMc5-8 and cloned into the low-copy-number plasmid pHSG576 (data not shown), excluding an increase in residual activities of mutant FhuA proteins by the high FhuA expression.

The FhuA sequence contains another hydrophilic region, Thr-Thr-Asp-Lys-Arg-Asp-Asp-Lys-Gln, extending from residues 467 to 475. Two amino acids were replaced to determine whether this region is also involved in ligand binding or translocation. Replacement of Arg-471 by Leu and of Asp-472 by Tyr did not affect the mutants' sensitivity to the three phages (PFU) and albomycin but resulted in a 10-fold reduction of colicin M sensitivity and a moderate reduction of the ferrichrome growth zone (30 mm). The results give no indication that this hydrophilic region itself forms a ligand-responsive domain, but it may instead influence true ligand-binding sites.

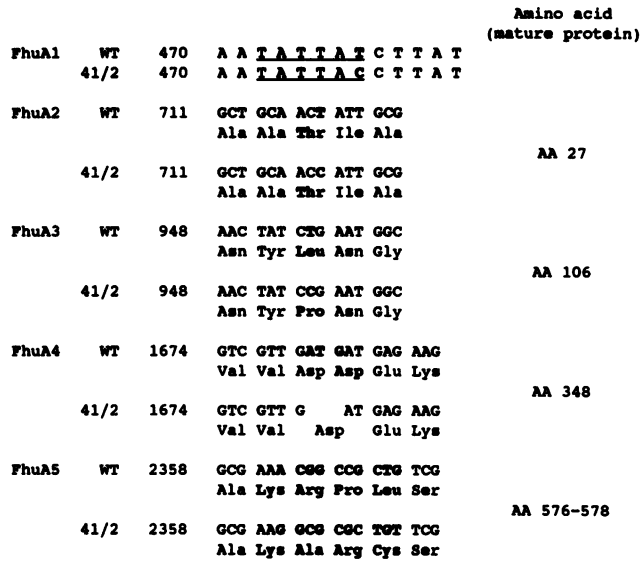

FIG. 1. Nucleotide substitutions and corresponding amino acid replacements found in the PCR product after amplification of the chromosomal *fhuA* gene of *E. coli* 41/2 *fhuA*. The start of the shown nucleotide and amino acid sequences and the positions of the replaced amino acids in the mature protein are indicated. WT, wild-type sequence; AA, amino acid.

Competition between ligand binding to FhuA and its derivatives. The spontaneous Asp-348 deletion mutant and the mutants constructed around Asp-348 displayed a reduced growth on ferrichrome, indicating a somewhat impaired binding and/or uptake of ferrichrome. To examine ferrichrome binding and to unravel differences in binding between the various mutants, inhibition of phage, colicin M, and albomycin activity by ferrichrome was determined. Tenfold dilutions of the ligand stock solutions were dropped onto ferrichrome-containing TY agar plates. Ferrichrome conferred resistance to albomycin in the wild-type and the mutant strains. Sensitivity to phages T1 and T5 remained unaltered. However, sensitivities to phage φ80 and colicin M were strongly and allele-specifically changed. The strain carrying the Asp-348-to-Tyr substitution (D348Y) and the strain expressing FhuA (E350V) became fully resistant to φ80 in the presence of ferrichrome, whereas sensitivity of these strains to colicin M was reduced only 10-fold. Binding of ferrichrome to FhuA (D349Y) reduced sensitivity to φ80 and colicin M 100-fold; binding to FhuA (K351M), FhuA (D472Y), and FhuA (R471L) and to wild-type FhuA diminished φ80 sensitivity 1,000-fold and sensitivity to colicin M 100-fold, respectively. The different responses to ferrichrome indicate altered binding properties of the mutants for ferrichrome and the ligands, which for phage φ80 and colicin M became not apparent in the experiments performed in the absence of ferrichrome (Table 2).

Fe^{3+} siderophore specificity of FhuA and FhuA derivatives. Additional siderophores of the hydroxamate type were tested to determine whether they reveal stronger defects of the *fhuA* mutants in iron uptake. Growth of *E. coli* UL3 *fhuA* was supported by a number of hydroxamates, suggesting uptake routes which differ from that of FhuA. Growth of H1857 *fhuABCD* was not supported by any of the siderophores, which indicates that translocation of all Fe^{3+} hydroxamates across the cytoplasmic membrane was dependent on the FhuCDB transport system and also excludes use of unknown alternative uptake pathways or leakage into the cells. The only known outer membrane receptors which, like FhuA, channel siderophores into FhuCDB are the FhuE protein, which transports Fe^{3+} coprogen and Fe^{3+} rhodotorulic acid (17, 26), and the IutA protein, through which Fe^{3+} aerobactin is taken up (3). The strains used contained the *fhuE* gene but not the *iutA* gene. To eliminate iron uptake via FhuE, the *fhuA fhuE* double mutant HK97 was constructed by phage P1 transduction (Materials and Methods). The receptor double mutant was unable to take up any of the siderophores, showing that FhuA and FhuE were the only iron hydroxamate receptors. After transformation with plasmid pHK582, carrying wild-type *fhuA*, all FhuA functions, including albomycin sensitivity and growth on ferrichrome, were quantitatively restored (data not shown), indicating that the *fhuA* mutation of HK97 had no detectable polar effect on the expression of the downstream *fhuCDB* genes. These requirements having been fulfilled, strain HK97 was transformed with the plasmids carrying the mutated *fhuA* genes. Controls were run with strains AB2847 *fhuA*$^+$ *fhuE*$^+$, MS172 *fhuA*$^+$ *fhuE*, B9/41 *fhuA fhuE*$^+$, and 41/2 *fhuA fhuE*$^+$. The siderophores were used at a concentration just sufficient to observe growth promotion (0.01 mM [Table 3] in contrast to 1 mM [Table 2]). Growth of the *fhuA* mutants was impaired to different extents on the various siderophores as sole iron sources. Strain UL3/pHK349 was the most active. It responded to ferrichrome and to all of the other siderophores listed. UL3/pHK348 was less active and displayed different growth responses to the various siderophores. UL3/pHK350 showed a low cell density in the growth zone, while UL3/pHK351 grew even more weakly. At the very low siderophore concentrations used, the mutants could be clearly differentiated phenotypically. The phenotype pattern did not change qualitatively when the siderophore concentration applied to the paper disk was increased from 0.01 mM to 0.03, 0.1, 0.3, and 1 mM (data not shown). However, at the higher siderophore concentrations, the alternative uptake route via FhuE became apparent with strain 41/2 (Table 3 and data not shown). Ferrichrome and the structurally closely related enantioferrichrome and ferricrocin were not taken up, excluding entry of these compounds via FhuE. Ferrichrysin (0.03 mM), propylferrichrome (0.1 mM), butylferrichrome (0.03 mM), tetraglycylferrichrome (1 mM), ferrichrome A (1 mM), ferrirubin (0.1 mM), and hexahydroferrirhodin (0.3 mM) stimulated growth. In the ferrichrome series, uptake via FhuE seemed to be correlated with the hydrophobicity of the compounds. Ferrichrome, which has no aliphatic side chains, was not taken up, but 0.3 mM propyl- and butylferrichrome gave rise to 10- and 18-mm growth zones, respectively. However, ferricrocin, in which one glycine residue of ferrichrome is replaced by a serine residue, is not taken up via FhuE, while ferrichrysin, which contains two glycine-to-serine substitutions, is taken up. Since the latter compound is more hydrophilic than ferrichrome, hydrophobicity does not seem to be an essential parameter; rather, the size of the siderophore may play a role. This assumption is supported by the fact that ferrirhodin did not enter cells but hexahydroferrirhodin could support growth (0.3 mM, 9 mm).

Characterization of mutant 41/2. A spontaneous mutant of strain B9/41, designated 41/2, which possessed complete resistance to phage T5 and still contained the FhuA protein in wild-type amounts was isolated (7). The mutated gene was amplified by PCR and sequenced. Mutations were found at five sites (designated *fhuA1,2,3,4,5*, Fig. 1). It contained the Asp deletion (*fhuA4*) of strain B9/41 from which it was

TABLE 3. Growth promotion of FhuA point mutants by Fe^{3+} hydroxamates

Fe^{3+} siderophore	Growth[a]									
	AB2847	MS172	B9/41	41/2[b]	HK97 transformed with:					
					pHK582	pHKD348	pHK348	pHK349	pHK350	pHK351
Ferrichrome	19	16	0	0	20	0	17	16	14	16
Coprogen	17	0	14	17	0	0	0	0	0	0
Enantioferri-chrome	(20)	20	0	0	(20)	0	(15)	20	(14)	(17)
Ferricrocin	19	18	0	0	(20)	0	17	18	(19)	(22)[c]
Ferrichrysin	18	17	0	0	20	0	(18)	18	(19)	(21)[c]
Propylferri-chrome	22	20	0	0	(24)[c]	0	(17)	(18)	(17)	(22)[c]
Butylferrichrome	(20)	17	0	0	(18)	0	18	18	(18)	(24)[c]
Tetraglycylferri-chrome	18	16	0	0	(22)	0	16	(19)	(18)	(20)
Ferrichrome A	12	(13)	0	0	(14)	0	(10)	11	0	(12)
Ferrichrome C	18	16	0	0	(16)	0	14	15	(15)	(16)
O-(Phenyl)-amino ferricro-cin	21	18	0	0	(20)	0	18	20	(19)	(24)[c]
Ferrirubin	19	15	0	0	(20)[c]	0	16	17	17	(20)[c]
Ferrirhodin	0	12	0	0	(20)[c]	0	0	(20)	0	(20)[c]
Hexahydroferr-irhodin	16	13	0	0	(16)	0	(16)	16	(14)	(20)[c]

[a] Expressed as the diameter (in millimeters) of growth zones around filter paper disks (6-mm diameter) onto which 10 μl of a 0.01 mM solution of each of the siderophores had been placed. Numbers in parentheses indicate low cell densities in the growth zones.
[b] See Fig. 1 for the amino acid replacements of the original chromosomal mutations *fhuA3,4,5*.
[c] Very low cell densities in the growth zones.

derived, supporting the previous conclusion that this deletion caused the phenotype of B9/41. One mutation is located upstream of the *fhuA* gene (*fhuA1*; Fig. 1 and Fig. 2). The T-to-C replacement at position 477 is located between the proposed −35 and −10 promoter regions (10). To examine whether the upstream mutation affects expression of the *fhuA* gene, total protein of strain UL3/pHK100 *fhuA1,2,3,4,5* and UL3/pHK101 *fhuA1* was separated by

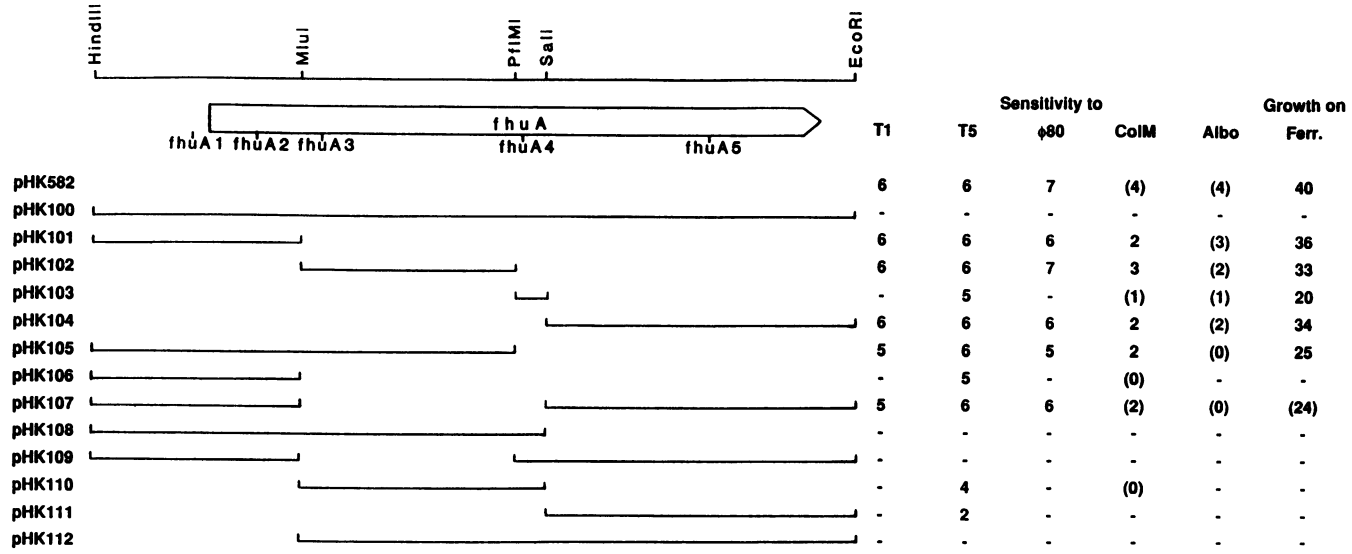

FIG. 2. Location of the *fhuA* gene (arrow) on the chromosome, as defined by the restriction site pattern, and locations of the *fhuA* mutations of the 41/2 PCR product. The designations *fhuA1* to *fhuA5* correspond to the protein designations FhuA1 to FhuA5 of Fig. 1. The plasmids used to identify the mutations on the *fhuA* gene which give rise to the phenotype of strain 41/2 are indicated at the left. DNA fragments of the wild-type *fhuA* gene on pHK582 (except plasmids pHK106 and pHK111, in which cases pHKD348 was used instead of pHK582) were replaced by fragments of the 41/2 PCR product, as indicated by the lines. Sensitivities of *E. coli* UL3 transformants carrying the pHK plasmids to phages T1, T5, and φ80, colicin M (ColM), and albomycin (Albo) and growth promotion by ferrichrome (Ferr) are listed at the right. The number 6, for example, indicates that a phage stock solution could be diluted 10^6-fold to yield single plaques. A 10^3-fold dilution of colicin M and albomycin stock solution resulted in a clear zone of growth inhibition; a 10^4-fold dilution resulted in a turbid zone (4 in parentheses). The growth stimulation zone of ferrichrome is presented in millimeters.

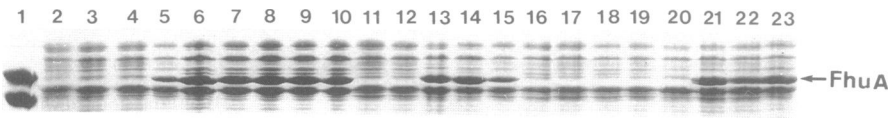

FIG. 3. Section of a stained polyacrylamide gel after SDS-PAGE of the total cell protein of *E. coli* UL3 *fhuA* (lane 2), B9/41 *fhuA* (ΔD348) (lane 3), 41/2 *fhuA3,4,5* (lane 4), UL3/pHK582 (lane 5), UL3/pHKD348 (lane 6), UL3/pHK348 (lane 7), UL3/pHK349 (lane 8), UL3/pHK350 (lane 9), UL3/pHK351 (lane 10), UL3/pHK100 (lane 11), UL3/pHK101 (lane 12), UL3/pHK102 (lane 13), UL3/pHK103 (lane 14), UL3/pHK104 (lane 15), UL3/pHK105 (lane 16), UL3/pHK106 (lane 17), UL3/pHK107 (lane 18), UL3/pHK108 (lane 19), UL3/pHK109 (lane 20), UL3/pHK110 (lane 21), UL3/pHK111 (lane 22), and UL3/pHK112 (lane 23). Lane 1, standard proteins (from top to bottom, human serum albumin [80 kDa], bovine serum albumin [67 kDa], ovalbumin [45 kDa], and carbonic anhydrase [29 kDa] [the latter two not shown]).

SDS-PAGE. No protein band was observed at the position of FhuA (Fig. 3, lanes 11 and 12). Apparently, the PCR had introduced the T-to-C replacement which reduced *fhuA* gene expression below the detection limit of the staining procedure. The −35 region starts at nucleotide 466 and reads TTCGAAT (identities with the consensus sequence are underlined), and the −10 region reads TATAAT. In addition, the consensus sequence proposed for Fur iron repressor-binding sites (26) overlaps with the −10 region. An alternative promoter region starts at nucleotide 448 and reads TTGCGAA at −35 and TATTAT at −10. Removal of the upstream *fhuA* sequence up to nucleotide 467 reduced iron-regulated *fhuA* expression at least fourfold (8), which suggests that this sequence functions as a *fhuA* promoter.

Restriction fragments of the wild-type *fhuA* gene were replaced by the corresponding fragments of the mutated *fhuA* gene to identify the mutations giving rise to the 41/2 phenotype (Fig. 2). Strain UL3 transformed with pHK101 *fhuA1*, in which the *Hin*dIII-*Mlu*I fragment of pHK582 (wild type) was replaced by the corresponding fragment of 41/2 (Fig. 2), synthesized no FhuA protein that could be detected by staining with Serva blue (Fig. 3, lane 12). Nevertheless, the transformants were fully sensitive to phages T1, ϕ80, and T5, sensitivity to colicin M was 100-fold reduced and that of albomycin was 10-fold reduced, and the growth zone around ferrichrome-loaded filter paper disks on NBD medium was smaller (compared with findings for UL3/pHK582 carrying wild-type *fhuA*; Fig. 2). The small number of FhuA molecules was apparently sufficient for phage infection, in that a single phage suffices to infect a cell, whereas many more molecules of colicin M, albomycin, and ferrichrome must enter to inhibit and promote cell growth. UL3/pHK102 *fhuA3*, in which the *Mlu*I-*Pfl*MI fragment of the wild-type *fhuA* gene was replaced by the corresponding 41/2 PCR product and in which leucine was replaced by proline, was also fully sensitive to the phages, 10 times less sensitive to colicin M, and 100 times less sensitive to albomycin (Fig. 2). The lower albomycin sensitivity corresponded to the ferrichrome-stimulated growth zone, which was smaller than that obtained with UL3 *fhuA1*. UL3/pHK103 *fhuA4* (*Pfl*MI-*Sal*I fragment of 41/2 in wild-type *fhuA*) was resistant to phages T1 and ϕ80, 10 times less sensitive to T5, and 1,000 times less sensitive to colicin M and albomycin (Fig. 2). UL3 *fhuA4* contains the same mutation as does B9/41 and showed the same phenotype. UL3/pHK104 *fhuA5* contained eight closely linked nucleotide replacements resulting in three amino acid substitutions (Fig. 1). Interestingly, these mutations, like the Asp deletion, are located in a charged region (Lys-Arg). The overall charge is, however, not altered by the mutations. UL3 *fhuA5* was fully sensitive to the phages and 100 times less sensitive to colicin M and albomycin; accordingly, growth promotion by ferrichrome was also smaller (Fig. 2). UL3/pHK111 *fhuA4,5* displayed nearly a 41/2 phenotype. Only sensitivity to T5 was higher (Fig. 2). A phenotype identical to that of 41/2 was obtained by combining mutations 3, 4, and 5 in UL3/pHK112 *fhuA3,4,5* (Fig. 2). The accumulation of such a high number of mutations was unexpected, since these mutations arose spontaneously by selection of T5-resistant colonies. UL3 *fhuA3,4* was resistant to T1, ϕ80, and albomycin and had a strongly reduced sensitivity to T5. Only undiluted colicin M inhibited growth. Combination of the *fhuA1* promoter mutation with mutations 3 and 4 in UL3/pHK108 *fhuA1,3,4* or with mutations 4 and 5 in UL3/pHK109 *fhuA1,4,5* resulted in full resistance to all agents used (Fig. 2). However, all *fhuA* genes carrying the *fhuA1* mutation expressed FhuA protein below the detection limit of staining (Fig. 3, lanes 11, 12, and 16 to 20), whereas all of the other *fhuA* mutants expressed wild-type FhuA levels (Fig. 3, lanes 6 to 10, 13 to 15, and 21 to 23). The FhuA activities displayed by these UL3 transformants did not correlate with the amount of FhuA protein formed. Rather, their phenotypes were determined by the mutations in the *fhuA* gene and the corresponding amino acid replacements (except *fhuA1*). In fact, very low amounts of wild-type FhuA protein suffice to display full sensitivities to all of the FhuA-related agents. When total cell proteins instead of outer membrane fractions were subjected to SDS-PAGE, as has been done in this study to facilitate examination of the many genetic constructions, chromosomally encoded FhuA protein of strains B9/41 (Fig. 3, lane 3), 41/2 (Fig. 3, lane 4), and AB2847 (data not shown) was not detected by staining. Previously, in these strains FhuA was determined by SDS-PAGE of outer membrane preparations (7). The phenotypes of the mutants were not affected by the location of the *fhuA3,4* and *fhuA4,5* genes on the high-copy-number vector pT7-6, since their cloning on the low-copy-number vector pHSG576 did not alter the properties of the transformants.

DISCUSSION

It is generally found that selection of phage-resistant mutants yields more than 99% derivatives which are lacking the receptor proteins. This is also our experience with FhuA, using phage T5, T1, or ϕ80. In a search for T1-resistant but T5-sensitive strains, thought to be mutated in *tonB*, we in fact obtained several *fhuA* mutants which, by closer inspection using lower phage titers, turned out to be only partially sensitive to T5. The important finding was that they produced wild-type amounts of the FhuA protein, which opened the way to characterizing missense mutations and correlating the genotypes with the phenotypes (7). All of the mutated FhuA derivatives studied in this investigation except those which carried the *fhuA1* mutation were produced in wild-type amounts and displayed wild-type stability. In contrast, insertion mutants and most notably deletion mutants frequently formed grossly altered amounts of FhuA and FhuA

degradation products, rendering it difficult to relate phenotype and genotype (8a, 9, 13, 28). The approach that we have chosen was to characterize chromosomal mutants. PCR made it possible to identify such mutations, although *Taq* polymerase, with an measured error rate of 2×10^{-4}, introduces additional mutations which becomes relevant when a DNA fragment of 2.8 kbp is amplified. However, by exchange of DNA fragments, the mutations determining the phenotype can be identified. In fact, this approach was necessary to ensure that the extensive mutations found on the cloned *fhuA* genes were originally contained on the chromosome. The phenotype of the partially T5 sensitive strain B9/41 is the result of a three-base deletion whereby one aspartate residue is lost in the hydrophilic sequence Asp-Asp-Glu-Lys. The same deletion was found in the fully resistant 41/2 derivative of B9/41, confirming the conclusion that the loss of the Asp residue caused resistance to T1 and φ80 and residual T5 sensitivity. The residual colicin M and albomycin sensitivities (10 and 0.1%, respectively) came from the use of solutions with higher activities and a more sensitive assay in this study compared with the former study (7). Additional single mutations in the Asp region caused 10- to 100-fold reductions in colicin M and albomycin sensitivity and also diminished ferrichrome uptake but left phage sensitivity nearly unaltered. These results suggest that the region defined by the Asp residue is involved in the interaction with all FhuA ligands. Because the ligands approach FhuA from outside the cell, we propose that the Asp domain is located at the cell surface.

The additional mutations rendering strain 41/2 resistant to all FhuA ligands were located at two sites far apart from the Asp site. They both involve a proline residue, which in UL3/pHK102 *fhuA3* has been created and in UL3/pHK104 *fhuA5* has been deleted. These proline changes should alter the conformations of the polypeptide chains. At the moment, it is difficult to predict whether these sites participate directly in binding-site formation or whether long-range conformational changes affect the proper binding site. The combination of the *fhuA3,4,5* mutations yielded exactly the phenotype of the chromosomal mutant and were therefore originally contained on the chromosome. In contrast, certainly mutation *fhuA1* and probably mutation *fhuA2* were the results of inaccurate *fhuA* gene amplification by PCR.

Previously, two experimental approaches were used to obtain information on FhuA outer membrane topology and ligand-binding domains: TAB linker insertions (9) and deletions using TAB linker restriction sites (8a). Two amino acid insertions after amino acids 69 and 128 abolished all FhuA functions, whereas those after residues 59 and 135 reduced receptor functions by several orders of magnitude. Insertion after residue 82 left only T5 sensitivity intact, and insertion after residue 321 eliminated only growth on ferrichrome. This technique clearly provided valuable information on possible ligand-binding sites. Regarding the deletion analysis, a large deletion extending from residues 129 to 168 left FhuA fully active, while the deletion from 21 to 128 reduced sensitivity to colicin M only somewhat (to one-eighth), and deletion from 60 to 69, just ahead of the above-mentioned insertion mutation, surprisingly did not alter FhuA activity. Apparently, such large deletions provide only limited information on FhuA active sites. Interpretation of the data is also hampered by proteolytic degradation of the majority of the deletion derivatives, so that the reduction of full-size protein and the degradation products could influence the phenotype. Nevertheless, functional domains, in a broad sense, could be ascribed to six regions: deletions from 21 to 59, 70 to 129, and 407 to 440 reduced colicin M sensitivity 8-fold, deletion from 169 to 195 reduced sensitivity to phages and colicin M 10-fold, and deletion from 196 to 223 affected strongly all FhuA activities, whereas deletion from 224 to 405 abolished phage and colicin M binding and diminished growth on ferrichrome.

Similar studies using linker insertions and deletions have been performed to elucidate ligand domains of the FepA protein, the receptor for ferric enterobactin, and for colicins B and D. Two amino acid insertions after residue 55, 142, or 324 strongly impaired the receptor activity for all three ligands (2). Insertions after residues 204 and 635 reduced sensitivity to colicins B and D only, leaving ferric enterobactin transport at near the wild-type level. These results demonstrated both shared and unique ligand-responsive domains. A further investigation used monoclonal antibodies to determine FepA epitopes located at the cell surface (21). Such epitopes were localized, one each in regions 27 to 37, 204 to 227, and 258 to 290 and two each in regions 290 to 339 and 382 to 400. Antibodies specific for the latter two regions inhibited binding of colicins and the uptake of ferric enterobactin.

The BtuB protein belongs to the same category of outer membrane receptors as do FhuA and FepA (19). Two amino acid insertions after residues 50, 252, and 412 reduced vitamin B_{12} uptake to less than 2%, in the first two cases by decreasing the rate of B_{12} binding in the last case by slowing its release from the receptor (12). The first two insertions also showed a reduced sensitivity to phage BF23 and colicin E3.

Though limited by the experimental approaches used, which do not provide random mutants, the data presented above, together with the results presented in this report, nevertheless add to a mosaic which must be completed in order to understand the membrane topology and functional sites of these receptors. For example, a functional site common to the receptors seems to be around residue 50, since two amino acid insertions in this site of FhuA, FepA, and BtuB impair receptor functions. Another common site has been defined between residues 224 to 405 in FhuA, at 324 (290 to 339) in FepA, and at 252 in BtuB. These experiments did not reveal another common site in these receptors, namely, the TonB box, a pentapeptide sequence located at residues 7 to 11 in FhuA, 12 to 16 in FepA, and 6 to 10 in BtuB which shows sequence similarity but not identity between all TonB-dependent receptors and colicins (5, 19, 22). According to a model describing the FepA transmembrane arrangement (21), the TonB box would be at the beginning of the first transmembrane β strand, starting from the periplasmic side. If the same model is applied to FhuA, the domain defined by the Asp-348 deletion is located in a loop at the cell surface where one would expect it. The Asp-348 site has not been localized before as an important region of FhuA activity, nor has an equivalent region been localized in FepA and BtuB. The binding sites for the various FhuA ligands overlap strongly, but mutations affecting only one ligand have been found. The binding sites are not formed by a sequence of consecutive amino acids at a single site but are composed of different segments of the polypeptide chain which in the linear sequence can be far apart. This makes mutational analysis a tedious task.

The totally inactive FhuA protein of strain 41/2 still contains typical features of the FhuA protein, since a T5 host range mutant (T5*h*) uses it as a receptor. FhuA deletion mutants are resistant to T5*h* which shows the FhuA specificity of T5*h* (7). The adsorption rate of T5*h* to wild-type

FhuA is faster than that of T5, and so the improved binding may suffice to infect 41/2 cells.

The two Fe^{3+} hydroxamate receptors FhuA and FhuE were functionally identified by the exclusive binding of ferrichrome to FhuA and of coprogen to FhuE. In this study, we found Fe^{3+} hydroxamates which bind to both receptors. Although FhuA is the preferred receptor, lack of FhuA still allows growth on butylferrichrome, ferrichrysin, ferrirubin, tetraglycylferrichrome, propylferrichrome, and hexahydroferrirhodin via FhuE. The first two compounds gave the largest and densest growth zones when taken up via FhuE. However, a 30-fold-higher concentration of butylferrichrome and a 10-fold-higher concentration of ferrichrysin were required to obtain the same growth zone on strain 41/2 *fhuA fhuE*$^+$ than on strain MS172 *fhuA*$^+$ *fhuE*. It is not clear why these ferrichrome derivatives are better recognized by FhuE than is unsubstituted ferrichrome. A larger hydrophobicity and size do not seem to be the only factors relevant for the uptake of hydroxamate derivatives via FhuE.

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