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Internal Deletions in the FhuA Receptor of *Escherichia coli* K-12 Define Domains of Ligand Interactions

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The ferrichrome-iron receptor encoded by the *fhuA* gene of *Escherichia coli* K-12 is a multifunctional outer membrane receptor required for the binding and uptake of ferrichrome and bacteriophages T5, T1, φ80, and UC-1 as well as colicin M. To identify domains of the protein which are important for FhuA activities, a library of 31 overlapping deletion mutants in the *fhuA* gene was generated. Export of FhuA deletion proteins to the outer membrane and receptor functions of the deletion proteins were analyzed. All but three of the deletion mutant FhuA proteins cofractionated with the outer membrane; no FhuA proteins were detected in outer membrane preparations or in cell extracts when the deletions spanned amino acids 418 to 440. Most deletion proteins were susceptible to cleavage by endogenous proteolytic activity; some degradation products were detected on Coomassie blue-stained gels and on Western blots (immunoblots). Receptor functions were measured with the mutated genes present on multicopy plasmids. Two deletion mutants, FhuAΔ060–069 and FhuAΔ129–168, conferred wild-type phenotypes: they demonstrated growth promotion by ferrichrome and the same efficiency of plating of bacteriophages as that of wild-type FhuA; killing by colicin M was also unaffected. For FhuAΔ021–128 and FhuAΔ406–417, reduced sensitivity to colicin M was detected; wild-type phenotypes were observed for all other FhuA functions. Deletions from amino acids 169 to 195 slightly reduced sensitivities to bacteriophages and to colicin M; ferrichrome growth promotion was unaffected. When deletions extended into the region of amino acids 196 to 405, all FhuA functions were either reduced or abolished. The results indicate that selected regions of the FhuA protein have receptor activities and demonstrate the presence of both shared and unique ligand-responsive domains.

Transport of iron from the siderophore ferrichrome into *Escherichia coli* K-12 is dependent upon the gene products encoded by the *fhu* (ferric hydroxamate uptake) operon located at approximately 3.8 min on the *E. coli* chromosome (2, 6). Because the ferrichrome-iron complex exceeds the molecular weight exclusion limit of porin channels in the outer membrane (29, 32), a receptor-mediated, energy-driven transport process is required to translocate ferrichrome-iron across the outer membrane and into the periplasm. This receptor is called the FhuA protein and is a minor component (approximately 10^3 copies per cell) of the outer membrane of *E. coli*. The copy number of FhuA can be modestly increased to 10^4 per cell when iron is limited or when iron transport proteins are constitutively synthesized in a regulation-deficient *fur* mutant (18). Subsequent translocation of iron to the cytoplasm is known to require at least three gene products encoded by *fhuB* (27) and *fhuC* and *fhuD* (9), as well as functions supplied by *exbB* (20) and *tonB* (33).

The FhuA protein is multifunctional. In addition to the binding and transport of ferrichrome, FhuA acts as receptor for the bacteriophages T5, T1, φ80, and UC-1. Both colicin M and albomycin, a structural analogue of ferrichrome, bind to FhuA. Infection by phage T5 and binding of colicin M require only FhuA; however, uptake of colicin M across the outer membrane and irreversible adsorption of phages T1, φ80, and UC-1 occur only in energized TonB$^+$ cells (7, 17). There may be some physical interaction between the FhuA protein and the *tonB* gene product, which is responsible for the energy coupling of the transport mechanism (19). In most cases, cell binding and killing by the lethal agents are competitively blocked by the presence of ferrichrome.

Our objective is to define the ligand-binding domains of FhuA and the domains required for the internalization of the ligands. These might include regions of FhuA which physically interact with FhuB, FhuC, FhuD, ExbB, or TonB. One genetic strategy was to create a library of 18 TAB (two amino acid Barany) linker insertion mutants in the *fhuA* gene (8). In characterizing these mutants, we observed that the insertion of two new amino acids following amino acid 69 or 128 of FhuA abolished all receptor functions. In cells that expressed mutant FhuAs with insertions after amino acid 59 or 135, sensitivity to the lethal agents was reduced by several orders of magnitude. Insertion at other selected sites decreased receptor functions only slightly. Noteworthy was an insertion after amino acid 321 which selectively eliminated ferrichrome growth promotion. Finally, a strain carrying a mutant *fhuA* gene in which the linker insertion occurred after amino acid 82 showed a *tonB* phenotype. We concluded that subtle perturbations were introduced into the FhuA protein and that they effected changes in the binding and uptake of its cognate ligands.

An extension of this genetic strategy exploits unique features of the library of linker insertions. Since each of our 18-insertion mutant *fhuA* genes contains a single *Sac*I restriction site, it was possible by pairwise ligation of restriction fragments to create defined overlapping deletions covering amino acids 21 to 440 of the mature FhuA. Localization studies revealed that despite extensive loss of amino acid sequences, many of the FhuA deletion proteins were translocated to the cell surface. We show here that some regions of the FhuA protein can be deleted without adverse effect on

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TABLE 1. *E. coli* K-12 strains, phages, and plasmids used in this study

Strain, phage, or plasmid	Genotype	Source or reference
Strains		
MC4100	F⁻ *araD* Δ(*argF-lac*)U169 *rspL thi relA flbB deoC pstF rbsR*	T. J. Silhavy
SG303	MC4100 *aroB*	S. Garrett
SG303*fhuA*	SG303 T5-resistant	Laboratory stock
RK4691	P1450 col M⁺	R. J. Kadner
Phages		
T5, T1, ϕ80*vir*		Laboratory stocks
UC-1		C. F. Earhart
Plasmids		
pGC01	*bla fhuA*⁺ pBR322 replicon, 6.3 kb	8
pGC020 to pGC440	*bla fhuA* pBR322 replicon, 6.3 kb	8
pGCΔ322–405	*bla fhuA* pBR322 replicon, 6.0 kb	8
pGCΔ021–082 to pGCΔ418–440	*bla fhuA* pBR322 replicon, 5.9 to 6.3 kb	This study

ferrichrome growth promotion or sensitivities to bacteriophages and to colicin M.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The *E. coli* K-12 strains used in this study were derived from MC4100. The bacterial strains, bacteriophages, and plasmids which were used are listed in Table 1. Plasmid pGC01 and the linker insertion plasmids pGC020 to pGC440 were described in a previous paper (8). Standard protocols used for the growth of bacteria, the propagation and handling of phages, and the manipulation of plasmids are according to Silhavy et al. (37).

Genetic procedures. Standard techniques for the isolation of plasmids, digestion with restriction endonucleases, separation by agarose gel electrophoresis, and the preparation and transformation of competent cells were described by Maniatis et al. (30).

Media, reagents, and enzymes. The liquid and solid media have been previously reported (10, 11). When needed, ampicillin was added to media at a concentration of 125 μg/ml. Restriction endonucleases were purchased from Boehringer-Mannheim Canada and Amersham Canada Ltd. T4 DNA ligase was obtained from Amersham Canada Ltd.

Deletion mutagenesis. The insertion of a 6-base oligonucleotide into the *Cfo*I and *Hpa*II sites of plasmid pGC01 (6,276 bp) created 18 linker insertion mutants (8). Insertion of a single linker resulted in the addition of two amino acids (3, 4). All these plasmids (pGC020 to pGC440) contain a unique *Sac*I restriction site at the various positions of the inserted linker as well as a unique *Eco*RI site derived from pBR322 at coordinate 0/0. Sequential digestion of plasmids of the pGC series was performed with *Sac*I and *Eco*RI. The pairs of fragments that resulted from each double digestion were purified from low-melting-point agarose gels. Fragments necessary to construct the defined deletions were then ligated. An aliquot of the ligated material was used to transform competent cells of SG303*fhuA* (16). Plasmids containing the anticipated deletions were isolated from stationary-phase cultures by the boiling method (25) and were analyzed by multiple digestions with restriction endonucleases to identify the deletions. A plasmid giving the predicted restriction fragments was used again to transform SG303*fhuA*, thereby segregating any anomalous plasmid from that carrying the desired deletion. The plasmids were reisolated and digested with restriction endonucleases, and the sizes of the resulting fragments confirmed the deletions. The deletions were routinely analyzed by digestion of the newly created plasmids with *Rsa*I. This enzyme cuts pGC01 nine times, producing fragments of 270 to 1,565 bp; the five fragments containing the *fhuA* coding region are between 270 and 700 bp in size. The deletion plasmids had either lost one *Rsa*I restriction site or one band was reduced in size according to the extent of deletion.

Electrophoresis of outer membrane proteins and immunoblotting. Outer membranes were prepared according to the method of Hantke (18) from plasmid-bearing cells grown in rich medium containing ampicillin. Proteins were solubilized by boiling them in 2% sodium dodecyl sulfate (SDS)–5% β-mercaptoethanol and were separated by electrophoresis on 8% polyacrylamide gels containing 0.1% SDS (28). The gels were either stained with Coomassie brilliant blue or were transferred electrophoretically (39) to nitrocellulose paper (Schleicher & Schuell, Inc., Keene, N.H.). The transferred proteins were reacted with a primary antibody followed by a secondary antibody conjugated to peroxidase. Immunoconjugants were detected by the addition of 4-chloro-1-naphthol and H_2O_2. The following two anti-FhuA antibodies were used in this study: a rat monoclonal antibody, 4AA-1, that recognizes amino acids 21 to 32 of mature FhuA, and rabbit anti-Fhu peptide antibodies that are specific for amino acids 636 to 651 of the mature FhuA (8).

Response of mutant proteins to bacteriophages, to colicin M, and to ferrichrome growth promotion. Sensitivities to the bacteriophages T5, T1, ϕ80, and UC-1 were determined for all strains which carried a deletion. Samples (5 μl) containing different dilutions of phage stock (2×10^9 to 2×10^3 PFU/ml) were spotted onto a lawn of cells. Sensitivity to phages was observed after an incubation at 37°C for 6 h. Results were scored as the highest dilution giving a distinct zone of lysis.

Colicin M was prepared from strain RK4691 according to the method of Schaller et al. (35). A 1-liter culture was centrifuged, and the cell pellet was resuspended in 15 ml of M9 salts supplemented with 0.2 mM $CaCl_2$, and 0.1% Triton X-100 to stabilize colicin M. The suspension was sonicated and centrifuged to remove cellular debris. Five microliters of each dilution of a twofold series was spotted to determine sensitivity to colicin M.

Ferrichrome growth promotion was determined as follows. Overnight cultures (2 ml, 37°C) of strains containing an *aroB* background were grown in nutrient broth. An aliquot (0.1 ml) was mixed with 3 ml of molten nutrient top agar. The mixture was poured onto a plate containing nutrient broth

agar and 40 μM ethylenediamine di(*o*-hydroxy)phenylacetic acid (EDDA). Spots (5 μl) of 10 mM, 1 mM, and 0.1 mM ferrichrome were placed on the surface of the plate and dried. Growth was observed after 7 h at 37°C. Strains able to transport iron from ferrichrome grew more densely under the spot than elsewhere. Strains SG303 and SG303*fhuA* (pGC01) served as positive controls; strain SG303*fhuA* was a negative control.

RESULTS

Construction of *fhuA* deletion mutations. Our library of TAB linker insertion mutants was described in a previous paper (8). Plasmids carrying insertion mutant *fhuA* genes were named pGC020 to pGC440. Each assigned number corresponds to the amino acid of mature FhuA (10) which precedes the two newly added amino acids. The two newly added amino acids were -Glu-Leu-, or -Ser-Ser-, or -Arg-Ala-.

Since linker insertion mutagenesis introduced unique *Sac*I restriction sites at 18 different positions within the coding region of the *fhuA* gene, it was possible to generate defined deletions by starting with this library of insertion mutants. As a general strategy, the plasmids were digested with *Eco*RI and *Sac*I restriction endonucleases, and the resulting fragments were purified. To create a particular deletion, an *Eco*RI-to-*Sac*I fragment containing the *fhu* promoter, signal sequence, and 5′-*fhuA* coding sequences was ligated to a selected *Sac*I-to-*Eco*RI fragment containing 3′-*fhuA* coding sequences, the pBR322 replicon, and the ampicillin resistance gene. In order to maintain the reading frame, this strategy was restricted to linker insertion mutants containing the same two newly added amino acids. Thus, one group of defined deletions was generated from linker insertion mutants having -Glu-Leu- as the two new amino acids; another group of defined deletions was generated for linker insertion mutants having -Ser-Ser- as the dipeptide inserted. Each FhuA deletion protein therefore conserved the two amino acids that originated from the linker mutagenesis. For example, to generate a deletion between amino acids 21 to 82, a DNA fragment coding for amino acids 1 to 20 was ligated to another fragment coding for amino acids 83 to 714. Amino acids 20 and 83 were separated by the dipeptide -Glu-Leu-.

By using this strategy, 31 overlapping deletion mutants of FhuA were constructed (Fig. 1). They covered a region spanning amino acids 21 to 440. The size of the deletions was as short as 10 amino acids (Δ060–069) and as long as 139 amino acids (Δ060–198). The deletions were not only overlapping, but in some cases they were subdivided into two smaller ones. For example, the deletion Δ060–135 is subdivided into the deletions Δ060–069 and Δ070–135, and the deletion Δ021–128 is subdivided into the deletions Δ021–082 and Δ083–128.

Identification of the deletion mutant FhuA proteins. The detection of deletion FhuAs in the outer membrane fraction was possible because the deletion genes were expressed from high-copy-number plasmids. Outer membrane vesicles were prepared (18) from the 31 strains, each containing a plasmid with some internal *fhuA* deletion. Outer membrane proteins were resolved by polyacrylamide gel electrophoresis (PAGE). When cells are grown in rich medium, the expression of the chromosomal *fhuA* gene is low, with only about a thousand copies of the FhuA protein being found in the outer membrane. SG303 contains a single copy of the wild-type *fhuA* gene in the chromosome; a faint band migrating at a M_r of 78,000 was detected (Fig. 2). This band was missing for the SG303*fhuA* strain. Cells that harbor the cloned wild-type *fhuA* gene produced amplified amounts of the receptor, and the overproduced protein was readily detected by staining electrophoretograms. Coomassie blue-stained gels of outer membrane preparations showed that the presence of plasmids containing the wild-type or internal-deletion *fhuA* genes did not alter significantly the relative amounts of major outer membrane proteins (Fig. 2). The following deletion mutants were readily detectable on the stained gels: Δ021–082, Δ021–128, Δ060–069, Δ060–135, Δ070–135, Δ083–128, and Δ406–417. Sometimes two or more bands were detected, or the amount of deletion FhuA as analyzed by Coomassie blue staining was very low. These might be FhuA-related bands since they were not present in SG303*fhuA*.

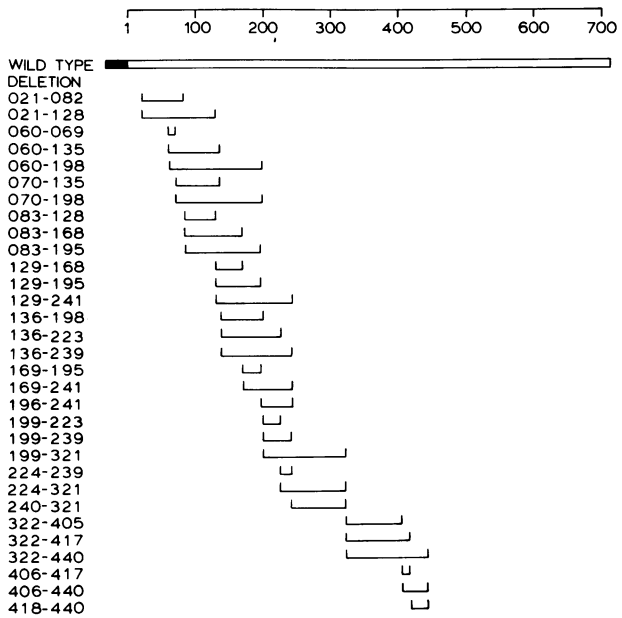

FIG. 1. Map of the defined overlapping deletions of the FhuA protein. Coordinates along the top show the scale of amino acids. The open box represents the 714 amino acids of the mature FhuA sequence. The filled box represents the signal sequence of FhuA.

To confirm this hypothesis, FhuA-specific antibodies were used in immunoblot analyses. Our immunological identification of the FhuA species employed two antibodies that recognize different termini of the FhuA protein. The monoclonal antibody 4AA-1 reacts with the amino terminus of the FhuA protein, whereas the anti-peptide antibody is directed against the carboxy terminus. For most of the deletion mutants, the monoclonal antibody 4AA-1 detected one FhuA-related species (Fig. 3). With the exception of Δ129–168, the M_rs of these species were as predicted on the basis of the amino acids deleted. No FhuA-specific species was detected by the monoclonal antibody for deletions Δ021–082, Δ021–128, Δ224–321, Δ322–440, Δ406–440, and Δ418–440 (Fig. 3). A very faint band was detected for FhuA deletion mutants Δ129–195 and Δ136–198. They were more readily detectable if 20 μg rather than 5 μg of outer membrane proteins was loaded onto the gels (data not shown). Since proteolytic degradation for carboxy-terminal deletions of the FhuA protein was observed by others (26, 36) and by us for some of our linker insertion mutants (8), we extended the characterization of FhuA deletion proteins by using anti-

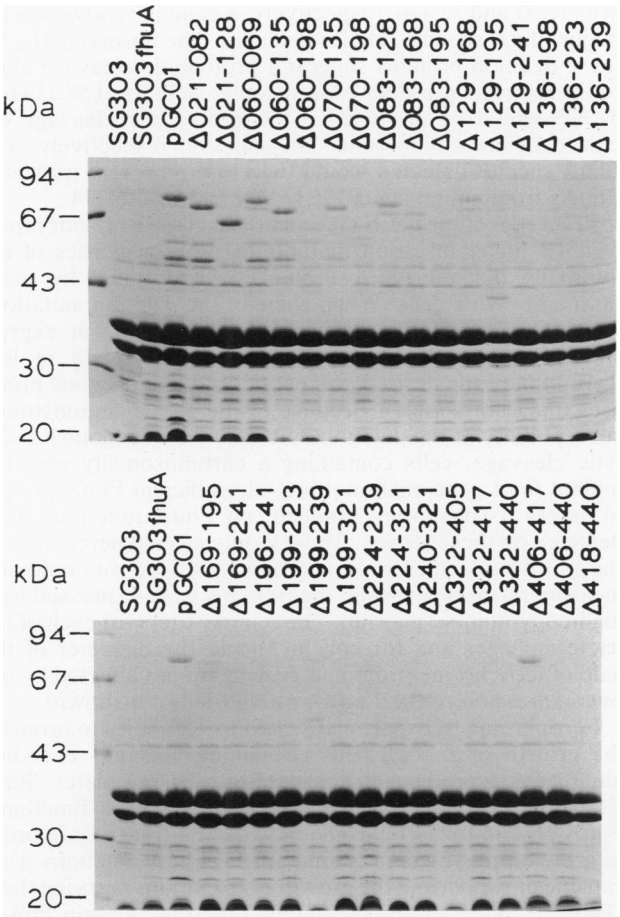

FIG. 2. Identification of the outer membrane proteins in a wild-type *fhuA* strain, in a *fhuA* mutant, and in strain SG303*fhuA* containing high-copy-number plasmids, wild-type *fhuA*, or the different deletion mutant *fhuA*s. Membrane proteins (20 μg) were subjected to SDS-PAGE and stained with Coomassie blue. Molecular mass marker proteins (Pharmacia) were phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; and soybean trypsin inhibitor, 20 kDa.

peptide antibodies (directed against amino acids 636 to 651 of the mature FhuA). Deletion mutants Δ021–082 and Δ021–128 were now detectable by the anti-peptide antibodies, and the estimated M_rs of these bands were as predicted on the basis of the deletions (Fig. 4). These two deletions did not contain the epitope recognized by the monoclonal antibody. For most of the deletion FhuAs, several bands were detected by the anti-peptide antibodies. A second band was detected by the anti-peptide antibodies for deletions Δ083–128, Δ083–168, Δ083–195, Δ129–168, Δ129–241, Δ136–223, Δ136–239, Δ169–195, Δ169–241, Δ196–241, and Δ199–239. These second bands were as intense as the band corresponding to the predicted M_r and migrated as species 5 to 7 kDa smaller. The faster-migrating bands were not detected by our amino terminus-specific monoclonal antibody. These results suggest that these species are derived by a proteolytic cleavage near the amino terminus of the deletion FhuAs, similar to what was observed for TAB linker insertions after amino acids 59, 69, 82, 128, and 300 (8).

The deletion FhuAs differ markedly in their stability. The following deletions in *fhuA* resulted in diminished levels of

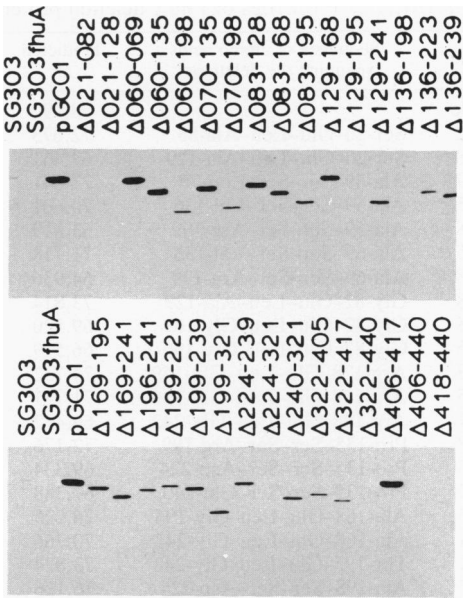

FIG. 3. Identification of wild-type and mutant FhuA proteins with monoclonal antibody 4AA-1. Outer membrane proteins (5 μg) were subjected to SDS-PAGE, transferred to nitrocellulose, and then probed with the FhuA-specific monoclonal antibody 4AA-1.

receptor protein: Δ060–198, Δ070–198, Δ129–195, Δ136–198, Δ199–223, Δ199–321, Δ224–321, Δ322–405, and Δ322–417. This could be attributed to protein instability. It is also possible that mRNA stability, lowered expression of the altered gene, and differences in plasmid copy number may account for the various levels of deletion FhuAs.

Twenty-eight of 31 FhuA deletion mutants could be identified by the reaction of monoclonal antibody and/or anti-

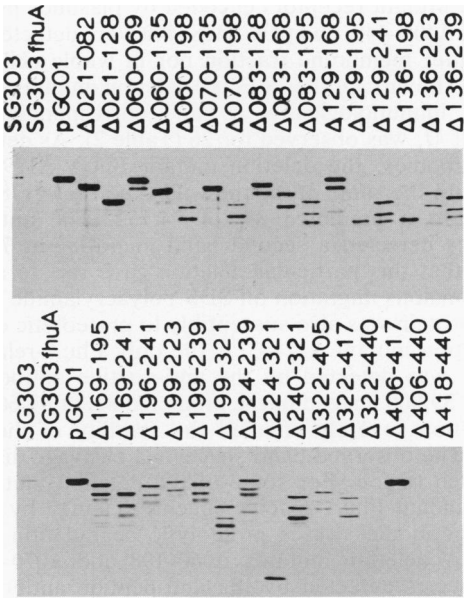

FIG. 4. Identification of wild-type and mutant FhuA proteins with anti-FhuA anti-peptide antibodies. Outer membrane proteins (5 μg) were subjected to SDS-PAGE, transferred to nitrocellulose, and then probed with the anti-FhuA anti-peptide antibodies.

TABLE 2. Properties of FhuA deletion proteins

fhuA allele	Sequence of amino acids spanning the deletions[a]	Predicted M_r	Observed M_r
Wild type		78,992	78,000
Δ021–082	Ser-20–Glu–Leu–Ala-83	72,673	73,000
Δ021–128	Ser-20–Glu–Leu–Ala-129	67,461	66,000
Δ060–069	Ala-59–Ser–Ser–Leu-70	77,841	77,000
Δ060–135	Ala-59–Ser–Ser–Val-136	70,601	71,000
Δ060–198	Ala-59–Ser–Ser–Arg-199	63,819	63,000
Δ070–135	Ala-69–Ser–Ser–Val-136	71,718	72,000
Δ070–198	Ala-69–Ser–Ser–Arg-199	64,936	64,000
Δ083–128	Gly-82–Glu–Leu–Ala-129	73,814	73,000
Δ083–168	Gly-82–Glu–Leu–Gly-169	69,520	70,000
Δ083–195	Gly-82–Glu–Leu–Gly-196	66,519	66,000
Δ129–168	Arg-128–Glu–Leu–Gly-169	74,732	78,000
Δ129–195	Arg-128–Glu–Leu–Gly-196	71,731	73,000
Δ129–241	Arg-128–Glu–Leu–Gly-242	66,472	66,000
Δ136–198	Pro-135–Ser–Ser–Arg-199	72,176	73,000
Δ136–223	Pro-135–Ser–Ser–Asp-224	69,334	71,000
Δ136–239	Pro-135–Ser–Ser–Glu-240	67,388	69,000
Δ169–195	Ala-168–Glu–Leu–Gly-196	76,025	75,000
Δ169–241	Ala-168–Glu–Leu–Gly-242	70,766	71,000
Δ196–241	Thr-195–Glu–Leu–Gly-242	73,824	74,000
Δ199–223	Ala-198–Ser–Ser–Asp-224	76,116	76,000
Δ199–239	Ala-198–Ser–Ser–Glu-240	74,170	74,000
Δ199–321	Ala-198–Ser–Ser–Ala-322	64,819	64,000
Δ224–239	Pro-223–Ser–Ser–Glu-240	77,012	77,000
Δ224–321	Pro-223–Ser–Ser–Ala-322	67,661	39,000
Δ240–321	Pro-239–Ser–Ser–Ala-322	69,607	69,000
Δ322–405	Pro-321–Ser–Ser–Val-406	69,499	70,000
Δ322–417	Pro-321–Ser–Ser–Ala-418	68,136	68,000
Δ322–440	Pro-321–Ser–Ser–Gln-441	65,578	ND[b]
Δ406–417	Pro-405–Ser–Ser–Ala-418	77,595	76,000
Δ406–440	Pro-405–Ser–Ser–Gln-441	75,037	ND
Δ418–440	Pro-417–Ser–Ser–Gln-441	76,400	ND

[a] Amino acid coordinates of FhuA, numbered from Ala-1 of the mature protein.
[b] ND, not detected.

peptide antibodies against preparations of outer membrane proteins. Mutant receptors encoded by plasmids pGCΔ322–440, pGCΔ406–440, and pGCΔ418–440 were detected neither in the outer membrane fraction nor in whole cell extracts (data not shown). Of the 28 FhuA deletion mutants that were detected immunologically, a band corresponding to the predicted M_r was observed for 26 (Table 2). As detected by both antibodies, the deletion mutant FhuA Δ129–168 migrated with the same M_r as the wild-type FhuA (78,000), as opposed to a predicted M_r of 74,732. The anti-peptide antibodies detected a second band migrating at 73,000. It appears that this particular deletion gave rise to a protein with anomalous migration on SDS-polyacrylamide gels; this mutant protein was also susceptible to proteolytic cleavage. For deletion mutant Δ224–321, the only FhuA-related species that was detected by the anti-peptide antibodies migrated at 39,000 instead of the predicted M_r of 67,661. Thus, this deletion severely affected the stability of the mutant protein. The observed band suggests a cleavage distal from the deleted region. For some of the deletion mutants, the most abundant FhuA-specific species detected by the anti-peptide antibodies was a proteolytic degradation product. For FhuA deletion mutants Δ060–198 and Δ070–198, the major species detected by the anti-peptide antibodies migrated at M_rs of 57,000 and 59,000, respectively. These observed M_rs represent proteins that are 6 to 7 kDa shorter than the proteins predicted on the basis of the amino acid sequence. For Δ129–195 and Δ136–198, the predicted M_rs are 71,731 and 72,176, respectively; a band of 58 kDa was the major FhuA-specific species detected. The observed M_rs for these deletion proteins suggest a proteolytic cleavage close to the region that is deleted. Molecular weights of 58,114 and 57,805 would be predicted if the proteolytic cleavage occurred before amino acids 196 and 199, respectively. The FhuA species detected would therefore represent truncated FhuAs from amino acids 196 to 714 and 199 to 714.

Properties of the deletion proteins. In contrast to our report of TAB linker insertion mutants (8), the properties of the internally deleted FhuA proteins were studied by using plasmid-bearing cells. When some of the deletion mutations were transferred onto the chromosome, the lower expression of the *fhuA* deletion genes did not result in less disruption of the desired polypeptides by endogenous proteases (data not shown). Because of the above-demonstrated susceptibility of the deletion mutants to endogenous proteolytic cleavage, cells containing a chromosomally encoded mutant *fhuA* gene might not provide sufficient FhuA protein to assess its phenotype. Indeed, no FhuA functions were detected on these strains. Thus, if only a small percentage of the FhuA molecules were transiently active, we could still measure phenotypes when the proteins were expressed by a high-copy-number plasmid. The relative titers for each of the bacteriophages and for colicin M and the diameter of the halo of ferrichrome-promoted growth are not affected by the overexpression of the FhuA protein (data not shown).

Ferrichrome transport was tested by its ability to promote the growth of *E. coli* cells containing plasmids encoding mutant FhuA proteins on nutrient broth-EDDA plates. Since EDDA is a strong iron chelator, strains need a functional FhuA protein for ferrichrome binding and transport in order to grow on this medium. Some of the FhuA deletions were capable of supporting the growth of the strains carrying them (Table 3). Nine strains containing different deletion FhuAs produced a halo of growth similar to that of SG303 and SG303*fhuA*(pGC01). This wild-type ferrichrome growth promotion was observed for strains containing the following deletions: Δ021–128, Δ060–069, Δ060–135, Δ070–135, Δ083–168, Δ129–168, Δ129–195, Δ169–195, and Δ406–417. An intermediate halo of ferrichrome growth promotion was observed for four strains carrying the following deletion FhuAs: Δ224–321, Δ240–321, Δ322–405, and Δ322–417. No detectable growth was observed for any other deletion mutants. Strains containing FhuA proteins with deletions that included amino acids 418 to 440 would not grow on nutrient broth-EDDA plates; no FhuA-related proteins were detected by SDS-PAGE or on Western blots (immunoblots). These results suggested that ferrichrome transport was abolished when the deletion extended into a segment bounded by amino acids 196 to 223; the receptor's activity in ferrichrome growth promotion was limited when some amino acids between 224 to 405 were deleted.

The responses of all the deletion mutants to the lytic phages T5, T1, φ80, and UC-1 were also determined. Tenfold serial dilutions of phages (5 μl) were spotted onto lawns of cells carrying the different plasmids with mutant *fhuA* genes. To calculate the relative phage titers or the efficiency of plating, the number of plaques formed on a lawn of mutant cells was divided by the number of plaques formed on the wild-type strains SG303*fhuA*(pGC01) and SG303. There was variation in the relative phage titers obtained for the different deletion strains. Deletion of four defined regions of the FhuA protein did not affect the response of these *fhuA* strains to any of the four phages. Titers similar to that of the wild-type strain could be measured with the following deletion alleles:

TABLE 3. Properties of *fhuA* deletion mutants

fhuA allele	FC[a]	Relative titer of[b]:				
		T5	T1	φ80	UC-1	Colicin M
Wild type	+	1	1	1	1	1
Δ021–082	−	0	10^{-5}	0	0	0
Δ021–128	+	1	1	1	1	1/8
Δ060–069	+	1	1	1	1	1
Δ060–135	+	0.01	0.1	0.01	10^{-4}	1/32
Δ060–198	−	0	0	0	0	0
Δ070–135	+	10^{-4}	0.01	0.01	10^{-4}	0
Δ070–198	−	0.01	0.1	0.01	10^{-3}	1/64
Δ083–128	−	10^{-5}	10^{-5}	10^{-4}	10^{-5}	0
Δ083–168	+	0.01	1	0.01	10^{-3}	1/8
Δ083–195	−	0.01	0.1	0.01	10^{-3}	1/16
Δ129–168	+	1	1	1	1	1
Δ129–195	+	0.01	0.1	0.1	0.01	1/8
Δ129–241	−	0	10^{-5}	0	0	0
Δ136–198	−	0.01	10^{-3}	10^{-4}	10^{-4}	1/32
Δ136–223	−	0.01	10^{-4}	0.01	10^{-4}	1/32
Δ136–239	−	10^{-5}	10^{-4}	10^{-5}	0	1/128
Δ169–195	+	0.1	0.1	0.1	0.1	1/16
Δ169–241	−	10^{-5}	10^{-3}	0	0	0
Δ196–241	−	10^{-5}	0	0	0	0
Δ199–223	−	10^{-3}	10^{-5}	10^{-3}	10^{-4}	1/128
Δ199–239	−	10^{-5}	10^{-3}	0	0	0
Δ199–321	−	10^{-5}	10^{-4}	10^{-5}	0	0
Δ224–239	−	10^{-5}	10^{-4}	0	0	0
Δ224–321	+/−	10^{-5}	10^{-5}	10^{-5}	0	1/128
Δ240–321	+/−	10^{-5}	10^{-5}	0	0	0
Δ322–405	+/−	0	0	0	0	0
Δ322–417	+/−	10^{-5}	0	0	0	0
Δ322–440	−	0	0	0	0	0
Δ406–417	+	1	1	1	1	1/8
Δ406–440	−	0	0	0	0	0
Δ418–440	−	0	0	0	0	0

[a] FC, ferrichrome growth promotion; +, same growth response as for pGC01; −, no ferrichrome growth promotion; +/−, diminished ferrichrome growth promotion as compared with that of wild type.

[b] Relative titers are the titers obtained with mutant cells divided by the titer obtained with the wild-type strain.

*fhuA*Δ021–128, *fhuA*Δ060–069, *fhuA*Δ129–168, and *fhuA* Δ406–417 (Table 3). A 10-fold reduction in relative titer was obtained for the mutant allele *fhuA*Δ169–195. For *fhuA*Δ129–195, 10-fold reduction in the relative titers was obtained for T1 and φ80, while a 100-fold reduction of the efficiency of plating was observed for T5 and UC-1 phages.

Compared with those of the other phages, the relative titers of phage T1 were usually less affected for any given FhuA deletion. For example, the efficiency of plating for T1 phage was 1 for Δ083–168; a 100- to 1000-fold reduction was observed for the other phages. Likewise, whereas only a 10-fold reduction was observed for the relative titers of T1 phage for deletions Δ060–135, Δ070–198, and Δ083–195, a 10^2- to 10^4-fold reduction was observed for the three other phages which use FhuA as their outer membrane receptor. The deletion alleles *fhuA*Δ021–082 and *fhuA*Δ129–241 were defective for all FhuA functions, but when spots containing high amounts of T1 phage (10^8 PFU/5 μl) were present, a clear zone of lysis was observed.

For FhuA deletions that extend between amino acids 224 and 405, phage binding seems to have been affected severely. Spots containing 10^8 phages were required to obtain a zone of growth inhibition for T5 phage. Phage UC-1 could not be grown on any strain containing plasmids encoding for deletions ranging from amino acids 224 to 405. For phages T1 and φ80, deletions ranging from amino acids 322 to 405 abolished plaque formation.

The sensitivity of every deletion mutant to colicin M was also investigated. Sensitivity to colicin M was determined by spotting 5 μl of a twofold dilution of a sonic extract from the colicin M-producing strain RK4691. The reciprocal of the highest dilution that gave a distinct inhibition of cell growth was used as a measure of the colicin M titer. Titers obtained for mutant strains were normalized to the titers obtained for the wild-type strains SG303*fhuA*(pGC01) and SG303. Wild-type titers were obtained only for two deletion mutants, Δ060–069 and Δ129–168. Lower titers (1/8) were observed for strains carrying the following deletions: Δ021–128, Δ083–168, Δ129–195, and Δ406–417. Titers of 1/16 were observed for strain SG303*fhuA* containing plasmids encoding for Δ083–195 and Δ169–195. With the exception of Δ224–321, no colicin M sensitivities could be observed when deletions covered a region spanning amino acids 224 to 405. Again, since no proteins were detected for deletions Δ322–440, Δ406–440, and Δ418–440, inhibition of growth by colicin was not observed.

DISCUSSION

One of the major challenges of research on cell surface proteins in bacteria is to determine which domains of a given receptor are responsible for interaction with its cognate ligand(s). In the case of the multifunctional FhuA receptor of *E. coli* K-12, it is of interest to identify not only the ferrichrome-binding site but also the binding sites for four bacteriophages and colicin M. To address this problem, we adopted strategies of molecular genetics and constructed a library of linker insertion mutants (8). Eighteen different FhuA mutants were created, each only two amino acids longer than the wild-type protein of 714 amino acids. The mutant FhuAs were found in the outer membranes in amounts similar to the amounts of wild-type cloned FhuA protein and migrated on SDS-polyacrylamide gels at a rate similar to that of the wild type. Some mutant FhuAs showed one or more altered functions.

In an attempt to gain more information about the domains of this multifunctional protein, this paper describes the construction of a library of defined deletions for the FhuA protein. The construction of deletions within outer membrane proteins may not always be successful because certain amino acid sequences may be important for export and outer membrane localization. For example, to obtain information about residues within the PhoE protein of *E. coli* required for export of the protein to the outer membrane, deletions throughout the *phoE* gene were generated (5). Immunocytochemical labeling on ultrathin cryosections revealed that polypeptides encoded by the mutant alleles were transported across the cytoplasmic membrane and accumulated in the periplasm. These results demonstrated that (i) no specific sequence within the PhoE protein is essential for transport across the cytoplasmic membrane and (ii) the truncated PhoEs cannot assemble into the outer membrane. The authors concluded that the overall structure of the protein, rather than a particular sequence of amino acids, seemed to be important for assembly into the outer membrane.

The deletion mutants described here provide evidence that segments of the mature FhuA between amino acids 21 and 417 can be deleted without apparent effect on outer membrane localization. Evidence for export information in FhuA was described by our use of FhuA′-′PhoA protein fusions (12). Fusions containing the FhuA signal sequence plus 32 or

88 amino acids of FhuA fused to PhoA were exported across the cytoplasmic membrane but remained in the periplasm as soluble proteins. The sequence of mature FhuA up to amino acid 88 was insufficient to ensure that the hybrid protein became strongly associated with the outer membrane. Fusions containing 180 amino acids or more of mature FhuA were identified in preparations of outer membrane. It was proposed that some information inherent in the sequences between amino acids 88 and 180 confers properties of stable association with the outer membrane. Our present results with internal FhuA deletions show that this export information located between amino acids 88 and 180 can be deleted without affecting outer membrane localization. The deletion mutant Δ070–198 was detected in the outer membrane fraction and was partly active in binding bacteriophages and colicin M, a clear indication of outer membrane integration. Thus, the FhuA receptor might represent a protein which contains several domains of export information, none of which is absolutely required. As with the OmpA protein, it is unlikely that the interaction of FhuA with the outer membrane is limited to a specific sequence within the protein. Analysis of the overlapping internal deletions in the *ompA* gene allowed the authors of one report to conclude that information required for export, if present, must be found repeatedly within the membrane part of the protein encompassing amino acid residues 1 to 177 (14).

When *fhuA* deletion genes were present on multicopy plasmids, some of their products were detected as major bands migrating at the predicted molecular weight. Those mutant proteins were found in the outer membrane in amounts similar to those for the wild type. These included FhuAΔ021–082, FhuAΔ021–128, FhuAΔ060–069, FhuAΔ 060–135, FhuAΔ070–135, and FhuAΔ406–417. A second faint band could be detected for FhuAΔ060–069, FhuAΔ060–135, and FhuAΔ070–135. Except for FhuAΔ021–082, these deletion mutants bind and transport ferrichrome and display various degrees of receptor recognition for the bacteriophages and colicin M, suggesting a conformation competent for FhuA export, for outer membrane integration, and for biological activities.

The deletion mutants FhuAΔ021–082 and FhuAΔ021–128 were not detected with our monoclonal antibody 4AA-1. Previous studies in our laboratory have shown that the monoclonal antibody could recognize a FhuA′-′PhoA fusion in which only the first 32 amino acids of the mature FhuA were fused in-frame to alkaline phosphatase (12). In this study, we demonstrate that the epitope recognized by this monoclonal antibody is located between amino acids 21 and 32, since proteins deleted for this sequence were not recognized by the monoclonal antibody. Attempts to make this antibody (and the anti-peptide antibodies) bind to whole cells or to purified outer membrane vesicles were unsuccessful (24). Presumably, the regions of antibody recognition are buried in the outer membrane. However, the possibility that the antibodies do not react against the native protein cannot be excluded.

For most of the deletions presented in this paper, more than one FhuA-related band was detected. The frequently observed double FhuA bands do not represent precursor and mature forms of the mutant proteins, since both the monoclonal antibody and the anti-peptide antibodies would have detected them. Therefore, the extra FhuA bands detected by the anti-peptide antibodies represent proteolytic products of the deletion FhuAs. Since all the cleaved FhuAs were detected in the outer membrane, the proteolytic cleavage likely occurred after translocation of the deletion pro-FhuAs across the cytoplasmic membrane. It is possible that lower-molecular-weight FhuA species were due to OmpT protease which cleaves between two basic residues (38). A scan of the FhuA amino acid sequence for potential OmpT cleavage sites indicates that such a possibility is unlikely. Other deletions in *fhuA* resulted in diminished levels of receptor protein. It has been demonstrated that *lon*, *pep*, and *degP* mutations increase the in vivo stability of aberrant proteins (15). These deletion mutant proteins may be susceptible to proteolysis during synthesis on the ribosome or while being translocated across the cytoplasmic membrane. They could be subject to degradation from both sides of the cytoplasmic membrane as long as they are not folded into export-competent or native conformations.

No protein could be detected in the outer membrane when a deletion encompassing amino acids 418 to 440 was created. When whole cells were subjected to SDS-PAGE and Western blotting, no protein could be detected by our FhuA-specific antibodies (data not shown). Experiments were also conducted to create internal deletions between amino acids 483 and 680. Plasmid pGC01 contains three *Hpa*I restriction sites located at the 3′ end of the *fhuA* coding region, at nucleotides 2082, 2382, and 2676 of our published sequence (10). Plasmid pGC01 was subjected to partial digestion with *Hpa*I followed by random religation of the digestion products. The following three deletions were created and characterized: Δ483–582, Δ583–680, and Δ483–680. For all three of these internal FhuA deletions, we were unable to detect any FhuA-related species by Western blotting, nor were any FhuA-related functions detectable (data not shown). This apparent absence of any protein resembles results obtained with the carboxy-terminal deletions of FhuA (26, 36) and more recently with the deletions of FhuE (34) and FepA (1). A small amount of carboxy-terminal truncated FhuA′ in the outer membrane was detectable in electrophoretograms (36). Transformation of these mutated *fhuA* genes into *E. coli lon* strains lacking ATP-dependent protease activity relieved FhuA degradation to a certain extent. Such deletions in FhuA not only caused protein degradation but resulted in an unusual Sarkosyl-soluble conformation of the protein in the outer membrane (36). A truncated FhuE protein (FhuE′) lacking 29 amino acids at the carboxy terminus but containing an additional 5 amino acids from the pACYC184 vector was described recently (34). Examination of the isolated outer membrane by SDS-PAGE revealed an absence of FhuE′. The precursor form of this protein could be detected only if the *fhuE* gene was under the control of a temperature-inducible promoter for T7 RNA polymerase. Deletions from the carboxy terminus also decreased amounts of FepA (1). Attempts to stabilize plasmid-encoded mutant FepA by using a *lon* host strain were unsuccessful. In addition, cells synthesizing a fragment of the vitamin B12 receptor lacking 12 amino acids at the carboxy terminus were still sensitive to phage BF23 but failed to bind and transport vitamin B12 (23). Only trace amounts of the truncated protein were detected in the outer membrane. Taking the results on FhuA, FhuE, FepA, and BtuB together, it appears that the carboxy terminus of these TonB-dependent receptors is important for their insertion into the outer membrane and for their binding of selected ligands.

The functions of the deleted FhuA proteins were tested in *E. coli* strains overexpressing the receptor. We also transferred the *fhuA* deletion genes onto the chromosome of a *polA*::Tn*10* strain (8) and found two classes of mutants. Either no FhuA functions could be measured on strains carrying a single copy of the mutated *fhuA* gene or the

chromosomal integration reconstituted a full-length wild-type FhuA protein as detected by Western blotting. No differences in the bacteriophage or colicin M titers for the *E. coli* strains SG303 and SG303*fhuA*(pGC01) are observed, suggesting that our in vivo tests are not influenced by the presence of chromosomally encoded or plasmid-encoded wild-type FhuA. The levels of full-length deletion mutant proteins were, in most cases, less than those of overproduced wild-type FhuA but more than those of chromosomally encoded FhuA.

When present on multicopy plasmids, two deletions, FhuAΔ060–069 and FhuAΔ129–168, behaved like wild-type protein for all FhuA functions; bacteriophage and colicin M titers obtained for cells harboring plasmids coding for these two deletions were not affected. For the deletion receptor mutants FhuAΔ021–128 and FhuAΔ406–417, only the colicin M titer was affected; an eightfold reduction in sensitivity was observed. These results suggest that there are domains of the FhuA protein specific for colicin M binding, these domains being delimited by amino acids 21 to 59, 70 to 128, and 406 to 417. These amino acid sequences can be deleted without affecting ferrichrome growth promotion or bacteriophage binding. They might comprise regions of the FhuA protein that are surface exposed. More dissection of these colicin M binding sites is needed to characterize the specific amino acids interacting with colicin M. Our TAB linker analyses (8) have also shown that a number of mutant receptors (FhuA195, FhuA198, and FhuA300) were only affected in their colicin M sensitivities. It suggests that the colicin M binding site on the FhuA protein is composed of numerous microdomains. Some of them can be disturbed by insertion or deletion mutagenesis, with moderate to severe effect on colicin M binding. Various *ompF-ompC*, *ompC-ompF*, and *ompF-ompC-ompF* chimeric genes were used to locate the domains of the OmpF protein involved in sensitivity to colicins A and N (13). As with colicin M sensitivity, several regions of OmpF are also required for colicin N action: (i) an amino-terminal domain located between residues 1 and 63, (ii) a central domain corresponding to residues 115 to 252, and (iii) a carboxy-terminal region which was mapped between residues 279 and 313 (13). A large domain corresponding to residues 1 to 260 of OmpF is involved in colicin A sensitivity.

The observed variation in colicin M sensitivity also suggests that the actual binding or uptake of colicin is an inefficient process. Many colicin M molecules may bind to FhuA, but few are correctly translocated to the periplasm for interactions which require accessory proteins or for the inhibition of the dephosphorylation of the bactoprenyl carrier lipid, the target of action for colicin M (21, 22).

When the FhuA deletions included segments of the protein from amino acids 21 to 195, most strains harboring plasmid-borne mutant *fhuA*s could grow in the presence of ferrichrome as the sole iron source. The exceptions were deletion mutants Δ021–082, Δ083–128, and Δ083–195. It is interesting that while FhuAΔ021–082 and FhuAΔ083–128 showed almost no FhuA functions, FhuAΔ021–128, a combination of these two smaller deletions, showed some wild-type phenotypes for FhuA functions. One interpretation of these results is that deletion of amino acids 21 to 82 or 83 to 128 may severely affect the proper integration of the deletion FhuAs into the outer membrane, thereby hindering access to surface-exposed regions for the FhuA-specific ligands. For the longer deletion mutant Δ021–128, the protein would then be properly integrated and all the surface-exposed domains required for ferrichrome, bacteriophage, and colicin M binding would be present. Some caution on interpretation is necessary when deletions are created in outer membrane proteins. One has to assess whether the deleted species assumes a near-native orientation in the outer membrane. Sensitive probes of the proper integration into the outer membrane, such as antibodies against surface-exposed epitopes, are needed to confirm this hypothesis.

Deletion mutants Δ129–195 and Δ169–195 could transport ferrichrome, but they were affected in other binding activities. It is particularly interesting that Δ129–195 could transport ferrichrome even if the major FhuA species detected by the anti-peptide antibodies was a degradation product. Proteolytic modification may somehow compensate for structural aberrations in the mutant proteins, enhancing their stability and restoring the normal physiology of the outer membrane. Since our immunoblot analysis showed that proteolytic degradation was from the amino terminus end, the phenotypes observed are predicted to be from our own genetic construction and not from the polypeptides that resulted from proteolysis, they become in the cell envelope. This is strengthened by reasoning that if the "TonB box" were removed from these polypeptides, they would become incompetent for ferrichrome uptake and for binding to bacteriophages T1, ϕ80, and UC-1, and to colicin M.

Although ferrichrome transport was abolished for FhuAΔ083–195, ligand binding was observed, although with reduced efficiency. Since deletion mutants Δ021–128 and Δ129–168 did not affect phage sensitivities, our results suggest that within the domain delimited by amino acids 169 to 195 of the FhuA protein, binding sites specific for the bacteriophages and colicin M are present.

Deletions extending beyond amino acid 195 affected all FhuA functions. No ferrichrome growth promotion was observed when deletions extended into the region bounded by amino acids 196 to 223. These include Δ060–198, Δ070–198, Δ129–241, Δ136–198, Δ136–223, Δ136–239, Δ169–241, Δ196–241, Δ199–223, Δ199–239, and Δ199–321. We cannot determine whether the mutations introduced local or global perturbations in the FhuA structure. It is possible that gross structural disturbances were created by some internal deletions, explaining the loss of receptor function. However, some functions of FhuA deletion mutants Δ070–198 (relative titer of 0.1 for T1 phage), Δ136–198 (relative titer of 0.01 for T5 phage), and Δ136–223 (relative titer of 0.01 for T5 and ϕ80 phages) were observed. This confirms the presence of those deletion FhuAs in the outer membrane and various degrees of competence for their functions. The absence of ferrichrome transport for these three mutants suggests an important role for amino acids 196 to 223 in the transport properties of FhuA. In addition, sensitivities to bacteriophages and colicin M were reduced. Therefore, it is proposed that amino acids 196 to 223 are part of a binding domain shared by all the ligands. Our results confirm the previous suggestion that FhuA contains overlapping binding sites for these very different agents (19). Such overlap would also explain why binding to the FhuA protein and killing by the lethal agents are blocked competitively by ferrichrome.

Some ferrichrome transport was observed for the following four deletion mutant FhuAs: Δ224–321, Δ240–321, Δ322–405, and Δ322–417. Since the predicted 67-kDa species for Δ240–321 was not detected by either antibody, we conclude that the amounts of biologically active protein are below the levels needed for immunodetection. For these deletion mutants, binding by the different bacteriophages and by colicin M was very low or not detectable by our assays. Our TAB linker insertion mutant FhuA321 was shown selectively to

TABLE 4. Functional domains of the FhuA protein as defined by deletion mutagenesis

Amino acids of mature FhuA	Remarks
21–59, 70–128	Colicin M sensitivity reduced 8-fold
169–195	Sensitivities to phages and colicin M reduced 10-fold
196–223	All FhuA functions severely affected
224–405	Diminished ferrichrome growth promotion; no binding of phages or colicin M
406–417	Colicin M sensitivity reduced 8-fold
418–440	No FhuA functions observed; no protein detected on gels

eliminate ferrichrome growth promotion. Since the inserted dipeptide -Ser-Ser- from FhuA321 is preserved in these four deletion mutants, our results demonstrate that deletion of amino acid sequences preceding or following -Ser-Ser- cause the mutant FhuA protein to revert to a partial wild-type phenotype for ferrichrome transport. Bacteriophage and colicin M binding were not affected by the TAB linker mutation, but the deletion of amino acids surrounding the two newly added amino acids severely affect their binding.

An understanding of the topology of FhuA as it traverses the outer membrane is central to an understanding of its function and its structural domains. In common with other outer membrane proteins, FhuA has no hydrophobic domains that are predicted to span the membrane (10). While our results do not define the topology of the FhuA protein in the outer membrane, they provide a detailed characterization of different FhuA domains involved in ligand binding (Table 4). Ferrichrome transport, bacteriophage binding, and colicin M binding are all affected when deletions extend into a "window" from amino acids 196 to 223; this domain is thus shared by every ligand that uses FhuA as its receptor. Amino acids 169 to 195 are included in a domain specific for binding of bacteriophages and colicin M; ferrichrome transport was unaffected when these amino acids were deleted. Although ferrichrome transport was diminished for deletions between amino acids 224 and 405, very low to undetectable sensitivities to bacteriophages or colicin M were observed. At the amino terminus, there are domains that seem to be specific for colicin M binding. These include amino acids 21 to 59 and 70 to 128. Another domain specific for colicin M is present between amino acids 406 and 417.

Murphy et al. recently reported monoclonal antibodies raised against surface epitopes of the ferric enterobactin receptor FepA (31). They also demonstrated that monoclonal antibodies directed against one surface-exposed domain could inhibit all FepA functions, suggesting a shared domain for ligand binding. Our results provide evidence that such a shared domain is present in FhuA. Deletion and insertion mutagenesis of the FepA receptor also identified key regions for activities of FepA and demonstrated the presence of both shared and unique domains for ligand binding (1).

Our studies define several regions of the FhuA protein that are important for substrate binding, transport of ligands, and protein stability. Because we have narrowed the possible range of important domains for ligand binding, it is now realistic to use site-directed mutagenesis of the *fhuA* gene in order to identify specific amino acids that are responsible for ligand binding. Mutations will be sought that affect only individual ligands. In this way, we hope to identify the residues involved in substrate binding and transport function.

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