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Genetic Insertion and Exposure of a Reporter Epitope in the Ferrichrome-Iron Receptor of *Escherichia coli* K-12

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The ferrichrome-iron receptor of *Escherichia coli* K-12 is FhuA (M_r, 78,992), the first component of an energy-dependent, high-affinity iron uptake pathway. FhuA is also the cognate receptor for bacteriophages T5, T1, ϕ80, and UC-1, for colicin M and microcin 25, and for albomycin. To probe the topological organization of FhuA which enables recognition of these different ligands, we generated a library of 16 insertion mutations within the *fhuA* gene. Each insertion spliced a 13-amino-acid antigenic determinant (the C3 epitope of poliovirus) at a different position within FhuA. Immunoblotting of outer membranes with anti-FhuA and anti-C3 antibodies indicated that 15 of 16 FhuA.C3 proteins were present in the outer membrane in amounts similar to that observed for plasmid-encoded wild-type FhuA. One chimeric protein with the C3 epitope inserted after amino acid 440 of FhuA was present in the outer membrane in greatly reduced amounts. Strains overexpressing FhuA.C3 proteins were subjected to flow cytometric analysis using anti-FhuA monoclonal antibodies. Such analysis showed that (i) the chimeric proteins were properly localized and (ii) the wild-type FhuA protein structure had not been grossly altered by insertion of the C3 epitope. Twelve of sixteen strains expressing FhuA.C3 proteins were proficient in ferrichrome transport and remained sensitive to FhuA-specific phages. Three FhuA.C3 proteins, with insertions after amino acid 321, 405, or 417 of FhuA, were detected at the cell surface by flow cytometry using anti-C3 antibodies. These three chimeric proteins were all biologically active. We conclude that amino acids 321, 405, and 417 are surface accessible in wild-type FhuA.

Under conditions of iron deprivation, many gram-negative bacteria derepress the expression of a class of surface receptors called iron-regulated outer membrane proteins. The function of these proteins is to bind specific siderophores and to initiate their internalization, thereby enabling the cell to satisfy its iron requirement (5). Siderophores have widely differing structures, but all share a high affinity for the ferric ion (24). Assimilation of the ferric siderophore requires a specific receptor as well as other periplasmic and cytoplasmic membrane proteins, including TonB (28). A cytoplasmic membrane protein complex including TonB, ExbB, and ExbD is thought to couple the electrochemical potential of the cytoplasmic membrane to the outer membrane siderophore receptor, a step which allows subsequent translocation of the bound ligand into the periplasm (37, 38).

The *Escherichia coli* ferrichrome-iron receptor is FhuA (14), an iron-regulated outer membrane protein with a molecular mass of 78,992 Da (13). FhuA is also the cognate receptor for bacteriophages T5, T1, ϕ80, and UC-1, for colicin M and microcin 25 (31), and for albomycin. Knowledge of the molecular organization of FhuA within the outer membrane will allow for a better understanding of its ability to bind these structurally diverse ligands and to promote their active translocation across the outer membrane. Like FepA, the enterobactin receptor of *E. coli* (26), FhuA contains multiple amphiphilic stretches within its primary sequence. Topological models have been proposed for several outer membrane proteins, including FepA (26), FhuA (23), FoxA (1), and LamB (9). These models incorporate some structural elements in common: amphiphilic sequences constitute antiparallel β sheets which traverse the outer membrane; intervening sequences are thought to constitute loops that are exposed at the cell surface or in the periplasm.

Predictions of outer membrane protein topology can be evaluated with a number of tools, including monoclonal antibodies (MAbs) (26, 34, 39), insertion and deletion mutagenesis (4, 6, 7, 23), and reporter protein fusions (27). One form of insertion mutagenesis termed epitope insertion requires the splicing of a foreign antigenic determinant into different sites within a protein. If the protein tolerates a particular insertion without gross alteration of function, the insertion site is termed a permissive site (11). Use of the C3 epitope of poliovirus VP1 capsid protein as a reporter epitope provided topological information for the outer membrane proteins LamB (8, 11) and TraT (41). Charbit et al. (11) identified 11 permissive sites for C3 epitope insertion within LamB. Anti-C3 antibody (Ab) accessibility studies of the LamB-C3 hybrids indicated that three permissive sites were exposed at the external surface of the cell, while three additional sites were proposed to face the periplasm. Of five sites in TraT that were permissive for C3 epitope insertion, three were found to be surface exposed on the basis of anti-C3 MAb reactivity with whole cells.

We wished to assess the surface accessibility of FhuA sequences which were proposed to face the external milieu (23). Through the use of epitope insertion mutagenesis, we spliced the C3 epitope into 16 different sites within FhuA. The stability, localization, and biological activities of the FhuA.C3 proteins were characterized. By flow cytometry, the exposure of the inserted epitopes in each of the 16 FhuA.C3 chimeric strains was evaluated.

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

Strain, phage, or plasmid	Relevant characteristics	Source or reference
E. coli 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
JWC202	SG303 (*fhuA'-'lacZ*) *polA*	7
GC01	JWC202 *bla fhuA*⁺	7
GC020 to GC646	JWC202 *bla fhuA*	7
BB020 to BB646	JWC202 *bla fhuA.C3*	This study
CS180	F⁻ *thr leuB6 proA argE his thi galK lacY1 trpE non mtl xyl ara-14*	21
CS2529	CS180 *rfaK2*::ΩKm^r	21
CS2529 *fhuA*	CS2529 T5 resistant	This study
CS2774	CS180 Δ*lac rfaQ9*::Tn*lac*	21
CS2774 *fhuA*	CS2774 T5 resistant	This study
Phages		
T5, T1, and ϕ80 *vir*		Laboratory stocks
UC-1		C. F. Earhart
Plasmids		
pGC01	*bla fhuA*⁺ pBR322 replicon, 6.3 kb	7
pGC020 to pGC646	*bla fhuA* pBR322 replicon, 6.3 kb	7
pBB020 to pBB646	*bla fhuA.C3* pBR322 replicon, 6.3 kb	This study

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The bacterial strains, phages, and plasmids used in this study are listed in Table 1. The *E. coli* K-12 strains used were all derived from the parental strain MC4100 (36). SG303, CS2529, and CS2774 were made FhuA⁻ by selection for spontaneous T5 phage resistance. To confirm the absence of FhuA in these strains, phage-resistant candidates were analyzed by immunoblotting and by flow cytometry using anti-FhuA MAbs.

Media, enzymes, and reagents. The media and concentrations of antibiotics used have been described previously (7). Restriction endonucleases, sequencing enzymes, and radiolabelled reagents were purchased from Boehringer-Mannheim Canada and Amersham Canada Ltd. Oligonucleotides encoding the C3 epitope and primers for DNA sequencing were synthesized by V. Manoloulis, Sheldon Biotechnology Centre, McGill University. Secondary Abs for immunological assays were from Gibco BRL, Burlington, Ontario, Canada.

C3 epitope insertion. Standard conditions were used for DNA manipulation and transformations (32). The C3 oligonucleotides for insertion (Fig. 1) were designed to maintain the correct reading frame of the *fhuA* gene and depended upon the position of the *Cfo*I or *Hpa*II restriction site within *fhuA* that had been previously used to construct TAB linker insertions (the pGC series of plasmids [7]). Duplication of either Ser-Ser or Glu-Leu flanking the C3 epitope was therefore necessary; for each insertion, 13 amino acids were added, 11 of which corresponded to the C3 epitope.

Plasmid DNA of the pGC series (pGC020 to pGC646) was linearized at the unique *Sac*I site within the TAB linker of each plasmid. Single-stranded oligonucleotides (C3EP.SER1 and C3EP.SER2, or C3EP.GLU1 and C3EP.GLU2; Fig. 1) were heated to 85°C for 30 min and then annealed by slow cooling. The unphosphorylated double-stranded C3 oligonucleotide was ligated to the *Sac*I-restricted plasmids in a molar ratio of 100:1. The ligated constructs were transformed into competent SG303 *fhuA*, with selection for ampicillin resistance. Transformant DNA was screened by two independent methods for the presence of the C3 oligonucleotide. (i) For dot blot hybridization, transformant cells were immobilized onto a nylon membrane by using a Milliblot-D system (Millipore Corp., Bedford, Mass.) and then lysed with 0.4 M NaOH. ^{32}P-end-labelled C3 oligonucleotide was used to probe the immobilized nucleic acids. The radiolabelled C3 probe hybridized to approximately 20% of screened transformants. (ii) For *Sal*I restriction analysis, greater than 90% of transformants that were positive by dot blot hybridization had acquired a novel *Sal*I restriction site, designed within the sequence of the C3 oligonucleotide (Fig. 1). The parent plasmids already contained a *Sal*I site at nucleotide 1770 of the *fhuA* gene (13). Upon digestion of candidate DNA with *Sal*I, the sizes of the two fragments on agarose or acrylamide gels corresponded exactly to those

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                    93                                  103
                    ASP ASN PRO ALA SER THR THR ASN LYS ASP LYS
C3EP.SER1       5'-CG GAT AAC CCG GCG TCG ACC ACT AAC AAG GAT AAG AGC T-3'
C3EP.SER2   3'-TCG AGC CTA TTG GGC CGC AGC TGG TGA TTG TTC CTA TTC-5'
                                  SalI

                    93                                  103
                    ASP ASN PRO ALA SER THR THR ASN LYS ASP LYS
C3EP.GLU1       5'-C GAT AAC CCG GCG TCG ACC ACT AAC AAG GAT AAG GAG CT-3'
C3EP.GLU2   3'-TC GAG CTA TTG GGC CGC AGC TGG TGA TTG TTC CTA TTC C-5'
                                  SalI
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FIG. 1. C3 oligonucleotides used in this study. The deduced amino acid sequence is indicated above the nucleotide sequence. The C3 epitope is contained within amino acids 93 to 103 of the VP1 protein of poliovirus, as indicated. The ends of each double-stranded oligonucleotide are *Sac*I cohesive ends.

predicted from the location of the *fhuA-SalI* site relative to the *C3-SalI* site.

DNA sequence determination. Oligonucleotides corresponding to seven regions of the *fhuA* gene were used as primers for the dideoxy method of DNA sequencing (33). The sequences of the seven primers were 5′-AAGCTTAAGCTT(639)GCG GTTGAACCGAAAGAA-3′, 5′-(769)AAAAAGTGCCACA GTCTATT-3′, 5′-(946)ATAACTATCTGAATGGCCTG-3′, 5′-(1153)GCCTGTTCCAGACTGGTTTT-3′, 5′-(1524)GTG CGTCAGAACCTGCGCTT-3′, 5′-(1776)TTTATGCGTAT GCGTAATGA-3′, and 5′-(2503)TCTTTGACGGTCCGCTT TCA-3′, where the number in parentheses identifies the position of the next nucleotide according to the published nucleotide sequence of *fhuA* (13). Rapid preparations of plasmid DNA (QIAprep-spin kit; Qiagen, Chatsworth, Calif.) from each of the candidate transformants were used as templates for sequencing reactions. An example of the nomenclature used to describe the chimeras is as follows: the genetic chimera *fhuA020.C3* contains the C3 oligonucleotide inserted in-frame into the TAB linker of the GC *fhuA020* allele. The TAB linker in this construct is within the codon for residue 20 of the mature FhuA protein. The resulting chimeric protein is termed FhuA020.C3.

Anti-FhuA MAbs. Rat anti-FhuA MAb 4AA-1, used in immunoblotting, has been described previously (15); its determinant lies between amino acids 21 to 32 of the mature FhuA sequence. MAb 4AA-1 was used at a final dilution of 1/5,000 (from a 0.3-mg/ml stock) for immunoblotting.

All mouse MAbs used in this study were purified from tissue culture supernatant by using an anti-mouse κ-chain-specific immunoaffinity column. Mouse anti-FhuA MAb Fhu6.9, which was used in immunoblotting (final dilution of 1/1,000 from a 0.1-mg/ml stock), recognizes amino acids C terminal to amino acid 417 of FhuA (25). Two mouse anti-FhuA MAbs, Fhu3.1 and Fhu4.1, were used in enzyme-linked immunosorbent assay (ELISA) and flow cytometry; both recognize cell surface-exposed conformational determinants of FhuA. MAb Fhu3.1 has been described previously (40); Fhu3.1 recognizes a determinant located C terminal to amino acid 417 of FhuA. MAb Fhu3.1 (stock concentration, 0.5 mg/ml) was used at a final dilution of 1/100 for both ELISA and flow cytometry. The determinant for Fhu4.1 is located between amino acids 321 and 417 of FhuA (25). MAb Fhu4.1 (stock concentration, 0.3 mg/ml) was used at a final dilution of 1/50 for both ELISA and flow cytometry.

Anti-C3 Abs. A peptide corresponding to amino acids 93 to 103 of the C3 epitope of poliovirus VP1 protein was synthesized by J. Hu, Sheldon Biotechnology Centre, McGill University. The C3 peptide was purified by reverse-phase high-pressure liquid chromatography and conjugated to keyhole limpet hemocyanin. A polyclonal anti-C3 antiserum (PAb 928) was generated by immunizing New Zealand White rabbits with keyhole limpet hemocyanin-conjugated C3 (17). To enhance the specificity of immunodetection of the C3 epitope, the anti-C3 serum was adsorbed against an acetone powder of *E. coli* MC4100 (17). The adsorbed PAb 928 was used in immunoblotting at a final dilution of 1/2,000 and in both ELISA and flow cytometry at 1/50.

Mouse anti-C3 MAb AS2 is a patented biological and was purchased from the Collection Nationale de Cultures de Microorganismes, Institut Pasteur, Paris. It has been described elsewhere (43). MAb AS2 (concentration, 0.5 mg/ml) was used at final dilutions of 1/2,000 for immunoblotting and 1/50 for ELISA and flow cytometry. Both monoclonal and polyclonal anti-C3 Abs reacted specifically with the C3 epitope in immunoblotting, ELISA, and flow cytometry.

Electrophoresis of outer membrane proteins and immunoblotting. Outer membranes were prepared by Tris-lysozyme-EDTA treatment of *E. coli* cells by the method of Hantke (16). Samples of 10 μg of protein were suspended in electrophoresis sample buffer containing 2% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol, heat denatured for 5 min at 100°C, and run on 8% polyacrylamide gels. The gels were either stained with Coomassie brilliant blue or used for immunoblotting as described previously (7). For immunoblotting, 2 μg of outer membrane proteins was resolved by SDS-polyacrylamide gel electrophoresis (PAGE). The secondary Abs for immunoblotting (final dilution, 1/4,000) were conjugated to alkaline phosphatase and were detected with 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium.

Phenotypic analyses. The *fhuA.C3* chimeras were transferred into the chromosome in order to examine the phenotypes of strains expressing *fhuA.C3* alleles at the single-copy level. Plasmids carrying defined C3 insertions within TAB linkers in *fhuA* were transformed into JWC202 (7) and then subjected to selection for resistance to ampicillin and tetracycline. Since the pBB series of plasmids, which contain a ColE1 origin of replication, cannot replicate in the *polA* strain JWC202, selection for ampicillin resistance forces plasmid integration into the chromosome by homologous recombination, thereby generating a *fhuA.C3* insertion mutation. The resulting 16 strains were termed BB020 to BB646. The absence of plasmids in these strains was confirmed by agarose gel electrophoresis. Sensitivity of all strains of the BB series to phages T5, T1, φ80, and UC-1 and the ability of the FhuA.C3 proteins in these strains to promote growth with ferrichrome as the sole iron source were determined by an endpoint dilution spot test as described previously (7).

Microcin 25 was a gift from R. A. Salomón and has been described elsewhere (30). To assay microcin 25 sensitivity, a partially purified sample of *E. coli* AY25 supernatant containing microcin 25 was spotted onto a lawn of top agar which had been seeded with 10^8 cells of the test strain (31). Sensitivity of the chimeric strains to all lethal agents was assessed after incubation at 37°C for 6 h.

Flow cytometry. Bacteria were grown at 37°C in L broth with antibiotics as required. At the mid-log phase of growth, cells were harvested and suspended to 5×10^8/ml in phosphate-buffered saline (PBS). Aliquots of 100 μl of cells were added to tubes containing 100 μl of primary Ab diluted in PBS. After a 30-min incubation at 4°C, cells were pelleted by microcentrifugation at 12,000 × *g* for 2 min at 4°C and then washed with 1 ml of PBS. The washed cell pellet was suspended in 100 μl of fluorescein isothiocyanate-labelled anti-immunoglobulin G Ab (either anti-mouse or anti-rabbit; final dilution, 1/200) and incubated for a further 30 min at 4°C. Cells were pelleted, suspended in 800 μl of PBS, and analyzed for green fluorescence intensity, using a FACScan flow cytometer (Becton Dickinson, Rutherford, N.J.) with LysisII software. The flow cytometer was configured to exclude lysed cells and cell aggregates from consideration. For each sample, 10^4 cells were analyzed.

ELISA with anti-FhuA and anti-C3 Abs. Outer membranes from strains expressing FhuA.C3 chimeric proteins from high-copy-number plasmids [SG303 *fhuA*(pBB series)] were suspended in 10 mM ammonium acetate–10 mM ammonium carbonate (pH 8.2) to 10 μg/ml. An aliquot of each suspension (50 μl per well) was then applied to polystyrene microtiter plates (Nunc MaxiSorp Certified; Nunc Intermed, Roskilde, Denmark), and the volatile salts were evaporated by incubation at 37°C. All subsequent incubations were at 25°C. Wells

were blocked with 5% skim milk; the primary Ab was then added, and the mixture was incubated for 45 min. Wells were washed four times with PBS containing 0.05% Tween 20 (PBST), and then an alkaline phosphatase-conjugated secondary Ab (anti-mouse κ light chain or anti-rabbit immunoglobulin G) was added. After 45 min, the plates were washed four times with PBST, and *p*-nitrophenyl phosphate (2 mg/ml in 10 mM diethanolamine–0.5 mM $MgCl_2$ [pH 9.5]) was added. After color development, the optical density at 405 nm was determined. ELISAs were performed in duplicate. Control assays used outer membranes from the $FhuA^+$ strain SG303 *fhuA*(pGC01) and from the $FhuA^-$ strain SG303 *fhuA* as antigens.

RESULTS

Insertion of the C3 epitope. The starting material for insertion of the C3 epitope was our library of TAB linker insertions within the *fhuA* gene (7). Since each member of this library (the pGC series of plasmids) contained a unique *Sac*I restriction site within the TAB linker, we were able to create a set of 16 C3 oligonucleotide insertions at defined locations within the *fhuA* gene. Two methods were used to screen for insertions of the C3 oligonucleotide in transformants of SG303 *fhuA*: (i) dot blot hybridization and (ii) *Sal*I restriction digestion. The results of screening could not exclude candidate transformants which possessed the C3 oligonucleotide inserted in reverse orientation. Outer membranes of transformants were prepared, and by immunoblotting with an anti-FhuA MAb, the presence of a protein which migrated near the level of wild-type FhuA was detected (data not shown). Plasmid DNA from candidates expressing full-length FhuA was then used for DNA sequencing. The nucleotide sequence and the deduced amino acid sequence of each of the 16 *fhuA.C3* alleles across the site of C3 oligonucleotide insertion are presented in Fig. 2.

Identification of the FhuA.C3 chimeric proteins. (i) SDS-PAGE and outer membrane protein staining. Outer membrane vesicles were prepared from cells lacking a functional chromosomal copy of *fhuA* but expressing either a FhuA.C3 chimeric protein (encoded by the pBB series of plasmids) or wild-type FhuA (encoded by pGC01 [7]). Outer membrane proteins were then resolved by SDS-PAGE and visualized by Coomassie brilliant blue staining. Compared with the protein profile of SG303 *fhuA*(pGC01), the overexpression of the FhuA.C3 chimeric proteins did not significantly alter the relative amounts of outer membrane proteins (Fig. 3). The FhuA.C3 proteins were detected in the outer membrane preparations, and they exhibited electrophoretic mobilities similar to that observed for wild-type FhuA (79 kDa; Fig. 3, lane 2). Furthermore, the amount of each chimeric protein was similar to that of wild-type FhuA, except for SG303 *fhuA* (pBB440), in which FhuA440.C3 was present in greatly reduced amounts.

(ii) Immunoblotting with anti-FhuA MAbs. To confirm that C3 epitope insertion had not affected the stability of FhuA, immunoblotting analysis of outer membranes using anti-FhuA MAbs was performed. MAb 4AA-1, which reacts with the amino terminus of FhuA (15), detected a single band in the outer membranes of each of the chimeric strains (Fig. 4A). Except for FhuA440.C3, the intensities of the bands corresponding to FhuA.C3 were equivalent, and none differed greatly from the intensity observed for plasmid-encoded wild-type FhuA. This was confirmed by immunoblotting with a second anti-FhuA MAb, Fhu6.9, which recognizes sequences carboxy terminal to amino acid 417 of FhuA (Fig. 4B). Probing outer membrane proteins with Fhu6.9 also revealed the presence of a second, abundant truncated FhuA species (72 kDa) for eight of the chimeras: FhuA069.C3, FhuA082.C3, FhuA128.C3, FhuA168.C3, FhuA195.C3, FhuA198.C3, FhuA239.C3, and FhuA241.C3. This analysis suggested that proteolytic cleavage of FhuA to the 72-kDa species resulted in the loss of the determinant recognized by MAb 4AA-1; both full-length FhuA (79 kDa) and the 72-kDa species were detected by MAb Fhu6.9.

(iii) Immunoblotting with anti-C3 Abs. Immunoblotting with either monoclonal or polyclonal Abs against the C3 epitope identified each of the 16 FhuA.C3 chimeras and confirmed that the electrophoretic mobilities of the chimeric proteins were similar to that observed for wild-type FhuA (Fig. 4C and D). The intensities of the bands corresponding to the FhuA.C3 chimeric proteins obtained by using the anti-C3 MAb AS2 were similar to the band intensities obtained by using PAb 928, a result which concurred with immunoblots prepared by using anti-FhuA MAbs (Fig. 4A and B). The pattern of anti-C3 Ab reactivity corresponded with Fhu6.9 reactivity in that the 72-kDa species was detected in the same eight chimeric strains. For each of the remaining eight FhuA.C3 chimeras (FhuA020.C3, FhuA135.C3, FhuA223.C3, FhuA321.C3, FhuA405.C3, FhuA417.C3, FhuA440.C3, and FhuA646.C3), the predominant C3-specific band had an electrophoretic mobility which was very similar to that of wild-type FhuA. Taken together, the results indicated that these latter chimeras were not significantly destabilized by insertion of the C3 epitope.

Assessment of FhuA.C3 protein folding. We wished to demonstrate that the FhuA.C3 chimeric proteins generated in this study had retained the ability to fold in the outer membrane in a manner equivalent to the folding of wild-type FhuA. The anti-FhuA MAbs used for this analysis, Fhu3.1 and Fhu4.1, each recognize a conformational epitope of FhuA that is accessible at the cell surface (25, 40). The rationale for this analysis is as follows: if C3 epitope insertion had distorted the proper folding of FhuA, the distortion might result in a loss of either Fhu3.1 or Fhu4.1 MAb binding to their respective conformational epitope. By using flow cytometry to analyze intact cells that were stained with the anti-FhuA MAbs, the proper localization of the FhuA.C3 chimeric proteins within the outer membrane was also confirmed. All strains overexpressing FhuA.C3 proteins demonstrated increases in fluorescence intensity after staining with either Fhu3.1 or Fhu4.1 (Table 2). Except for SG303 *fhuA*(pBB440), the fluorescence shifts were similar to the increase in fluorescence of the positive control strain SG303 *fhuA*(pGC01) over the negative control strain SG303 *fhuA* when stained with the same two MAbs (Table 2). Moreover, the fluorescence histogram profiles of all FhuA.C3 chimeric strains except SG303 *fhuA* (pBB440) matched the profile of SG303 *fhuA*(pGC01), indicating a similar pattern of recognition of each of these strains by Fhu3.1 and Fhu4.1 (Fig. 5). The mean fluorescence intensity of SG303 *fhuA*(pBB440) when stained with either Fhu3.1 or Fhu4.1 was approximately fivefold lower than that of SG303 *fhuA*(pGC01). All 16 parental FhuA TAB linker insertion strains (SG303 *fhuA* containing the pGC series of plasmids) also bound both Fhu3.1 and Fhu4.1 in flow cytometry as well as did the positive control strain SG303 *fhuA*(pGC01) (data not shown).

In vivo biological activities of the chimeras. The phenotypes associated with the 16 *fhuA.C3* alleles were examined at the single-copy level, since reduced biological activity of a partially defective chimeric protein may be compensated for by its overexpression from a high-copy-number plasmid (7). We assayed the ability of chromosomally encoded FhuA.C3 pro-

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                    19  20          93              103          21  22
                   glu ser glu leu ASP ASN ... ASP LYS glu leu ala trp
pBB020          693-gaa agc gAG CTC GAT AAC ... GAT AAG GAG CTC Gca tgg-704

                    68  69                                      70  71
                   glu ala ser ser ASP ASN ... ASP LYS ser ser leu ser
pBB069          840-gaa gcg AGC TCG GAT AAC ... GAT AAG AGC TCG ctt agc-851

                    81  82                                      83  84
                   arg gly glu leu ASP ASN ... ASP LYS glu leu ala ser
pBB082          879-cgt ggc gAG CTC GAT AAC ... GAT AAG GAG CTC Gca tcc-890

                   127 128                                     129 130
                   glu arg glu leu ASP ASN ... ASP LYS glu leu ala glu
pBB128         1017-gaa cgc gAG CTC GAT AAC ... GAT AAG GAG CTC Gct gaa-1028

                   134 135                                     136 137
                   gly pro ser ser ASP ASN ... ASP LYS ser ser val ser
pBB135         1038-ggc cCG AGC TCG GAT AAC ... GAT AAG AGC Tcg gtt tcc-1049

                   167 168                                     169 170
                   lys ala glu leu ASP ASN ... ASP LYS glu leu gly thr
pBB168         1137-aaa gcC GAG CTC GAT AAC ... GAT AAG GAG CTc ggt act-1148

                   194 195                                     196 197
                   leu thr glu leu ASP ASN ... ASP LYS glu leu gly leu
pBB195         1218-ctg acC GAG CTC GAT AAC ... GAT AAG GAG CTc ggt ctt-1229

                   197 198                                     199 200
                   leu ala ser ser ASP ASN ... ASP LYS ser ser arg ser
pBB198         1227-ctt gcg AGC TCG GAT AAC ... GAT AAG AGC TCG cgt tct-1238

                   222 223                                     224 225
                   arg pro ser ser ASP ASN ... ASP LYS ser ser asp asp
pBB223         1302-cgt cCG AGC TCG GAT AAC ... GAT AAG AGC Tcg gat gat-1313

                   238 239                                     240 241
                   glu pro ser ser ASP ASN ... ASP LYS ser ser glu thr
pBB239         1350-gag cCG AGC TCG GAT AAC ... GAT AAG AGC Tcg gaa acc-1361

                   240 241                                     242 243
                   glu thr glu leu ASP ASN ... ASP LYS glu leu gly tyr
pBB241         1356-gaa acC GAG CTC GAT AAC ... GAT AAG GAG CTc ggt tat-1367

                   320 321                                     322 323
                   asp pro ser ser ASP ASN ... ASP LYS ser ser ala asn
pBB321         1596-gat cCG AGC TCG GAT AAC ... GAT AAG AGC Tcg gcg aat-1607

                   404 405                                     406 407
                   asn pro ser ser ASP ASN ... ASP LYS ser ser val asn
pBB405         1848-aat cCG AGC TCG GAT AAC ... GAT AAG AGC Tcg gtg aat-1859

                   416 417                                     418 419
                   asp pro ser ser ASP ASN ... ASP LYS ser ser ala asn
pBB417         1884-gat cCG AGC TCG GAT AAC ... GAT AAG AGC Tcg gca aac-1895

                   439 440                                     441 442
                   gln ala ser ser ASP ASN ... ASP LYS ser ser gln trp
pBB440         1953-cag gcg AGC TCG GAT AAC ... GAT AAG AGC TCG cag tgg-1964

                   645 646                                     647 648
                   asp pro ser ser ASP ASN ... ASP LYS ser ser ala asn
pBB646         2571-gat cCG AGC TCG GAT AAC ... GAT AAG AGC Tcg gct aac-2582
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FIG. 2. Sequences of *fhuA.C3* chimeric genes. Plasmids encoding FhuA.C3 chimeric proteins are designated pBB, followed by the position of the last FhuA residue before the TAB linker insertion. The first nucleotide of the local sequence is preceded by a number indicating its position in the published sequence of *fhuA* (13); the position of the last nucleotide is also indicated. The *fhuA* nucleotide sequence is indicated in lowercase letters; uppercase letters correspond to nucleotides of the TAB linker. Uppercase boldface letters represent inserted C3 oligonucleotides. The corresponding amino acid sequences are indicated above the nucleotide sequence; the first and last numbers identify residues of the mature FhuA protein. Only the first two and last two residues of the 93–103 C3 epitope are indicated.

teins to promote growth of an *aroB fhuA* strain (JWC202 [7]) on nutrient broth-ethylenediamine di(*o*-hydroxy)phenyl acetic acid plates with ferrichrome as the sole iron source. For growth on this medium, strains require a functional FhuA protein to bind ferrichrome and to transport iron. Twelve of the sixteen chimeric strains demonstrated the wild-type phenotype of growth promotion by ferrichrome (Table 3). Insertion of the C3 epitope after amino acid 69, 82, 128, or 135 of FhuA eliminated the ability of the FhuA.C3 chimeric proteins to promote growth of the strain on ferrichrome.

The sensitivities of the BB series of strains to the FhuA-specific lytic bacteriophages T5, T1, ϕ80, and UC-1 were analyzed by using an endpoint dilution spot test. Ten of the FhuA.C3 chimeric strains retained wild-type sensitivities to all phages (Table 3). Strains BB321 and BB417 displayed wild-type sensitivities to T5, ϕ80, and UC-1 but 1,000- and 10-fold-reduced sensitivities to T1 phage, respectively. Insertion of the C3 epitope after amino acids 69, 82, 128, and 135 abolished sensitivity to all FhuA-specific phages, just as C3 insertions at these sites had eliminated ferrichrome growth promotion. The phage resistance of BB069, BB082, BB128, and BB135 was FhuA specific, since each of these four strains remained susceptible to three bacteriophages (data not shown) which use BtuB, OmpC, and PhoE as specific receptors, namely, BF23, TuIb, and TC45, respectively (18).

Assays were performed to determine the sensitivities of the

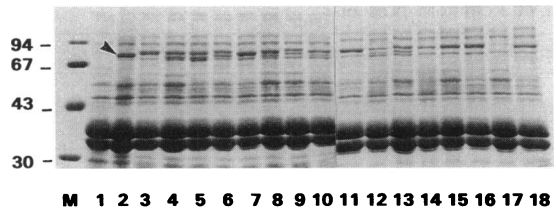

FIG. 3. Identification of outer membrane proteins in strain SG303 *fhuA* containing high-copy-number plasmids encoding wild-type FhuA and FhuA.C3 chimeric proteins. Outer membrane proteins were subjected to SDS-PAGE and stained with Coomassie brilliant blue. The arrowhead indicates the position of wild-type FhuA. Lanes: M, marker proteins with masses as indicated in kilodaltons; 1, no plasmid; 2, pGC01; 3, pBB020; 4, pBB069; 5, pBB082; 6, pBB128; 7, pBB135; 8, pBB168; 9, pBB195; 10, pBB198; 11, pBB223; 12, pBB239; 13, pBB241; 14, pBB321; 15, pBB405; 16, pBB417; 17, pBB440; 18, pBB646.

chimeric strains to the recently reported antibiotic microcin 25 (30). This agent uses the FhuA protein as its specific surface receptor (31). With one exception, the pattern of microcin 25 sensitivity matched the pattern of phage sensitivities of the chimeric strains; strains which were susceptible to killing by phage were as sensitive to microcin 25 as was GC01 (Table 3). The notable exception was the *fhuA.C3* chimeric strain BB020, which demonstrated complete resistance to microcin 25 while displaying wild-type sensitivity to all FhuA-specific phages.

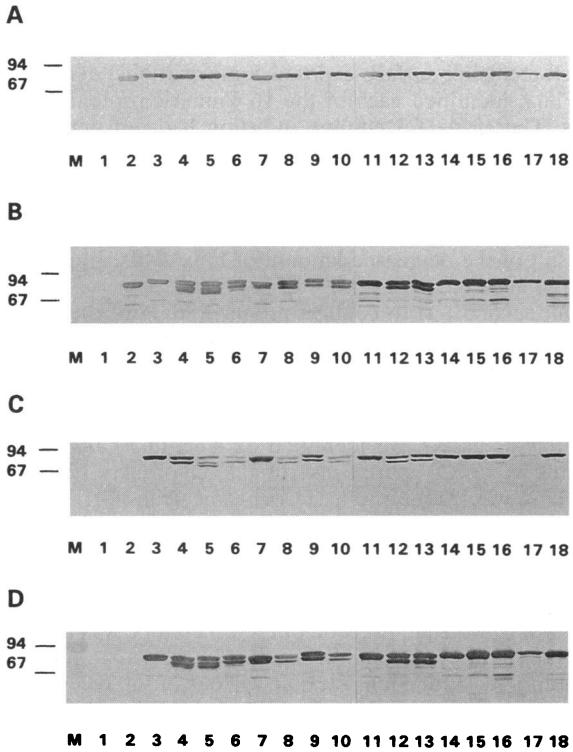

FIG. 4. Immunoblotting of wild-type FhuA and FhuA.C3 proteins. Outer membrane proteins were subjected to SDS-PAGE, transferred to nitrocellulose, and then probed with anti-FhuA MAb 4AA-1 (A) or Fhu6.9 (B), anti-C3 MAb AS2 (C), or anti-C3 PAb 928 (D). Lane designations are identical to those in Fig. 3.

Strains BB069, BB082, BB128, and BB135 were completely resistant to microcin 25.

The sensitivities of cells carrying TAB linker insertion mutations within the *fhuA* gene at the single-copy level (the GC series of strains [7]) to microcin 25 were also assessed. Strains GC069, GC082, GC128, GC135, and GC440 all demonstrated complete resistance to microcin 25. GC020 and GC239 displayed twofold-reduced sensitivity to microcin 25. The remaining strains of the GC series, GC168, GC195, GC198, GC223, GC241, GC321, GC405, GC417, and GC646, all displayed wild-type microcin 25 sensitivity.

Exposure of the C3 epitope in chimeric strains. To determine the accessibility of the C3 epitope at the cell surface, antibody recognition of FhuA.C3 was analyzed in strains overexpressing the chimeric proteins. Two anti-C3 Abs, MAb AS2 and PAb 928, were used to stain SG303 *fhuA* cells containing one of the pBB plasmids. Relative fluorescence intensity of the strains was assessed by using a fluorescein isothiocyanate-labelled secondary Ab followed by flow cytometry. Since only intact cells were counted, any significant increase in fluorescence over that observed with the negative control strain SG303 *fhuA*(pGC01) was indicative of C3 exposure at the cell surface. Strains expressing FhuA321.C3, FhuA405.C3, and FhuA417.C3 demonstrated substantial increases in fluorescence over the control strain when stained with MAb AS2 and PAb 928 (Fig. 5; Table 2). The magnitude of the fluorescence shift was 30- to 80-fold over the relative fluorescence of SG303 *fhuA*(pGC01).

C3 epitope exposure in CS2529 *fhuA* and CS2774 *fhuA* cells (22) which were transformed with the pBB series of plasmids was also analyzed. Both strains express truncated lipopolysaccharide (LPS). The *rfaK* mutation in CS2529 severely hinders the completion of the outer core (22, 35); the *rfaQ* mutation in CS2774 results in a partial deep rough phenotype (22, 35). Staining of transformants with anti-FhuA MAbs Fhu3.1 and Fhu4.1 followed by flow cytometry revealed that the chimeric proteins were expressed in the outer membrane at levels equivalent to the level of FhuA in SG303 *fhuA*(pGC01) (data not shown). Flow cytometric analysis with anti-C3 Abs verified the exposure of the C3 epitope in FhuA321.C3, FhuA405.C3, and FhuA417.C3 (data not shown). The remaining 13 of 16 FhuA.C3 chimeric proteins were not detected at the cell surface with anti-C3 Abs.

Results from flow cytometry were confirmed by an ELISA using outer membrane vesicles from SG303 *fhuA* cells which overexpressed the FhuA.C3 chimeric proteins. Anti-FhuA MAbs Fhu3.1 and Fhu4.1 identified FhuA.C3 in the outer membranes of each strain in approximately equivalent amounts, except in the outer membrane vesicles of SG303 *fhuA*(pBB440), a strain which expressed diminished amounts of the chimeric protein (Fig. 3 and 4). Signals of 8 to 16 times over background were obtained with MAb AS2 and PAb 928 staining of outer membrane vesicles containing FhuA321.C3, FhuA405.C3, and FhuA417.C3 (Table 2). The ELISA results therefore correlated well with those from C3-specific Ab recognition of intact cells by flow cytometry (Table 2).

DISCUSSION

The goals of this study were to insert a foreign antigenic determinant at various positions within FhuA of *E. coli* and then to assess the properties of the novel proteins at the bacterial cell surface. By such analyses, it was possible to deduce information about topology from the chimeric protein and extrapolate to wild-type FhuA. We chose to insert the C3 epitope of poliovirus VP1 protein because it is a well-charac-

TABLE 2. Antibody recognition of FhuA.C3 chimeric proteins

Plasmid[a]	Signal intensity							
	Flow cytometry[b]				ELISA[c]			
	Fhu3.1	Fhu4.1	AS2	928	Fhu3.1	Fhu4.1	AS2	928
none	−	−	−	−	−	−	−	+
pGC01	+++	+++	−	−	+++	+++	−	+
pBB020	+++	+++	−	−	++++	+++	−	+
pBB069	+++	++	−	−	+++	+++	−	+
pBB082	+++	+++	−	−	+++	+++	−	+
pBB128	+++	+++	−	−	+++	+++	−	+
pBB135	+++	++	−	−	++++	++++	−	+
pBB168	+++	++	−	−	+++	+++	−	+
pBB195	+++	+++	−	−	+++	+++	−	++
pBB198	+++	++	−	−	+++	+++	−	+
pBB223	+++	+++	−	−	+++	++++	−	+
pBB239	+++	++	−	−	+++	+++	−	++
pBB241	+++	+++	−	−	+++	+++	−	++
pBB321	+++	+++	++	+++	+++	++++	+++	++++
pBB405	+++	+++	+++	+++	+++	+++	+++	++++
pBB417	+++	+++	+++	+++	+++	+++	++++	++++
pBB440	+	+	−	−	+	+	−	++
pBB646	+++	++	−	−	+++	+++	−	+

[a] High-copy-number plasmid in *E. coli* SG303 *fhuA*.

[b] Flow cytometry on intact cells, using either anti-FhuA MAbs or anti-C3 Abs. Signals are categories of mean fluorescence values (for 10,000 cells) as follows: −, less than 8; +, 8 to 32; ++, 32 to 128; +++, 128 to 512.

[c] ELISA on outer membrane vesicles, using either anti-FhuA MAbs or anti-C3 Abs. Signals are categories of A_{405} values as follows: −, less than 0.15; +, 0.15 to 0.25; ++, 0.25 to 0.5; +++, 0.5 to 1; ++++, 1 to 2.

terized determinant (19, 42, 43) against which MAbs are available (3, 12). In addition, its utility as a reporter epitope for insertion into outer membrane proteins is well documented (8, 10, 11, 41).

Combining strategies of TAB linker insertion mutagenesis followed by C3 oligonucleotide insertion into the unique *Sac*I restriction site within the TAB linker proved effective in generating defined insertions in the *fhuA* gene. For all 16 *fhuA.C3* alleles, the C3 oligonucleotide was inserted without alterations to flanking sequences, thereby avoiding a possible consequence of random insertion mutagenesis performed with DNase I (4, 8, 11).

The insertion of 13 additional amino acids encoding the C3 epitope did not disrupt the export competence of the chimeric proteins. The FhuA.C3 protein was detected in the outer membrane fraction of each strain by Coomassie blue staining and by immunoblotting with anti-FhuA and anti-C3 Abs. Flow cytometry with MAbs recognizing surface-exposed determinants of FhuA confirmed the faithful localization of each FhuA.C3 protein in the outer membrane. The insertion of the C3 epitope was therefore tolerated at all 16 sites in FhuA: the FhuA.C3 proteins were not toxic to the cell, and all were export and localization competent.

Anti-FhuA MAbs identified FhuA.C3 in amounts similar to that of wild-type FhuA when FhuA and the chimeric proteins were expressed from high-copy-number plasmids. The one exception was FhuA440.C3; it was present in the outer membrane but in reduced amounts. However, the FhuA$^+$ phenotypes of SG303 *fhuA*(pBB440) indicate that FhuA440.C3 was still able to function as a wild-type FhuA receptor, and so residue 440 of FhuA is permissive for C3 epitope insertion.

Eight of the 16 FhuA.C3 proteins generated in this study displayed susceptibility to proteolytic cleavage. Truncation of FhuA to a 72-kDa species was also observed for some TAB linker insertion mutants of FhuA (7). Apparently, insertion mutations at selected sites near the N terminus of FhuA cause sensitivity to cleavage, resulting in production of a truncated FhuA species which appeared resistant to further degradation.

It was then necessary to address the possibility that insertion of the C3 epitope might alter the folding of FhuA in the outer membrane. Flow cytometric analysis using anti-FhuA MAbs which recognize surface-exposed conformational determinants of FhuA identified each of the 16 chimeric proteins in intact cells. Therefore, C3 epitope insertion had not detrimentally altered the folding of the FhuA.C3 proteins, at least not in the regions of the receptor proximal to the determinants which are recognized by MAbs Fhu3.1 and Fhu4.1. The diminished fluorescence shift of SG303 *fhuA*(pBB440) was most probably a result of the decreased amount of FhuA440.C3 in the outer membrane.

Our second test to confirm proper folding of the FhuA.C3 proteins analyzed the biological functions provided by the chimeric *fhuA.C3* alleles when expressed in single copy. By definition, C3 insertions which did not eliminate the FhuA$^+$ phenotypes occurred at permissive sites within FhuA. Using phenotypic assays, we identified 12 sites in FhuA that were permissive for C3 epitope insertion. Each of these 12 FhuA.C3 proteins was proficient in ferrichrome transport, and each bound all four FhuA-specific phages. Except for FhuA020.C3, each of these chimeric proteins also provided sensitivity to the FhuA-specific antibiotic microcin 25. Of the 12 permissive C3 insertion mutations, 9 were phenotypically silent. This observation suggests that insertion of the C3 epitope was in a region of FhuA that could readily accept 13 additional amino acids without loss of function. Flexible regions of the receptor, such as exposed loops, would most likely be able to accommodate the C3 epitope without disruption of the wild-type FhuA folding pattern necessary for biological activity.

C3 insertion into the TAB linker at amino acid 321 or 417 selectively reduced phage T1 sensitivity by 1,000- or 10-fold, respectively. These mutations may have resulted in local perturbation of the T1 phage binding site in FhuA321.C3 and

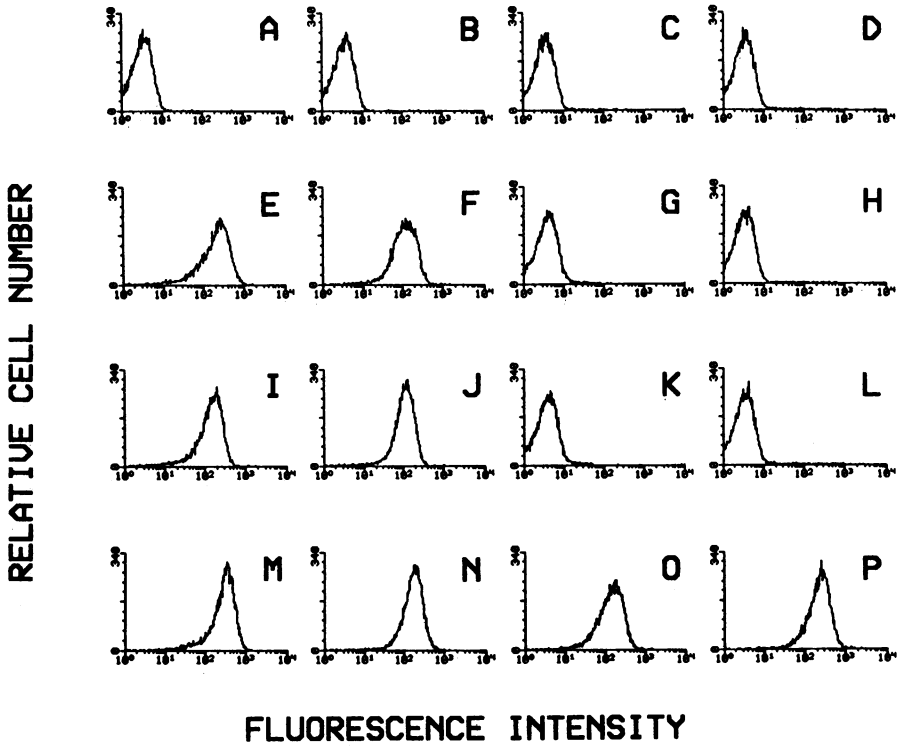

FIG. 5. Flow cytometric analysis of intact cells expressing FhuA.C3 chimeric proteins. SG303 *fhuA* cells containing either no plasmid (A to D), pGC01 (E to H), pBB195 (I to L), or pBB417 (M to P) were stained with an anti-FhuA or anti-C3 Ab as described in Materials and Methods. The primary Abs used were anti-FhuA MAb Fhu3.1 (A, E, I, and M), anti-FhuA-MAb Fhu4.1 (B, F, J, and N), anti-C3 MAb AS2 (C, G, K, and O), and anti-C3 PAb 928 (D, H, L, and P).

FhuA417.C3. This conclusion contrasts with results demonstrating complete T1 resistance of strains expressing 4- or 16-amino-acid insertions at residue 321 of FhuA (23). The discrepancy may be explained by the particular amino acid sequence of the insert: if the 13-residue polypeptide which encodes the C3 epitope is flexible, it may assume a conformation in FhuA321.C3 which allows T1 phage still to bind and lyse BB321 cells, albeit with reduced efficiency.

TABLE 3. Properties of strains expressing chromosomal *fhuA.C3* alleles

fhuA.C3 allele	FC[a]	Relative titer[b]				
		T5	T1	φ80	UC-1	Mcc25
Δ*fhuA*	−	0	0	0	0	0
Wild type	+	1	1	1	1	1
fhuA020.C3	+	1	1	1	1	0
fhuA069.C3	−	0	0	0	0	0
fhuA082.C3	−	0	0	0	0	0
fhuA128.C3	−	0	0	0	0	0
fhuA135.C3	−	0	0	0	0	0
fhuA168.C3	+	1	1	1	1	1
fhuA195.C3	+	1	1	1	1	1
fhuA198.C3	+	1	1	1	1	1
fhuA223.C3	+	1	1	1	1	1
fhuA239.C3	+	1	1	1	1	1
fhuA241.C3	+	1	1	1	1	1
fhuA321.C3	+	1	0.001	1	1	1
fhuA405.C3	+	1	1	1	1	1
fhuA417.C3	+	1	0.1	1	1	1
fhuA440.C3	+	1	1	1	1	1
fhuA646.C3	+	1	1	1	1	1

[a] FC, ferrichrome growth promotion.

[b] The titer obtained on mutant cells divided by the titer obtained on the wild-type strain; 0 indicates that no plaque or zone of inhibition was detected, even for the most concentrated spot of phage or microcin 25 (Mcc25).

Strain BB020 was fully competent in ferrichrome uptake and FhuA-specific phage binding. The complete resistance of this strain to microcin 25 serves to identify a region around amino acid 20 of FhuA which is important for microcin 25 sensitivity.

Four *fhuA.C3* chimeric strains, expressing FhuA069.C3, FhuA082.C3, FhuA128.C3, or FhuA135.C3, could not utilize ferrichrome and were completely resistant to T5, T1, φ80, UC-1, and microcin 25. The FhuA$^-$ phenotypes of these four strains are shared by the corresponding parental TAB linker insertion mutants of FhuA (7). Insertions of 4, 8, or 16 amino acids at positions between residues 69 and 135 of FhuA also drastically reduced or eliminated the biological activities of the insertion mutant FhuAs (23). We conclude that residues 69, 82, 128, and 135 of FhuA are nonpermissive for C3 epitope insertion. This result and those from other studies (7, 23) suggest that insertions into this region of the N terminus of FhuA disrupted a segment which is required for FhuA to fold in an active conformation.

Anti-C3 Abs identified FhuA321.C3, FhuA405.C3, and FhuA417.C3 on intact cells by flow cytometry. Since these three FhuA.C3 chimeric proteins were all (i) localized in the outer membrane, (ii) bound by MAbs recognizing conformational determinants of FhuA, and (iii) biologically active, we conclude that amino acids 321, 405, and 417 are surface accessible in wild-type FhuA. A model of FhuA organization in the outer membrane was constructed by Koebnik and Braun (23) on the basis of insertion mutants of FhuA which demonstrated susceptibility to proteolytic cleavage. Our approach of

C3 epitope insertion followed by direct identification of the epitope by using C3-specific Abs serves to confirm independently the surface exposure of residues 321, 405, and 417 of FhuA. In addition, our use of flow cytometry to evaluate accessibility of the C3 epitope at the cell surface ensured that only intact bacteria, as opposed to lysed cells, were analyzed. Residue 321 of FhuA was postulated (23) to be in a large surface-exposed loop which includes residues 315 to 356. The accessibility of this proposed loop of FhuA at the cell surface is also supported by observations that (i) deletion of residues 322 to 355 of FhuA transformed the receptor from a ligand-specific active transporter into a nonspecific passive diffusion channel (20) and (ii) deletion of Asp-348 of FhuA severely impaired the ligand responsiveness of FhuA (21).

Our flow cytometric analyses with anti-C3 Abs indicated that the C3 epitope in the remaining 13 of 16 FhuA.C3 proteins is not accessible at the cell surface. This was surprising, since according to the topological model of FhuA in the outer membrane (23), 10 of 16 of the FhuA.C3 chimeric proteins should present the C3 epitope at the cell surface. There are at least three possible explanations for this apparent discrepancy. (i) The site of C3 insertion may lie within a transmembrane strand of FhuA such that the epitope is inaccessible. This possibility is improbable for the 12 permissive C3 insertions within FhuA, since the splicing of 13 amino acids into a transmembrane segment would likely disrupt the gross topology and therefore the activity of the chimeric protein. For the four nonpermissive C3 insertions, FhuA069.C3, FhuA082.C3, FhuA128.C3, and FhuA135.C3, the C3 epitope may be inserted into a membrane-spanning region of FhuA. However, these proteins were identified in the outer membrane with the anti-FhuA MAbs Fhu3.1 and Fhu4.1 and must therefore be folded in a conformation that matches the conformation of wild-type FhuA, at least in the regions which display the MAb determinants. It is possible that the disruption of FhuA folding caused by C3 epitope insertion at amino acid 69, 82, 128, or 135 of FhuA is localized, as opposed to extending throughout the protein. Long-range disruptions of protein structure would probably result in loss of MAb binding to conformational epitopes of FhuA. Ideally, analysis of protein conformation would use many MAbs, each of which recognizes a conformational determinant located in a different region of the protein.

(ii) The site of C3 insertion may lie within a loop of FhuA exposed to the periplasm. To address this possibility, we developed an ELISA in which anti-FhuA and anti-C3 Abs were used to probe outer membrane vesicles from SG303 *fhuA* expressing FhuA.C3 proteins. The ELISA data confirmed the accessibility of the C3 epitope in FhuA321.C3, FhuA405.C3, and FhuA417.C3. However, the data did not indicate C3 exposure to the periplasm for any of the remaining 13 of 16 FhuA.C3 chimeric proteins. Since outer membrane vesicles contain embedded protein in both right-side-out and inside-out orientations, anti-C3 Ab binding to outer membrane vesicles in ELISA but not to intact cells in flow cytometry would have indicated C3 exposure to the periplasm. Based upon the model presented for FhuA topology (23), the C3 epitope in FhuA069.C3, FhuA128.C3, and FhuA223.C3 is expected to be exposed to the periplasm. The lack of anti-C3 Ab reactivity in ELISA with outer membrane vesicles from SG303 *fhuA*(pBB069), SG303 *fhuA*(pBB128), and SG303 *fhuA*(pBB223) does not support the proposed periplasmic exposure of residues 69, 128, and 223.

(iii) The site of C3 insertion may be directed toward the outside of the cell but masked from Ab recognition. At least two mechanisms of steric hindrance of the C3 epitope are possible. First, long extracellular loops of FhuA may assume a conformation which blocks Ab-epitope interaction. This possibility may be addressed by assessing epitope exposure in stable internal deletion mutants (6, 29). Second, the core oligosaccharide of LPS may sterically hinder otherwise accessible epitopes. Successive truncation of LPS from strains containing an intact O antigen (smooth strains) to those containing severely truncated core oligosaccharides (deep rough strains) has been shown to reveal epitopes of outer membrane proteins to MAbs (2, 26, 29). We therefore elected to assess the exposure of C3 epitopes in *rfa* mutant strains of *E. coli* (22, 35) which had been transformed with the pBB series of plasmids. While confirming the exposure of the C3 epitope in FhuA321.C3, FhuA405.C3, and FhuA417.C3, the data did not suggest any further exposure of the remaining 13 of 16 C3 epitopes in these strains. It may be that further truncation of LPS, for example to the inner core, is necessary to reveal masked epitopes.

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