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Topological Analysis of the *Escherichia coli* Ferrichrome-Iron Receptor by Using Monoclonal Antibodies

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Ferrichrome-iron transport in *Escherichia coli* is initiated by the outer membrane receptor FhuA. Thirty-five anti-FhuA monoclonal antibodies (MAbs) were isolated to examine the surface accessibility of FhuA sequences and their contribution to ligand binding. The determinants of 32 of the MAbs were mapped to eight distinct regions in the primary sequence of FhuA by immunoblotting against (i) five internal deletion FhuA proteins and (ii) four FhuA peptides generated by cyanogen bromide cleavage. Two groups of MAbs bound to FhuA in outer membrane vesicles but not to intact cells, indicating that their determinants, located between residues 1 and 20 and 21 and 59, are exposed to the periplasm. One of the 28 strongly immunoblot-reactive MAbs bound to FhuA on intact cells in flow cytometry, indicating that its determinant, located between amino acids 321 and 381, is cell surface exposed. This MAb and four others which in flow cytometry bound to cells expressing FhuA were tested for the ability to block ligand binding. While no MAb inhibited growth promotion by ferrichrome or cell killing by microcin 25, some prevented killing by colicin M and were partially able to inhibit the inactivation of T5 phage. These data provide evidence for spatially distinct ligand binding sites on FhuA. The lack of surface reactivity of most of the immunoblot-reactive MAbs suggests that the majority of FhuA sequences which lie external to the outer membrane may adopt a tightly ordered organization with little accessible linear sequence.

The uptake of ferric siderophores and vitamin B_{12} across the outer membrane (OM) of gram-negative bacteria is driven by a poorly understood energy-coupled mechanism which involves the cytoplasmic membrane-associated proteins TonB, ExbB, and ExbD (reviewed in references 21, 26, 33). TonB-dependent transport systems are characterized by OM receptors with high affinity and substrate specificity for their cognate siderophore or for vitamin B_{12}. There are additional requirements for proteins within the periplasm and in the cytoplasmic membrane to complete the transport process of siderophore or vitamin B_{12}. The *Escherichia coli* OM receptor for ferrichrome-iron is FhuA (M_r, 78,992; 714 amino acids [11]), a protein which also acts as the receptor for phages T1, T5, UC-1, and φ80, for colicin M, and for the peptide antibiotics albomycin and microcin 25. The periplasmic binding protein FhuD (5, 10) and the cytoplasmic membrane-associated proteins FhuB and FhuC are required for internalization of hydroxamate siderophores (reviewed in reference 3). FhuB and FhuC display characteristics of binding protein-dependent ATP-binding cassette transporters (18).

A prerequisite to understanding the mechanism of active transport of ferrichrome-iron is to understand the molecular organization of FhuA within the OM. As with other OM proteins (OMPs), amphiphilic sequences of FhuA are thought to constitute membrane-spanning beta sheets, while intervening sequences (which often display a strongly hydrophilic character) probably form extramembranous loops. Experimental evidence generally supports these structural predictions and has provided some basis on which to model the topological organization of FhuA. Insertions of tetra- to hexadecapeptides at some sites within FhuA induced susceptibility to exogenously added proteases (28). Differential protease susceptibility of the insertion mutant FhuAs in whole cells as opposed to spheroplasts led to a prediction of the transmembrane arrangement of FhuA (28). Cells expressing FhuAΔAsp-348 demonstrated reduced sensitivity to killing by phage T5 and complete resistance to T1, φ80, and colicin M (23). These data supported the proposed cell surface exposure of a loop containing amino acids 316 to 356 of FhuA. Excision of amino acids 322 to 355 from FhuA transformed the ferrichrome-specific active transporter into a protein which displayed characteristics of a TonB-independent channel: cells expressing FhuAΔ322–355 became sensitive to bacitracin and sodium dodecyl sulfate (SDS) and formed stable conductance channels in black lipid membranes (22). It was proposed that the transmembrane strands of FhuA assume the conformation of a large beta barrel, with the predicted loop of amino acids 316 to 356 forming a "gate" that somehow regulates ferrichrome transport through the channel (4, 22). Recently, Killmann et al. (24) identified amino acids within the proposed gating loop of FhuA which contribute to the binding of phages T1, T5, and φ80 and of colicin M. Preincubation of phages or colicin M with selected synthetic hexapeptides representing sequences between amino acids 316 and 356 of FhuA reduced the efficiency of plating of the lethal agents by up to 7 orders of magnitude. It was concluded that three regions of the gating loop were involved in the binding of T1, T5, φ80, and colicin M and that the regions probably formed a single binding region for the ligands.

To examine directly the surface accessibility of FhuA sequences which were proposed to face the external milieu, we inserted a foreign antigenic determinant (the C3 epitope of poliovirus) at 16 different positions within FhuA, forming FhuA.C3 (29). Anti-C3 antibodies, when added to intact cells or to OM vesicles containing the FhuA.C3 proteins, bound the C3 epitope when it was inserted at positions 321, 405, and 417 of FhuA. This analysis confirmed the proposed cell surface accessibility of amino acids 321, 405, and 417 of FhuA and

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suggested that other extramembranous sequences might be masked from antibody recognition.

We isolated a library of monoclonal antibodies (MAbs) against FhuA as probes of protein topology. MAbs recognize their determinants with exquisite specificity and are ideal tools with which to deduce topological organization because they bind to short linear sequences or to conformational determinants (31, 38, 41). We produced and characterized 35 FhuA-specific MAbs, mapped their determinants, and measured their abilities to inhibit the interactions between the native FhuA receptor and its cognate ligands.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *E. coli* K-12 strains PL-6 (*aroB thi* λ^r *cir feuB* TuII^{*r} [9]), MC4100 [F-*araD* Δ(*argF-lac*)*U169 rspL thi relA flbB deoC pstF rbsR* (40)], SG303 (MC4100 *aroB* [7]), and AW740 (*hisG4 thr-1 fhuA31 tsx-78* Δ*ompF zcb*::Tn*10* Δ*ompC* [20]) were used as hosts for protein expression. *E. coli* CS2529 (F$^-$ *thr leuB6 proA argE his thi galK lacY1 trpE non mtl xyl ara-14 rfaK2*::ΩKm^r [27]) expresses truncated lipopolysaccharide (LPS) and was used to enhance recognition of FhuA on intact cells by MAbs. Derivatives PL-6*fhuA*, SG303*fhuA*, and CS2529*fhuA* were isolated as resistant to phage T5. To confirm the FhuA$^-$ phenotype of these strains, derivatives were analyzed by immunoblotting and by flow cytometry using anti-FhuA MAbs. Plasmid pGC01 encodes the entire *fhuA* gene of *E. coli* K-12 on a pBR322-based replicon (7). *Bacillus subtilis* IH6140 is a prototrophic, sporulation-deficient derivative of Marburg strain 1A298 from the Bacillus Genetic Stock Center, Ohio State University, Columbus, which secretes reduced amounts of exoproteases. The *B. subtilis* expression vector used was pKTH288 (4.5 kbp). It was derived from pKTH239 (32) by insertion of a 14-nucleotide linker at its *Eco*RI site. *E. coli* strains were grown either in L medium or in M63 minimal glucose medium (40) with the *aroB* supplements phenylalanine, tyrosine, and tryptophan. *B. subtilis* was grown in 2× L medium without NaCl. Where required, the antibiotics ampicillin, kanamycin, and tetracycline were added to final concentrations of 125, 30, and 10 μg/ml, respectively.

Purification of Eco.FhuA. Vesicles containing OMPs were prepared by using Tris-lysozyme-EDTA treatment of cells according to the method of Hantke (15). For purification of *E. coli* FhuA (Eco.FhuA) by ion-exchange chromatography, OMPs from PL-6*fhuA*(pGC01) were solubilized directly by addition of an equal volume of 20 mM Tris-HCl (pH 8.0)–4% Triton X-100–20 mM EDTA (TTE). Following dialysis to remove EDTA, the Triton-soluble extract was treated with BioBeads SM-2 adsorbent (Bio-Rad, Hercules, Calif.) to remove excess Triton X-100. FhuA was purified by fast protein liquid chromatography (FPLC; Pharmacia Biotech, Uppsala, Sweden). A 2-mg sample of solubilized OMPs was made 0.1% in Zwittergent Z-3,14 (Calbiochem, San Diego, Calif.) and applied to an analytical Mono Q HR 5/5 column which had been equilibrated with buffer containing 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, and 0.1% Z-3,14. The salt concentration of the buffer was raised to 1 M NaCl by using a linear gradient. A major peak containing purified FhuA was eluted at 0.6 M NaCl. Coomassie blue staining of pooled, FhuA-containing fractions (Fig. 1A, lane 4) indicated that FhuA was the major protein in the eluate. Minor amounts of protein with an electrophoretic mobility of approximately 38 kDa were also present. In preparation for immunizations, fractions containing Eco.FhuA were pooled and dialyzed against 10 mM Tris-HCl (pH 7.9)–150 mM NaCl–0.1% Z-3,14.

For immunoaffinity purification of FhuA, OM vesicles from AW740(pGC01) were solubilized in 50 mM Tris-HCl (pH 8.0)–6 M urea–2% Triton X-100 for 30 min at room temperature (19). The insoluble fraction was collected by centrifugation, solubilized as described above in TTE, and dialyzed against 10 mM Tris-HCl (pH 8.0)–0.1% Triton X-100 to remove EDTA. An immunoaffinity column was constructed by coupling 3 mg of purified MAb Fhu3.1 (42) to 1 ml of protein A-agarose, using an Affinica Ab Orientation Kit (Schleicher & Schuell, Keene, N.H.). A 1,000-μg sample of Triton X-100-solubilized OMPs was added to 1 ml of Fhu3.1-protein A-agarose, which had been equilibrated against 10 mM Tris-HCl (pH 8.0)–0.1% Triton X-100, and mixed gently in batch format at room temperature for 1 h. The affinity matrix containing bound FhuA was collected by centrifugation and poured into a 5-ml polypropylene column. After extensive washing with equilibration buffer, FhuA was eluted as a single peak, using high-pH buffer (17) containing 100 mM triethylamine (pH 11.5) and 0.05% Triton X-100. Purified FhuA was detected as a single band by Coomassie blue staining (Fig. 1B, lane 4) and also as a single band by more sensitive silver staining of 1,000 ng (data not shown). Immunoblotting of protein samples shown in Fig. 1B with MAb Fhu8.3 confirmed the identity of the purified protein as FhuA (Fig. 1C, lane 4). For immunizations, fractions containing immunoaffinity purified Eco.FhuA were dialyzed against 10 mM Tris-HCl (pH 8.0)–0.05% Triton X-100.

Molecular cloning of *fhuA* in *B. subtilis*. The *fhuA* gene was amplified from plasmid pGC01 by PCR. The first primer, 5′-AAG CTT AAG CTT GCG GTT GAA CCG AAA GAA-3′, corresponds to two tandem *Hin*dIII sites plus codons for the first six amino acids of the mature *fhuA* sequence (11). The second primer, 5′-AAG CTT AAG CTT TTA ATG ATG ATG ATG ATG ATG GAA ACG GAA GGT TGC GGT-3′, corresponds to two tandem *Hin*dIII sites, the termination codon, six consecutive histidine codons, and codons for the penultimate six amino acids of FhuA. Reaction conditions for amplification of the recombinant *fhuA* gene were identical to conditions previously described (42). Recombinant DNA work followed established protocols (36). The resulting PCR fragment was trimmed with *Hin*dIII and then ligated at twofold molar excess into the *B. subtilis* expression vector pKTH288 which had been restricted with *Hin*dIII. In the resulting plasmid, codons for the first seven amino acids of α-amylase plus four amino acids of a linker were fused in frame to codons for the 714 residues of the mature form of FhuA plus six histidine codons. Transformation of *B. subtilis* IH6140 and selection of transformants containing recombinant plasmid pGM01 of anticipated size (6.6 kbp) were performed as described previously (42).

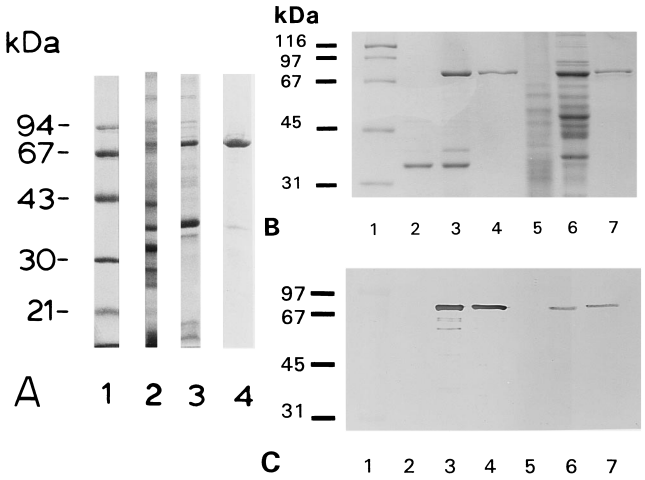

FIG. 1. (A) Purification of Eco.FhuA by ion-exchange chromatography. Lanes: 1, marker proteins with masses; 2, OM vesicles from strain PL-6*fhuA*(pGC01); 3, Triton X-100-solubilized OM vesicles; 4, FPLC-purified Eco.FhuA. (B) Purification of Eco.FhuA by immunoaffinity chromatography and of Bac.FhuA(His)$_6$ by Ni-NTA chromatography. Lanes: 1, marker proteins with masses as indicated; 2, solubilized OM vesicles from *E. coli* AW740; 3, solubilized OM vesicles from AW740(pGC01); 4, immunoaffinity-purified Eco.FhuA; 5, inclusion bodies proteins from *B. subtilis* IH6140(pKTH288); 6, inclusion bodies proteins from IH6140(pGM01); 7, Bac.FhuA(His)$_6$ after Ni-NTA chromatography. Proteins in panels A and B were resolved by SDS-PAGE (10% gels) and stained with Coomassie brilliant blue. (C) Identification of purified proteins. Proteins in panel B were transferred to nitrocellulose after SDS-PAGE and probed with anti-FhuA MAb Fhu8.3. Lane designations are identical to those in panel B.

Purification of Bac.FhuA(His)$_6$. Inclusion bodies containing recombinant FhuA from *B. subtilis* [Bac.FhuA(His)$_6$] were isolated by lysis of protoplasts as described previously (42) and solubilized in denaturing buffer (8 M urea, 100 mM $NaHPO_4$, 10 mM Tris-HCl [pH 8.0]). Inclusion bodies isolated from *B. subtilis* IH6140(pGM01) contained large amounts of recombinant FhuA in addition to a number of other proteins (Fig. 1B, lane 6). Bac.FhuA(His)$_6$ was purified on Ni-nitrilotriacetate (NTA) resin (Qiagen, Chatsworth, Calif.), using the following procedure. Aliquots (10 mg) of solubilized inclusion bodies proteins were loaded onto 1 ml of Ni-NTA resin in an HR 5/5 column (Pharmacia) attached to an FPLC system. The resin was washed extensively with denaturing buffer, and Bac.FhuA(His)$_6$ was eluted at pH 6.0, using a linear pH gradient from pH 8.0 to 4.5. The recombinant protein (Fig. 1B, lanes 6 and 7) exhibited an electrophoretic mobility similar to that observed for Eco.FhuA (Fig. 1B, lanes 3 and 4). Slight differences in mobilities of FhuA protein produced in *B. subtilis* are related to amounts of protein loaded and not to differences in molecular mass. These differences extend also to the immunoreactivities of wild-type and recombinant FhuA. The identity of Bac.FhuA(His)$_6$ was confirmed by immunoblotting with anti-FhuA MAb Fhu8.3 (Fig. 1C, lane 7). The slightly weaker staining of Bac.FhuA(His)$_6$ may also reflect the presence of its 11-amino-acid N-terminal extension which is adjacent to the determinant of MAb Fhu8.3, located between residues 1 and 20 of mature Eco.FhuA. For immunizations, fractions containing purified Bac.FhuA(His)$_6$ were pooled, and the protein was precipitated with 2 volumes of absolute ethanol. The Bac.FhuA(His)$_6$ pellet was solubilized in 1% SDS, diluted to 0.1% SDS with sterile saline, and then boiled for 5 min to ensure complete denaturation of the sample.

Anti-FhuA hybridomas. Purified Eco.FhuA or Bac.FhuA(His)$_6$ was combined 1:1 with either Freund's complete adjuvant or Hunter's TiterMax adjuvant (CytRX Corporation, Norcross, Ga.). Female BALB/c mice were injected intraperitoneally with 20 μg of FhuA in adjuvant. Handling and care of experimental

mice conformed to the guidelines of the Canadian Council on Animal Care. Immune sera were collected after 14 days and tested for anti-FhuA antibodies by immunoblotting, enzyme-linked immunosorbent assay (ELISA), and flow cytometry. Mice were boosted intravenously with 5 μg of antigen in saline 4 days prior to fusion. Mice were splenectomized, and 5×10^7 spleen cells were combined 1:1 with the myeloma fusion partner Sp2/o according to the method of Galfrè and Milstein (14). Fused cells at a concentration of 5×10^5/ml were distributed into 96-well plates already containing a feeder layer of 10^5 murine macrophages.

Hybridoma screening. Hybridoma supernatants were screened by either (i) ELISA against OM vesicles of SG303*fhuA* and SG303*fhuA*(pGC01) (29), (ii) flow cytometry against intact cells of strains CS2529*fhuA* and CS2529*fhuA* (pGC01) (29), or (iii) dot blotting against denatured Bac.FhuA$(His)_6$. For dot blotting, inclusion bodies from *B. subtilis* IH6140(pKTH288) or IH6140(pGM01) were solubilized in urea and aspirated onto nitrocellulose, using a Milliblot-D apparatus (Millipore, Bedford, Mass.). The nitrocellulose was blocked with 1% bovine serum albumin in Tris-buffered saline (10 mM Tris-HCl [pH 7.4], 150 mM NaCl); then supernatants were added, and the mixture was incubated 45 min at room temperature. The blot was washed, and an anti-mouse kappa light-chain MAb (MAb 187.1 [45]) conjugated to alkaline phosphatase was added. The dot blot was developed with 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium. Positive clones were expanded and subcloned by limiting dilution. The isotypes of the MAbs were determined in an ELISA using alkaline phosphatase-conjugated anti-isotype-specific antibodies (Southern Biotechnology Associates, Birmingham, Ala.). MAbs Fhu3.1, Fhu3.3, Fhu4.1, Fhu7.2, and Fhu8.1 were purified from tissue culture supernatants, using a 187.1 affinity column. For immunoblotting, affinity-purified MAbs were diluted to between 0.05 and 1 μg/ml; for ELISA, flow cytometry, and MAb-ligand competition assays, the MAbs were used at concentrations of 1 to 5 μg/ml. MAbs in tissue culture supernatants were diluted 1:20 to 1:500 for immunoblotting and between 1:2 and 1:10 for ELISA and flow cytometry.

Analytical procedures. SDS-polyacrylamide gel electrophoresis (PAGE), immunoblotting, ELISA, and flow cytometry were performed as described previously (29). All ELISAs were performed in triplicate, with minimal variation in A_{405} readings from experiment to experiment.

Cleavage of FhuA by CNBr. Immunoaffinity-purified Eco.FhuA (20 μg) was solubilized in 25 μl of 70% formic acid. CNBr (1250 μg) was added to the reaction vial and incubated in the dark for 18 h at room temperature. After completion of the reaction, the CNBr and formic acid were removed by dilution with water followed by vacuum concentration. Protein pellets were resuspended in electrophoresis sample buffer, boiled for 5 min, and resolved on SDS–15% polyacrylamide gels. Cleavage products were either visualized by silver staining (30), transferred to nitrocellulose membranes, and detected with anti-FhuA MAbs or transferred to polyvinylidene difluoride membranes for N-terminal sequence analysis (Sheldon Biotechnology Centre, McGill University).

Ligand binding and MAb competition assays. To assess the ability of the MAbs to interfere with phage T5 binding activity to FhuA, a modification of the T5 phage inactivation assay (6) was used. A total of 100 ng of solubilized OMPs from SG303*fhuA*(pGC01) in octylglucoside-EDTA buffer (50 mM Tris-HCl [pH 7.8], 1 mM EDTA, 1% octylglucoside) was diluted to 100 μl with sterile water. Increasing amounts of MAbs (range, 0.05 to 5 μg) were added to the solubilized FhuA sample and incubated for 30 min at 37°C. Phage T5 (1,000 PFU) was added, and the mixture was incubated for a further 30 min. The mixture was diluted to 1 ml with sterile Tris-buffered saline, and then 100 μl was mixed with the indicator strain SG303*fhuA*(pGC01) and plated in triplicate. The ability of each of the MAbs to inhibit T5 phage inactivation was assessed by comparing the residual PFU against a control sample to which no MAb was added.

To determine if the MAbs could protect *E. coli* MC4100 cells from killing by colicin M or by microcin 25, we used a protocol based on that described by Murphy et al. (31). Briefly, 100 μl of MC4100 cells at a concentration of 10^4/ml was incubated with dilutions of MAbs, and a dilution of colicin M or of microcin 25 which killed greater than 90% of the cells within 30 min was added. After 30 min of incubation at 37°C, the number of surviving cells was determined by plating the mixture onto L medium in triplicate. The percentage of surviving cells was then calculated by determining the ratio of colonies in the presence of colicin M or microcin 25 divided by the number of colonies in the absence of the lethal agents.

To assess whether the MAbs could prevent ferrichrome uptake, a siderophore nutrition assay was used. A total of 10^7 cells of the *aroB* strain SG303 which had been cultured in nutrient broth plus 40 μM ethlyenediamine di(*o*-hydroxyphenylacetic acid) (NB/EDDA) were mixed with dilutions of MAbs in phosphate-buffered saline and incubated for 30 min at room temperature. Cells were mixed with 3 ml of molten NB/EDDA top agar and poured onto NB/EDDA plates. A sterile filter paper disc was placed on the agar, and 3 μl of a dilute ferrichrome solution (range, 0.01 to 1 mM) was dropped onto the disc. The presence or absence of a growth halo around each disc was assessed after 5 and 8 h. The control MAb for all MAb-ligand competition assays was GM.E5, a surface-reactive, non-FhuA-specific MAb which was isolated during the course of this study.

RESULTS

Generation and characterization of anti-FhuA MAbs. When the prefusion screens on mouse immune sera indicated maximum anti-FhuA reactivity, mice were boosted and splenocytes were isolated for fusion. The supernatants of hybridomas generated by using Eco.FhuA as the immunogen were screened for anti-FhuA specificity by (i) ELISA against OM vesicles from *E. coli* SG303*fhuA* and SG303*fhuA*(pGC01) or (ii) flow cytometry against both CS2529*fhuA* and CS2529*fhuA*(pGC01). Of the nine MAbs isolated by using Eco.FhuA as the immunogen, Fhu3.1, Fhu3.3, Fhu8.1, Fhu8.2, and Fhu8.6 recognized conformational determinants, since their ability to bind FhuA was diminished or abolished upon denaturation with heat and SDS. Considering that the immunogen used was purified under mild conditions in the presence of nonionic detergents, the isolation of MAbs which recognized conformational determinants of FhuA was to be expected. In contrast, the determinants bound by MAbs Fhu4.1, Fhu8.3, Fhu8.4, and Fhu8.5 are proposed to be linear, since denaturation of FhuA did not diminish their reactivity in immunoblotting. FhuA produced in *B. subtilis* [Bac.FhuA$(His)_6$] was a potent immunogen as assessed by dot blotting of immune sera against the denatured recombinant protein. Twenty-six hybridomas were raised by using Bac.FhuA$(His)_6$. The majority of these MAbs reacted strongly in immunoblotting, a characteristic which correlates with the denatured form of antigen used as the immunogen. Surprisingly, MAbs Fhu7.2 and Fhu7.6 did not react with denatured FhuA but instead bound to FhuA in OM vesicles by ELISA, suggesting that they recognize conformational determinants. We propose that the determinants of these two MAbs are formed by polypeptides which have strong tendencies to adopt a secondary structure present in the native protein.

A library of 35 anti-FhuA MAbs was assembled from eight different fusions, and their isotypes were determined (Table 1). All MAbs were of kappa light-chain isotype, since each was detected by the anti-mouse kappa light-chain MAb 187.1 (45). All MAbs against Eco.FhuA demonstrated FhuA-specific reactivity in ELISA against OM vesicles of SG303*fhuA*(pGC01) (Table 1). Anti-FhuA MAbs Fhu4.1, Fhu8.3, Fhu8.4, and Fhu8.5 were also strongly reactive in immunoblotting against OM vesicles containing cloned FhuA but not against vesicles devoid of FhuA (Table 1).

Of the 26 MAbs raised using Bac.FhuA$(His)_6$ as the immunogen, 24 demonstrated FhuA-specific reactivity in both (i) dot blotting using denatured inclusion bodies proteins from *B. subtilis* IH6140(pGM01) and (ii) immunoblotting against OM vesicles containing FhuA expressed from plasmid pGC01 (Table 1). The two anti-Bac.FhuA$(His)_6$ MAbs which did not react in immunoblotting, Fhu7.2 and Fhu7.6, bound specifically to FhuA in OM vesicles as assessed by ELISA.

Our observation that 30 of 35 MAbs did not bind to intact cells in flow cytometry (see below) suggested that their determinants may be located within loops exposed to the periplasm or in transmembrane segments of FhuA. We examined whether those MAbs recognized periplasmically exposed determinants by ELISA against OM vesicles containing FhuA. As expected, all MAbs which bound intact cells as measured by flow cytometry also reacted strongly in ELISA. Strikingly, all MAbs whose determinants mapped between amino acids 1 and 20 and between amino acids 21 and 59 (see below) bound to FhuA in OM vesicles (Table 1). This result suggests that the determinants of these MAbs are exposed to the periplasm in native FhuA. Strong reactivity against FhuA in OM vesicles but not against intact cells was also demonstrated by MAbs Fhu6.3 and Fhu6.5, which bound to determinants within amino

TABLE 1. Anti-FhuA MAb characteristics

MAb	Antigen[a]	Isotype	MAb reactivity				Determinant[d]
			ELISA		Immunoblot reactivity[b]	Flow cytometry[c]	
			OM[e]	Cells[f]			
Fhu3.1	EF	IgG2b[g]	+++	++++	w	+	417–550
Fhu3.3	EF	IgG2b	+++	++++	w	+	417–550
Fhu4.1	EF	IgG1	+++	+++	+	+	321–381
Fhu5.1	BF	IgG3	+++	−	+	−	21–59
Fhu6.1	BF	IgM	−	−	+	−	241–281
Fhu6.2	BF	IgG1	++++	−	+	−	21–59
Fhu6.3	BF	IgM	++	−	+	−	383–417
Fhu6.4	BF	IgG1	−	−	+	−	417–550
Fhu6.5	BF	IgG1	++	−	+	−	417–550
Fhu6.6	BF	IgG1	−	−	+	−	417–550
Fhu6.7	BF	IgG1	−	−	+	−	417–550
Fhu6.8	BF	IgG1	+	−	+	−	321
Fhu6.9	BF	IgG1	+	−	+	−	417–550
Fhu6.10	BF	IgG1	+	−	+	−	417–550
Fhu6.11	BF	IgG1	+	−	+	−	417–550
Fhu6.12	BF	IgG1	−	−	+	−	417–550
Fhu6.13	BF	IgM	+	−	+	−	417–550
Fhu6.14	BF	IgG1	+++	−	+	−	21–59
Fhu6.15	BF	IgG1	+	−	+	−	417–550
Fhu6.16	BF	IgG1	−	−	+	−	417–550
Fhu6.19	BF	IgG1	+	−	+	−	417–550
Fhu7.1	BF	IgG2b	+	−	+	−	21–59
Fhu7.2	BF	IgM	+++	+++	−	+	ND
Fhu7.3	BF	IgM	−	−	+	−	151–199
Fhu7.5	BF	IgG1	+	−	+	−	21–59
Fhu7.6	BF	IgG1	+	−	−	−	ND
Fhu7.10	BF	IgG1	++	−	+	−	21–59
Fhu7.11	BF	IgG1	+	−	+	−	321
Fhu7.12	BF	IgG1	−	−	+	−	417–550
Fhu8.1	EF	IgA	++++	++++	w	+	417–550
Fhu8.2	EF	IgM	+	−	w	−	417–550
Fhu8.3	EF	IgG2a	++++	−	+	−	1–20
Fhu8.4	EF	IgG1	+++	−	+	−	1–20
Fhu8.5	EF	IgG3	+++	+	+	−	1–20
Fhu8.6	EF	IgM	+	−	w	−	ND

[a] The antigen used for immunization was either Eco.FhuA (EF) or Bac.FhuA(His)$_6$ (BF).
[b] +, strongly positive; w, weakly positive; −, no reaction.
[c] Binding of MAbs to CS2529*fhuA*(pGC01) cells in flow cytometry.
[d] Amino acids required for MAb binding as determined by immunoblot analysis against five internal deletion mutant FhuAs and against four CNBr fragments of FhuA. ND, the determinant could not be mapped onto the primary sequence of FhuA because the MAb was not reactive in immunoblotting.
[e] ELISA on OM vesicles. Values are ratios of A_{405} readings for SG303*fhuA*(pGC01) OM vesicles divided by the readings for SG303*fhuA* OM vesicles. Ratios are presented as categories of values: <5, −; 5 to 20, +; 20 to 80, ++; 80 to 320, +++; >320, ++++.
[f] ELISA on whole cells. Values are ratios of A_{405} readings for SG303*fhuA*(pGC01) cells divided by the readings for SG303*fhuA* cells. Ratios are presented as categories of values: <4, −; 4 to 8, +; 8 to 16, ++; 16 to 32, +++; >32, ++++.
[g] IgG2b, immunoglobulin G2b.

acids 383 to 417 and 417 to 550, respectively. To eliminate the possibility that the positive ELISA reactions were due to exposure of otherwise buried transmembranous determinants upon binding of antigen to the microtiter plate, we repeated the ELISA with intact SG303*fhuA* and SG303*fhuA*(pGC01) cells. Since only the cell surface-reactive MAbs bound strongly to intact cells (Table 1), we concluded that in ELISA, the receptor retained its native structure within OM vesicles. This assay also confirmed the surface reactivities of the MAbs as determined by flow cytometry. The ability to bind FhuA in OM vesicles was not a characteristic of all MAbs. This feature indicated that some regions of the receptor, likely transmembrane beta strands, remained inaccessible to MAb binding.

Mapping of MAb determinants on FhuA. Two approaches were used to localize the determinants recognized by each of the anti-FhuA MAbs which were reactive in immunoblotting. MAbs were first tested by immunoblotting for their reactivities with a set of five mutant FhuA proteins carrying the following internal deletions: amino acids 21 to 128, 60 to 135, 129 to 241, 199 to 321, and 322 to 417 (6). For each deletion mutant, the amount of full-length FhuA was normalized by using an anti-N-terminal MAb (Fhu8.3), thereby accounting for differences of each mutant protein in level of expression and sensitivity to proteolytic degradation. OM vesicles devoid of FhuA and OM vesicles containing wild-type FhuA (expressed from the multicopy plasmid pGC01) were used as controls for the specificity of each MAb. An example of the immunoblotting strategy used to localize the anti-FhuA MAb determinants is depicted in Fig. 2. In this example, both MAbs Fhu8.3 (Fig. 2A) and Fhu6.16 (Fig. 2D) reacted with wild-type FhuA and with all five deletion FhuA proteins. On the basis of (i) the susceptibility of the deletion mutant proteins to N-terminal proteolysis (6) and (ii) the immunoreactivity (data not shown) of a previously isolated anti-N-terminal MAb (4AA-1 [12]) compared with that of an

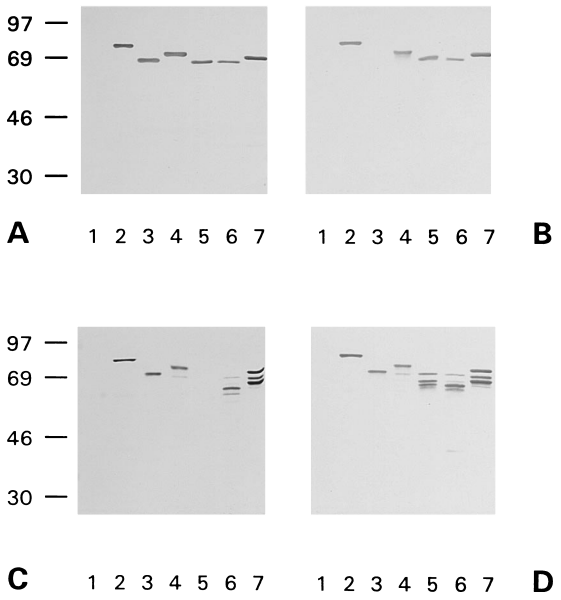

FIG. 2. Identification of MAb determinants by using deletion FhuAs. OMPs from strain SG303*fhuA* (lane 1) or from SG303*fhuA* containing high-copy-number plasmids encoding wild-type FhuA (lane 2) or internal deletion FhuAs (lane 3, FhuΔ021–128; lane 4, FhuΔ060–135; lane 5, FhuΔ129–241; lane 6, FhuΔ199–321; lane 7, FhuΔ322–417) were subjected to SDS-PAGE, transferred to nitrocellulose, and then probed with anti-FhuA MAb Fhu8.3 (A), Fhu5.1 (B), Fhu7.3 (C), or Fhu6.16 (D). Sizes are indicated in kilodaltons.

anti-C-terminal polyclonal antibody (specific for a peptide corresponding to Gly-636 to Lys-651 of FhuA [7]), the pattern of reactivity of MAb Fhu8.3 indicates that its determinant is located between amino acids 1 and 20, while that of MAb Fhu6.16 is located C-terminal to amino acid 417.

Probing the set of five FhuA deletion mutant proteins with MAb Fhu5.1 (Fig. 2B) showed that this MAb did not react with FhuAΔ021–128. Therefore, the determinant recognized by Fhu5.1 must be contained within amino acids 21 to 128 of FhuA. However, since Fhu5.1 bound FhuAΔ060–135, its determinant could be further delimited to amino acids 21 to 59 of FhuA. Using similar reasoning, the determinant for MAb Fhu7.3 (Fig. 2C) was shown to lie between amino acids 136 and 198. Reactivity with FhuAΔ060–135 defined amino acid 136 as the N-terminal boundary of the region containing the determinant for Fhu7.3, while reactivity with FhuAΔ199–321 set its C-terminal boundary to amino acid 198.

In summary, immunoblot analysis using the internally deleted FhuA proteins revealed six distinct immunoreactive regions of FhuA: amino acids 1 to 20, 21 to 59, 136 to 198, 241 to 321, and 417 to 714 and a region which includes residues from both regions 241 to 321 and 322 to 417, that is, a region centered around amino acid 321.

A second, complementary approach was used to delineate further the determinants recognized by all of the MAbs in our library. Each was tested for its reactivity in immunoblotting against four prominent CNBr cleavage fragments of FhuA, designated f1, f2, f3, and f4. The identities of these four immunoreactive fragments were confirmed by submitting each fragment to microsequencing by Edman degradation. Ten amino acids at the N terminus of each fragment were matched to the FhuA sequence (11). The CNBr digestion of Eco.FhuA was thus demonstrated to be incomplete because f1 also contained internal methionine residues which were not readily cleaved under the reaction conditions used. f2, f3, and f4 contained no internal methionine residues. The region of primary sequence recognized by every anti-FhuA MAb, originally identified with deletion FhuAs, was confirmed by MAb reactivity against either f1, f2, f3, or f4. In some cases, boundaries of reactivity were narrowed. Data are summarized graphically in Fig. 3.

Cell surface-reactive MAbs. Further characterization revealed that MAbs Fhu3.1, Fhu3.3, Fhu4.1, and Fhu8.1 bound to SG303*fhuA*(pGC01) cells but not to SG303*fhuA* cells in flow cytometry, indicating that they recognize determinants which are surface accessible in K-12 strains of *E. coli*. The mean fluorescence intensity on SG303*fhuA*(pGC01) cells with each of these four MAbs was 40- to 100-fold greater than on cells of the negative control strain SG303*fhuA*. To identify epitopes which may have been masked from MAb recognition by LPS, we evaluated MAb binding to CS2529*fhuA* and to CS2529*fhuA* (pGC01) cells which express truncated LPS (27). A fifth surface-reactive MAb, Fhu7.2, was characterized by this analysis: Fhu7.2 demonstrated very weak binding to SG303*fhuA*(pGC01) cells, yet it recognized CS2529*fhuA*(pGC01) cells with a mean

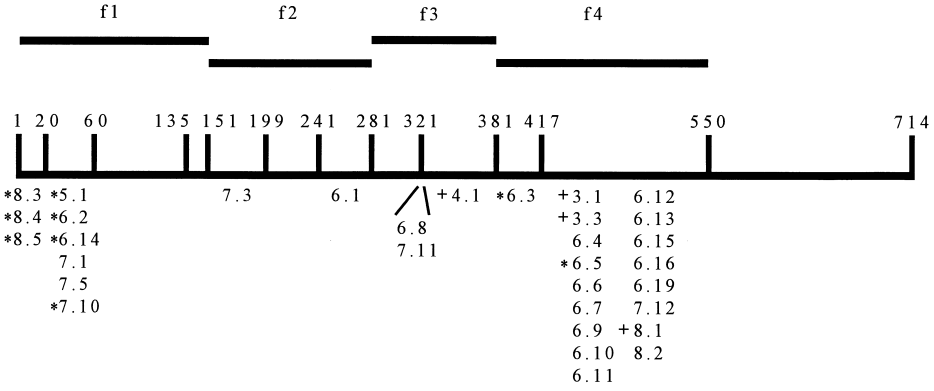

FIG. 3. Identification of MAb determinants on FhuA. Data from immunoblot analysis of anti-FhuA MAbs by using deletion FhuAs or FhuA polypeptides from CNBr cleavage (f1 to f4) were compiled into a linear map of MAb determinants superimposed on a line representing the 714 amino acids in the mature FhuA protein. MAbs (without their "Fhu" prefixes) are grouped underneath. Vertical bars are landmarks in the FhuA primary sequence from the five internal deletion FhuA proteins and from the endpoints of CNBr fragments f1 to f4, as determined by N-terminal sequence analysis. *, the MAb bound very strongly to FhuA in OM vesicles of strain SG303*fhuA*(pGC01) but not to OM vesicles of SG303*fhuA* as measured by ELISA; +, the MAb (i) bound specifically to FhuA on intact cells of strain CS2529*fhuA*(pGC01) as measured by flow cytometry and (ii) bound strongly to OM vesicles of strain SG303*fhuA*(pGC01) in ELISA.

TABLE 2. Inhibition of colicin M killing and of T5 phage inactivation by anti-FhuA MAbs

MAb	Inhibition[a]			
	Colicin M	T5 phage	Ferrichrome	Microcin 25
Fhu3.1	++++	++	−	−
Fhu3.3	++++	++	−	−
Fhu4.1	−	−	−	−
Fhu7.2	−	++	−	−
Fhu8.1	−	−	−	−
Fhu8.3	−	−	−	−
GM.E5[b]	−	−	−	−

[a] The abilities of the MAbs to inhibit (i) the killing of MC4100 cells by colicin M or by microcin 25, (ii) the inactivation T5 phage, or (iii) growth promotion by ferrichrome as described in Materials and Methods were scored as follows: 0 to 20%, −; 20 to 40%, +; 40 to 60%, ++; 60 to 80%, +++; 81 to 100%, ++++.

[b] A non-FhuA-specific, surface-reactive MAb used as a negative control for inhibition of FhuA-specific ligand binding.

fluorescence intensity which was consistently 5- to 10-fold greater.

Using flow cytometry, we addressed the possibility that the cell surface-reactive MAbs which bind conformational determinants of FhuA (MAbs Fhu3.1, Fhu3.3, Fhu7.2, and Fhu8.1) bind to an FhuA-LPS complex instead of to FhuA alone. Strong flow cytometric reactivities were observed for these MAbs not only with *E. coli* CS2529*fhuA*(pGC01), of genotype *rfaK2*, but also with four other *E. coli* strains which express successively deeper truncations in LPS. Strains CS2775, CS2774, CS2175, and CS2429 have been genetically characterized as having *rfaS*, *rfaQ*, *rfaB*, and *rfaC* mutations (27, 39) and were used as host strains to harbor pGC01 and to overexpress FhuA in amounts comparable to those found in an *E. coli* K-12 background. Because of the equally strong signals of fluorescence intensity against all of these *rfa* strains, we consider it unlikely that the above-listed surface-reactive MAbs react with an FhuA-LPS complex.

MAb-ligand competition. MAbs Fhu3.1 and Fhu3.3 prevented killing of cells by colicin M (Table 2) in a manner which was dependent on the amount of MAb added. MAb Fhu8.1 did not protect cells from colicin M killing, even though its determinant was mapped to within the same region, amino acids 417 to 550, as those of the colicin M-protective MAbs Fhu3.1 and Fhu3.3. Fhu8.1 must therefore bind a determinant which is distinct from Fhu3.1 and Fhu3.3. Neither Fhu4.1, Fhu7.2, nor a surface-reactive control MAb (GM.E5) which is not FhuA specific was able to inhibit the action of colicin M. Such a test confirmed the MAb-specific inhibition by Fhu3.1 and Fhu3.3. None of the surface-reactive MAbs inhibited cell killing by microcin 25 or growth promotion by ferrichrome (Table 2). The inability of Fhu7.2 to inhibit cell killing by colicin M or microcin 25, or growth promotion by ferrichrome, may reflect its weak binding to the K-12 strains (MC4100 and SG303) used in these assays.

We tested the ability of each of the MAbs to prevent irreversible adsorption of T5 phage to FhuA by incubating octylglucoside-solubilized OMPs with MAbs and then counting the residual number of PFUs of T5. MAbs Fhu3.1, Fhu3.3, and Fhu7.2 all inhibited the inactivation of T5 phage by 40 to 60%, while no other MAb was able to inhibit T5 inactivation by more than the background controls (Table 2).

DISCUSSION

In this study, we generated a library of MAbs against the ferrichrome-iron receptor of *E. coli*. The MAbs served as probes of FhuA topology and of FhuA-ligand interactions. We delineated the MAb determinants by immunoblotting and defined eight immunoreactive segments of FhuA. Three regions in particular, comprised of residues 1 to 20, 21 to 59, and 417 to 550, contained the determinants for most (26 of 32) of the MAbs in the immunoblot-reactive panel. Notably, we did not isolate any MAbs with determinants in the C-terminal quarter of the FhuA protein (amino acids 551 to 714). Similarly, of the 29 anti-FepA MAbs characterized in the study of Murphy et al. (31), none with determinants C terminal to amino acid 400 of the 723-residue receptor were isolated.

All MAbs raised against Eco.FhuA reacted by ELISA specifically with FhuA in OM vesicles. Since the antigen in ELISA is predicted to be partially buried in the OM, as is the native receptor in the cell, these MAbs might bind determinants which are located in extramembranous loops. Our flow cytometric analyses on intact cells revealed three distinct surface-accessible epitopes bound by MAbs (i) Fhu4.1, (ii) Fhu3.1, Fhu3.3, and Fhu8.1, and (iii) Fhu7.2. Our study represents the first analysis of surface-accessible FhuA sequences by using MAbs and flow cytometry against cells expressing wild-type FhuA. The determinant recognized by Fhu4.1 lies between amino acids 321 and 381 of FhuA, possibly within the prominent surface-accessible loop proposed to include residues 316 to 356 (28). Hexapeptides corresponding to sequences within this loop were recently shown to inhibit infection of cells by phages T1, T5, and ϕ80 (24). MAb Fhu4.1 was unable to inhibit the inactivation of T5 phage by FhuA. This result refutes the idea that a MAb bound to its surface-located determinant would sterically inhibit the binding of all ligands. Because the determinant recognized by Fhu4.1 is proposed to be linear, it follows that there exists some linear sequence within amino acids 316 to 356 which acts as the determinant for Fhu4.1, despite the requirement for a phage binding region with secondary structure (24). It is possible that the binding of Fhu4.1 to its determinant prevents the inactivation of T5 phage by inducing or preventing a conformational change in FhuA. However, this possibility is unlikely given that Fhu4.1 did not inhibit the killing of cells by colicin M or the growth promotion of cells by ferrichrome. These two processes are thought to occur through a conformational change in FhuA induced by energized TonB (16, 19, 26, 33).

The determinants for MAbs Fhu3.1, Fhu3.3, and Fhu8.1 were mapped between amino acids 417 to 550. Some of these MAbs may bind to a loop which was proposed to include amino acids 404 to 433 (28). We showed that sequences in this region are exposed at the cell surface, given the accessibility of a C3 epitope inserted at either position 405 or position 417 of FhuA to anti-C3 antibodies in flow cytometry (29). Fhu3.1 and Fhu3.3 inhibited the inactivation of T5 phage, indicating that the determinants bound by Fhu3.1 and by Fhu3.3 are in proximity to the T5 phage binding site within amino acids 316 to 356. MAb Fhu8.1 binds a determinant which is also located between amino acids 417 and 550, but which is functionally separable from those of Fhu3.1 and Fhu3.3 on the basis that it is unable to inhibit T5 phage inactivation.

The determinant bound by Fhu7.2 is distinct from those bound by MAbs Fhu3.1, Fhu3.3, Fhu4.1, and Fhu8.1, since it is almost completely masked from flow cytometric recognition in K-12 strains of *E. coli*. This is the first direct demonstration of the role for LPS in the shielding of FhuA determinants. Steric hindrance of OM protein sequences by LPS was also noted for the ferric enterobactin receptor FepA (2, 31, 34) and for the major OMP OmpF (25). The application of flow cytometry to assay MAb reactivities against an OMP on intact cells, K-12 or expressing truncated LPS, is a powerful strategy to define ex-

posed sequences and shielded sequences of the target protein. This technology can therefore be used to analyze surface characteristics of gram-negative bacteria as they apply to immune recognition.

Like Fhu3.1 and Fhu3.3, Fhu7.2 partially inhibited the inactivation of T5 phage, suggesting that its determinant is close to the T5 binding site. We propose that the T5 phage and colicin M binding sites are in proximity to one another, since Fhu3.1 and Fhu3.3 each inhibited the binding of these ligands to FhuA. This view is supported by the study which identified overlapping binding sites for phages and colicin M within the gating loop of amino acids 316 to 356 (24). Like Fhu3.1 and Fhu3.3, an anti-FhuA MAb which was described previously (MAb AJ216 [13]) inhibited the irreversible adsorption of T5 phage to FhuA and prevented cell killing by colicin M. This MAb was unable to inhibit ferrichrome-iron uptake. However, in that study, the MAb determinant on the primary sequence of FhuA was not identified. Our mapping of MAb determinants onto the primary sequence of FhuA allowed us to identify regions of the receptor which play a role in determining the accessibility of surface-exposed sequences to ligands of FhuA.

None of the MAbs within our library prevented cell killing by microcin 25. In examining the microcin 25 phenotypes of our FhuA.C3 insertion mutants (29), we identified a role for residue 20 of FhuA in conferring microcin 25 sensitivity: the *E. coli* strain expressing the C3 epitope at position 20 of FhuA demonstrated wild-type sensitivity to FhuA-specific phages and growth promotion by ferrichrome but was completely resistant to microcin 25. Because Fhu3.1 and Fhu3.3 inhibited the binding of colicin M and T5 to FhuA but did not inhibit cell killing by microcin 25, we conclude that the binding of this peptide antibiotic occurs at a site distinct from that used by colicin M and by T5 phage. Another possibility is that the small molecular mass of microcin 25 (approximately 2,100 Da [35]) allows it to penetrate the MAb-FhuA complex with greater ease than colicin M with a molecular mass of 27 kDa (37).

A striking finding was the strong reactivity of a group of MAbs (Fhu8.3, Fhu8.4, Fhu8.5, Fhu5.1, Fhu6.2, Fhu6.14, and Fhu7.10; Fig. 3) against FhuA in OM vesicles but not against FhuA on intact cells. Given the strong reactivity of these MAbs in immunoblotting, it follows that their determinants are probably linear. Since the determinants of these MAbs mapped to the N terminus of FhuA, we propose that sequences between amino acids 1 and 20 and between amino acids 21 and 59 are accessible from the periplasm. N-terminal sequences of FhuA might be exposed to the periplasm, since the cytoplasmic membrane-anchored TonB protein is thought to physically interact with FhuA and with other TonB-dependent OM receptors through the TonB box located near their N termini. Furthermore, TonB-dependent transport processes were inhibited by a soluble, synthetic TonB box consensus pentapeptide (44). In FhuA, the TonB box includes residues 7 to 11 (11). In the model of FhuA transmembrane arrangement by Koebnik and Braun (28), amino acids 7 to 15 of FhuA are predicted to form the first transmembrane strand of the receptor. Since none of their FhuA mutants carrying tetra- or hexadecapeptide insertions N terminal to amino acid 134 displayed increased sensitivity to proteases (28), their prediction of N-terminal FhuA topology relied heavily on computer-assisted sequence analysis. Our direct analysis of FhuA topology by using MAbs suggests that the TonB box of FhuA is located within a loop accessible from the periplasm and not within the first transmembrane strand of FhuA as proposed previously (28).

MAbs Fhu6.3 and Fhu6.5 bound strongly to FhuA in OM vesicles but not to FhuA on intact cells, suggesting that their determinants (located between amino acids 381 and 417 and amino acids 417 and 550, respectively) are also accessible from the periplasm. Several other immunoblot-positive MAbs bound weakly to FhuA in OM vesicles but not to intact cells in ELISA, again indicating that their determinants may be exposed to the periplasm. While not all of the MAbs which bind weakly to FhuA in OM vesicles necessarily have their determinants exposed in the periplasm, our results may indicate structural differences between TonB-dependent receptors and the porins, whose periplasmic loops are generally quite short. Siderophore and vitamin B_{12} receptors may require additional structural elements that extend into the periplasm, since their active transport mechanism most likely occurs through a conformational change induced by the cytoplasmic membrane-anchored TonB protein. To understand more completely the accessibility of FhuA sequences responsible for MAb binding, further delineation of their determinants is required. MAb epitope mapping using overlapping synthetic hexapeptides corresponding to the entire sequence of *Haemophilus influenzae* type b porin was successfully used to define the cell surface-exposed determinants recognized by anti-porin MAbs (41). Knowledge of the amino acid sequence which comprises the MAb determinant will assist in placement of that peptide with respect to the OM: MAb determinant peptides with a strongly hydrophilic character would likely be exposed to the aqueous periplasm or external milieu, while amphiphilic peptides should belong to beta sheets within the OM. The advantage of MAbs which recognize linear determinants of FhuA (28 of the 35 MAbs in our library) is that their determinants can be mapped with precision to short stretches within the primary sequence.

Given the number of MAbs generated against FhuA, it is likely that some would recognize linear determinants in flow cytometry if extracellular loops of FhuA were freely accessible. We demonstrated a role for LPS in masking a determinant from recognition by MAb Fhu7.2. It is possible that further truncation of LPS will reveal surface-exposed determinants of other MAbs in our library. The paucity of linear FhuA sequences accessible to MAbs from the external milieu may also be explained if extracellular loops of FhuA adopt a packed configuration with a limited number of accessible linear peptides. This feature would explain the low susceptibility of the native receptor to exogenously added proteases and the acquired instability of the loops resulting from insertions of 4 to 16 amino acids (28). The longest loops in the proposed transmembrane organization of FhuA of 41, 38, and 30 amino acids would likely be targets for proteolysis unless they adopted some secondary structure or were folded into the lumen of the proposed pore. Some loops greater than 30 amino acids of the OMPs PhoE (1) and LamB (8) were found to be cleaved by proteases.

Our mapping of MAb determinants to FhuA, coupled with our characterization of the MAb reactivities versus intact cells and against OM vesicles, has provided essential links between primary sequence and structural organization of this polytopic membrane protein.

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J. D. Klena provided LPS-deficient *E. coli* strains.

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