JOURNAL OF BACTERIOLOGY, May 1990, p. 2736–2746
0021-9193/90/052736-11$02.00/0

Vol. 172, No. 5

Surface Topology of the *Escherichia coli* K-12 Ferric Enterobactin Receptor†

CHRISTOPHER K. MURPHY, VALDIS I. KALVE, AND PHILLIP E. KLEBBA*

Department of Microbiology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226

Received 6 October 1989/Accepted 9 February 1990

Monoclonal antibodies (MAb) were raised to the *Escherichia coli* K-12 ferric enterobactin receptor, FepA, and used to identify regions of the polypeptide that are involved in interaction with its ligands ferric enterobactin and colicins B and D. A total of 11 distinct FepA epitopes were identified. The locations of these epitopes within the primary sequence of FepA were mapped by screening MAb against a library of FepA::PhoA fusion proteins, a FepA deletion mutant, and proteolytically modified FepA. These experiments localized the 11 epitopes to seven different regions within the FepA polypeptide, including residues 2 to 24, 27 to 37, 100 to 178, 204 to 227, 258 to 290, 290 to 339, and 382 to 400 of the mature protein. Cell surface-exposed epitopes of FepA were identified and discriminated by cytofluorimetry and by the ability of MAb that recognize them to block the interaction of FepA with its ligands. Seven surface epitopes were defined, including one each in regions 27 to 37, 204 to 227, and 258 to 290 and two each in regions 290 to 339 and 382 to 400. One of these, within region 290 to 339, was recognized by MAb in bacteria containing intact (*rfa*$^+$) lipopolysaccharide (LPS); all other surface epitopes were susceptible to MAb binding only in a strain containing a truncated (*rfaD*) LPS core, suggesting that they are physically shielded by *E. coli* K-12 LPS core sugars. Antibody binding to FepA surface epitopes within region 290 to 339 or 382 to 400 inhibited killing by colicin B or D and the uptake of ferric enterobactin. In addition to the FepA-specific MAb, antibodies that recognized other outer membrane components, including Cir, OmpA, TonA, and LPS, were identified. Immunochemical and biochemical characterization of the surface structures of FepA and analysis of its hydrophobicity and amphilicity were used to generate a model of the ferric enterobactin receptor's transmembrane strands, surface peptides, and ligand-binding domains.

In response to iron deprivation, *Escherichia coli* induces the expression of numerous outer membrane (OM) proteins that function in the transport of ferric siderophores (30). Among these is FepA, which facilitates the uptake of the native *E. coli* siderophore, ferric enterobactin, across the OM. FepA also acts as the cognate OM receptor for colicins B and D (12, 34). Passage of all three molecules through the cell envelope requires the function of TonB and ExbB (14, 34, 47). The specific details of the interaction of FepA with its ligands are unknown, but the colicin- and siderophore-binding domains of the receptor have been separated genetically (23) and by their thermal denaturation properties (16). The physical characteristics of FepA are also unknown, but its predicted structural features (11, 20) indicate that it is a protein dominated by β-sheets, with highly antigenic stretches of hydrophilicity interspersed throughout its sequence. In this sense, FepA resembles several other *E. coli* OM proteins, including OmpA (15, 37), OmpF (10, 27, 38), PhoE (17), and LamB (3, 9, 39). Models of bacterial OM protein structure, which are largely based on analysis of porins (3, 10, 17, 38, 39, 46) and OmpA (37, 46), emphasize the likelihood of membrane-spanning β-strands and -sheets that are perpendicular to the plane of the bilayer. These conclusions result from the study of bacteriophage resistance mutations (9), antibody-binding domains (39), gene fusions (45), and spectrophotometric data (10, 17, 25, 27).

In this study we have used immunochemical analysis of monoclonal antibody (MAb)-binding sites and antibody inhibition of colicin killing and siderophore uptake to identify cell surface-exposed peptides of the ferric enterobactin receptor. Antibodies that recognize FepA surface epitopes fall into three distinct categories: those that block ligand interaction with FepA and bind it in bacteria with a complete lipopolysaccharide (LPS) core; those that inhibit ligand interactions and react with their epitope only if the LPS core structure of the host strain is truncated; and those that recognize their epitope if the LPS core is truncated but nevertheless do not inhibit ligand binding. The ferric enterobactin- and colicin-binding domains of FepA are not discriminated by the antibody binding studies we report herein and in fact seem identical to one another.

MATERIALS AND METHODS

Bacterial strains and media. Bacteria were grown in LB medium (24) or T medium (18). Bacterial strains, plasmids, and phage used in this study are listed in Table 1. KDF29, a CL29 derivative (4), was made *recA* by transduction with a P1 lysate grown on *E. coli* K-12 strain JC10240 (5, 24). Tetracycline-resistant, UV-sensitive transductants were made FepA$^-$ by selection for spontaneous colicin B resistance and subsequently immunoblotted with rabbit anti-FepA serum to confirm the absence of the protein. Phage K3h1, a host range mutant of K3, was used to select KDF101, an OmpA$^-$ mutant of SA8, as described previously (22). Lack of OmpA in this strain was verified by immunoblot with polyclonal anti-OmpA serum. pITS449RV8, an *Eco*RV deletion mutant of pITS449, was constructed by using standard molecular genetic techniques (21). pFP24 was constructed as described previously (26) and encodes a FepA::PhoA fusion protein containing the first 24 mature FepA amino acids.

The level of *fepA* expression in the bacteria was controlled

* Corresponding author.

† This paper is dedicated to the memory of Donald R. Harris, who was involved in many helpful discussions during its preparation.

TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Genotype or phenotype	Source or reference
E. coli strains		
KDL118	*fepA* CC118	26
DM1187(pCLB1)	Colicin B^+	M. A. McIntosh
CA53	Colicin Ia^+	David Brunner
CA23	Colicin D^+	David Brunner
32T19	Colicin M^+	David Brunner
BN1071	*entA* AB1515 derivative	18
AN193	*tonA* BN1071 derivative	18
BN3040	*cir* BN1071 derivative	15
UT2300	*fepA* BN1071 derivative	23
RWB18-60	*recA* RWB18 derivative (*fepA*)	M. A. McIntosh; 48
SA8	*fur* BN3040 derivative	S. K. Armstrong
KDF101	SA8 K3h1 resistant ($OmpA^-$)	This study
CL29	*rfaD* (Re chemotype LPS)	4
KDF29	*recA* CL29 $FepA^-$	This study
JC10240	Hfr PO45 *srl-300*::Tn*10* *recA56*	5
Plasmids		
pITS449	pUC18 $fepA^+$	S. K. Armstrong[a]
pFP2 to 710	pITS449 *fepA*::Tn*phoA*	26
pFP24	pITS449 *fepA*::Tn*phoA*	This study
pITS449RV8	pITS449 *fepA* (204–339)	This study
Phages		
K3h1	Broad-host-range K3 mutant	U. Henning
P1 *recA*	Tet^r *recA*	W. Reznikoff

[a] S. K. Armstrong and M. A. McIntosh, submitted.

by manipulating the concentration of available Fe^{3+} in the media (18). For iron-rich medium, L broth or minimal medium with ferrichrome (10 μM) was used. For iron-deficient culture, L broth with deferriferrichrome A (100 μM) or minimal medium without added iron was used.

Colicins. Colicin B was purified from strain DM 1187(pCLB1), a gift from Mark A. McIntosh. Colicins D and Ia were prepared from crude lysates of CA53 and CA23, respectively, after induction with mitomycin C (35). Colicin M was purified from cultures of 32T19 (34).

Antigen preparation. FepA was prepared in a variety of different forms for immunization and assay. Live bacteria with either wild-type K-12 LPS (BN1071 and its derivatives) or *rfaD* (Re chemotype) LPS (CL29 and its derivatives) were used as a source of the ferric enterobactin receptor in its most native, OM-resident state.

In addition, FepA and other OM proteins were studied in French pressure cell-generated OM vesicles (FPOM [26]), as a sodium dodecyl sulfate (SDS)-solubilized, nondenatured protein (nFepA) and as a partially (95%) purified (16), Triton X-100-extracted complex that contained small quantities of LPS and other OM proteins (TXFepA). Triton X-100 in TXFepA was removed by sequential ethanol precipitations at −20°C. Fully denatured FepA was obtained by boiling the protein in 1% SDS and removing the detergent by acetone precipitation at −20°C (dFepA).

Anti-FepA serum. Polyclonal anti-FepA serum was prepared as described previously (26).

Anti-FepA hybridomas. BALB/C mice were immunized with TXFepA (100 μg) adsorbed to alum or with FPOM (200 μg) on days 1, 7, and 9 and splenectomized on day 10. Cell fusions were performed by the method of Oi and Herzenberg (1, 32). Hybridomas were selected in hypoxanthine-aminopterin-thymidine medium with splenic feeder cells (10^6/ml) and assayed for antigen specificity by enzyme-linked immunosorbent assay (ELISA) against a mixture of TXFepA and dFepA. Positive clones were expanded to 24-well plates and assayed by ELISA and Western blot (immunoblot) at confluence. Cells from positive wells were subcloned twice and preserved at −70°C.

Subcloning. An aliquot of cell suspension (10^6/ml) was diluted 100-fold, and 50 μl was deposited in the wells of the first column on a 96-well microtiter plate that had been filled with 50 μl of hypoxanthine-thymidine medium plus splenic feeder cells. The hybridoma suspension was subjected to 11 twofold dilutions across the columns of the plate, using a Costar octapette. Wide-bore plastic tips (Outpatient Services, Petaluma, Calif.) were used to avoid cell lysis. This procedure was almost always successful in yielding monoclonal hybridoma growth. Subclones were assayed by ELISA after 2 weeks, and positive clones were expanded and evaluated for specificity by immunoblot. Two subclonings were generally sufficient to produce monoclonal hybridomas, which were grown as ascitic tumors and frozen.

Ascitic tumors. Monoclonal anti-FepA hybridomas (10^6 to 10^7 cells) were washed with Hanks balanced salt solution and injected intraperitoneally into mice that had been primed the previous day with 0.5 ml of Freund incomplete adjuvant. Ascitic fluid was removed after 10 to 15 days, clarified by centrifugation, and stored at 4°C with 0.2% sodium azide.

ELISA. Intact bacteria (5×10^7 cells per ml), FPOM (10 μg/ml), TXFepA (10 μg/ml), or dFepA (10 μg/ml) was suspended in 0.01 M ammonium acetate–0.01 M ammonium carbonate (pH 8.2). A 50-μl sample of each suspension was dispensed into the wells of polystyrene microtiter plates (Immulon II; Dynatech), and the volatile salts were evaporated by incubation at 37°C. All subsequent incubations were at 25°C. The wells were blocked with phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) (3%) for 30 min, and the buffer was replaced with 50 μl of PBS-BSA (1%). Hybridoma supernatant (5 μl) was transferred to the assay plate by using a Clonemaster liquid transfer device (Immusine Inc., San Leandro, Calif.) and incubated for 1 h. The wells were washed three times with PBS containing 0.05% Tween 20, and goat anti-mouse immunoglobulin-alkaline phosphatase (GaMIg-AP; 0.1%) was added. After 1 h, the plates were washed as described above, and *p*-nitrophenyl phosphate (1 mg/ml) was added. The reaction was terminated after 1 h by the addition of 50 μl of 1 N NaOH, and the optical density of each well at 410 nm was determined.

Electrophoresis and immunoelectroblots. For denaturing SDS-polyacrylamide gel electrophoresis (PAGE), samples were suspended in SDS-containing sample buffer plus 3% β-mercaptoethanol, heated at 100°C for 5 min, and electrophoresed (1, 26) at room temperature. For nondenaturing SDS-PAGE, samples were incubated in sample buffer at 20°C for 30 min before electrophoresis at 4°C. Western immunoblots were performed as described previously (26). Protein was transferred to nitrocellulose paper, blocked in 50 mM Tris chloride (pH 7.5)–0.5 M NaCl (TBS) plus 1% gelatin, and incubated with ascites fluid (0.5%) in TBS plus 2.5% gelatin for 3 h. The nitrocellulose paper was washed in TBS plus 0.05% Tween 20, incubated with GaMIg-AP for

2 h, washed, and developed in bromochloroindolyl phosphate (2).

Antibody isotypes and concentrations. The immunoglobulin heavy-chain class of each MAb was determined by ELISA. Plates were coated with a mixture of TXFepA (10 μg/ml) and dFepA (10 μg/ml), blocked with 1% BSA for 1 h, and incubated with anti-FepA ascitic fluid (1%) for 1 h. Class-specific GaMIg (Zymed) was added, the preparation was incubated for 1 h, and the assay was developed with rabbit anti-goat AP (Sigma).

Antibody concentrations were also determined by ELISA. Microtiter plates were coated with GaMIg (Fc specific) by incubation with a 0.1% solution of the sera in 0.01 M $NaHPO_4$ (pH 7.5) at 4°C for 2 h. Serial 10-fold dilutions of anti-FepA ascitic fluid were added to the washed plates and incubated for 1 h. The assay was developed with GaMIg-AP–*p*-nitrophenyl phosphate. Monoclonal mouse immunoglobulins of each heavy-chain class, quantitated by protein determination (19), were assayed as standards. For flow cytometry and assays of antibody inhibition of colicin or siderophore binding, MAb were adjusted to approximately 1 μg/ml.

Flow cytometry. Whole cells or OM vesicles were reacted with diluted ascites fluid and fluorescein isothiocyanate (FITC)-conjugated GaMIg as described previously (26), except that vesicles were centrifuged at 11,000 × *g* for 30 min. Stained material was analyzed on a Coulter EPICS V flow cytometer/cell sorter for green fluorescence.

Ferric siderophores. Purified enterobactin (1.5 μmol) was dissolved in 0.5 ml of methanol, and $FeCl_3$ (0.5 μmol) dissolved in 0.4 ml of 0.05 N HCl was added. For preparation of [^{59}Fe]enterobactin, $^{59}FeCl_3$ was used (0.5 μmol; 5.5 mCi/μmol; Dupont, NEN Research Products, Boston, Mass.). The mixture was incubated for 30 min, and 0.1 ml of 0.5 M $NaHPO_4$ (pH 7.0) was added to form the iron complex. The solution was analyzed spectrophotometrically to determine its exact concentration of ferric enterobactin ($\varepsilon_{495}^{mM} = 5.6$) and adjusted to 0.5 mM if necessary by addition of distilled water.

Ferrichrome, purified from *Ustilago sphaerogena* (29), was reconstituted in 0.05 M $NaHPO_4$ (pH 7.0) at 0.5 mM and diluted as necessary. [^{59}Fe]ferrichrome was prepared in the following manner. Ferrichrome (25 mg in 1.25 ml of distilled water) was deferrated by incubation with 1 N NaOH (1.25 ml) for 30 min at 0°C. Insoluble $Fe(OH)_n$ was removed by centrifugation, and the solution of deferriferrichrome was adjusted to neutrality by addition of 1 N HCl at 0°C. [^{59}Fe]ferrichrome was formed by the addition of 1.5 μmol of deferriferrichrome to 0.5 μmol of $^{59}FeCl_3$ in 0.05 N HCl. The solution was analyzed spectrophotometrically ($\varepsilon_{425}^{mM} = 2.9$) to determine its exact concentration of ferrichrome and adjusted to 0.5 mM if necessary by addition of distilled water.

Siderophore uptake experiments. Two methods were used to assess the ability of MAbs to inhibit siderophore uptake. As an initial screen of the antibody test panel, a modification of the siderophore nutrition assay (48) was used. A total of 10^7 cells of the *entA* strain BN1071, grown previously in nutrient broth containing 100 μM deferriferrichrome A, were incubated with 0.01 μg of anti-FepA MAb for 30 min. Then 1 ml of molten nutrient agar containing 100 μM deferriferrichrome A was added, and the cells were plated in 45-mm-diameter dishes. A sterile disk containing 2 μl of 50 μM siderophore (either ferric enterobactin or ferrichrome) was applied to the center of the dish, which was incubated at 37°C. The presence or absence of a growth halo around the disk was scored after 4, 6, and 8 h. To evaluate the specificity of antibody inhibition of siderophore uptake, inhibition of ferrichrome transport was also tested.

To quantitatively assess the ability of anti-FepA MAb to inhibit ferric enterobactin transport, ^{59}Fe-siderophore uptake experiments were performed (23). BN1071 or KDF29 was cultured in L broth and subjected to iron stress at a density of 8×10^7 cells per ml by the addition of 100 μM deferriferrichrome A (18). After 3 h, the bacteria were pelleted by centrifugation and then washed with and suspended in uptake medium (M63 minimal medium [24]) at 4×10^9/ml. Bacteria (10^8) were incubated with ascitic fluid containing individual MAb (10 μg in 25 μl) for 30 min at 0°C; 1 ml of uptake medium was added, and the bacteria were incubated for 10 min at 37°C with shaking (250 rpm). [^{59}Fe]enterobactin or [^{59}Fe]ferrichrome was added to 1 μM, and the bacteria were incubated for 15 min at 37°C with shaking. The cells were diluted to 4 ml with 10 mM EDTA, filtered onto 0.2-μm-pore-size filters, and washed with 10 ml of 0.9% saline. The filters were counted in a Packard gamma scintillation counter.

Colicin binding experiments. Anti-FepA MAb were tested for their ability to block colicin killing by a modification of the method of Guterman (12, 13). BN1071 or its *fepA*, *cir*, or *tonA* derivatives were diluted in L broth to 10^4 cells per ml, and 100 μl was incubated with 0.01 μg of MAb for 15 min. A dilution of appropriate colicin (in L broth) that gave approximately one hit per cell was added and incubated for 15 min. Soft agar (2.5 ml [24]) was added, and the mixture was poured onto LB plates and incubated overnight at 37°C. Colonies were counted, and the number of hits per cell in the presence or absence of the anti-FepA MAb was determined by the relationship $S/S_0 = e^{-k}$, where S_0 is the number of cells plated (colonies in the absence of colicin), S is the number of cells that survive exposure to colicin (colonies in the presence of colicin), and k is the number of hits per cell (12, 13).

RESULTS

Generation and characterization of anti-FepA MAbs. Anti-FepA hybridomas were identified initially by ELISA, using microtiter plates coated with a mixture of TXFepA and dFepA, even though these preparations contained minor amounts of other OM antigens, including TonA, OmpA, Cir, OmpF, and LPS. Positive hybridomas were expanded and immediately assayed by Western immunoblot to determine their specificity. Roughly 80% of the hybridomas isolated in this manner recognized FepA, and the remainder reacted with the other antigens noted above. Western analysis at this stage was essential to the ultimate isolation of monoclonal hybridomas and the understanding of MAb specificity, because several of the initial isolates were not monoclonal and produced multiple different antibodies that recognized TonA, OmpA, OmpF, or LPS in addition to FepA. Each hybridoma was subcloned (usually twice) until monoclonal specificity could be demonstrated by immunoblot.

Hybridomas were grown as ascitic tumors, and ascitic fluids were collected, diluted, and assayed by ELISA and Western blot. A test panel containing 36 antibodies of interest was assembled. MAb in ascitic fluids were characterized with respect to immunoglobulin isotype and concentration (Table 2); the antibodies in the panel were normalized to 0.1 μg/ml for cytofluorimetric studies.

MAb specificity. The panel of 36 MAb was tested by immunoblot for reactivity with FepA::PhoA fusion proteins (Fig. 1). These chimeric proteins, generated previously by

TABLE 2. MAb characteristics

Epitope[a]	MAb	Heavy chain	81K*	FACS[b]		
				SA8wc	KDF29(pITS449)	SA8FPOM
FepA						
2–24	29	G2b	−	−	−	−
27–37a	1	G1	−	−	−	−
27–37b	6	G1	−	−	+	−
27–37a	20	G1	−	−	−	−
27–37a	26	G2b	−	−	−	−
27–37a	47	G2b	−	−	−	−
100–178	2	G2b	+	−	−	−
	3	G2a	+	−	−	−
	4	ND[c]	+	−	−	−
	5	G2b	+	−	−	−
	7	G2b	+	−	−	−
	10	G1	+	−	−	−
	11	G2a	+	−	−	−
	17	G1	+	−	−	−
	27	ND	+	−	−	−
	30	G2b	+	−	−	−
	38	G1	+	−	−	−
	39	ND	+	−	−	−
	41	G3	+	−	−	−
204–227	33	G1	+	−	+	−
258–290a	16	G2b	+	−	−	−
258–290b	34	G1	+	−	+	−
290–339a	31	G1	+	+	+	+
290–339a	35	G3	+	+	+	+
290–339b	37	G2b	+	±	+	±
290–339a	44	G2b	+	+	+	+
290–339a	45	G2b	+	+	+	+
382–400a	23	G2b	+	−	+	−
382–400b	24	G2a	+	−	+	−
OmpA	19	G1	−	−	−	+[d]
Cir	9	G2b	−	−[e]	−	−
TonA	32	G2b	−	−	−	−
	36	G3	−	−	−	−
LPS	46	G2b	−	+[f]	−	+
	53	G1	−	±	+	±

[a] FepA epitopes were defined by MAb by using immunoblot analysis, cytofluorimetry, and ligand inhibition experiments as described in Materials and Methods.
[b] Flow cytometry and fluorescence-activated cell sorting (FACS) were carried out as described previously (26). Values were considered positive if the mean peak fluorescence was significantly higher than for the background or control population (see Fig. 2). Control populations were as follows: for SA8 whole cells (SA8wc), RWB18-60; for KDF29(pITS449), plasmidless KDF29; for SA8FPOM, RWB18-60FPOM.
[c] ND, Not done.
[d] MAb19 was reactive with both SA8FPOM and RWB18-60FPOM by flow cytometry. The negative control population used for this MAb was KDF101FPOM (OmpA$^-$; see Fig. 2D).
[e] MAb9 was reactive with RWB18-60 whole cells (Cir$^+$) but not SA8 (Cir$^-$).
[f] MAb46 was reactive with whole cells of SA8 and RWB18-60 and with FPOM derived from both of these strains. It failed to react with both *rfaD* and *galE* (KDL118) whole cells by flow cytometric analysis (data not shown).

transposition of Tn*phoA* into the *fepA* structural gene of pITS449, contain various lengths of the amino terminus of the FepA protein (26). Since the level of FepA expression and its susceptibility to endogenous proteolysis varied among the mutant strains (26), the amount of full-length mutant protein in each sample was initially estimated by immunoblot with polyclonal anti-PhoA or anti-FepA serum, and sample concentrations were appropriately normalized for further experiments (Fig. 1). Immunoblot analysis of the FepA::PhoA fusion polypeptides revealed seven distinct domains of the receptor (residues 2 to 24, 27 to 56, 100 to 178, 178 to 227, 258 to 290, 290 to 352, and 382 to 400) that were recognized by antibodies within the test panel (Table 2). An example of the immunoblot mapping procedure is shown in Fig. 1 for MAb reactive with regions 2 to 27, 27 to 56, 258 to 290, 290 to 352, and 382 to 400; antibodies that recognized regions 100 to 178 and 178 to 227 were also identified (not shown).

MAb that reacted with regions 178 to 227 and 290 to 352 were assayed by immunoblot against a mutant FepA polypeptide containing the internal, *Eco*RV-generated in-frame deletion of amino acids 204 to 339 to further refine the boundaries of their epitopes. All MAb in both groups were unreactive with the FepA deletion (not shown), indicating that the epitopes recognized by these antibodies reside within regions 204 to 227 and 290 to 339, respectively.

Mature FepA contains four potential OmpT cleavage sites (43), at positions 37, 147, 172, and 680. When FepA was truncated by OmpT (8, 43), antibodies that reacted with epitopes within regions 2 to 24 (MAb 29) and 27 to 56 (MAb 1, 6, 20, 26, and 47) did not bind the resultant 81K* (16) polypeptide (Table 2). MAb that recognized residues within region 100 to 178 or further downstream, on the other hand, bound both 81K* and FepA on immunoblots (Table 2). These results define epitopes that are upstream from or include the OmpT cleavage site within FepA. They are consistent with OmpT cleavage of FepA at position 37.

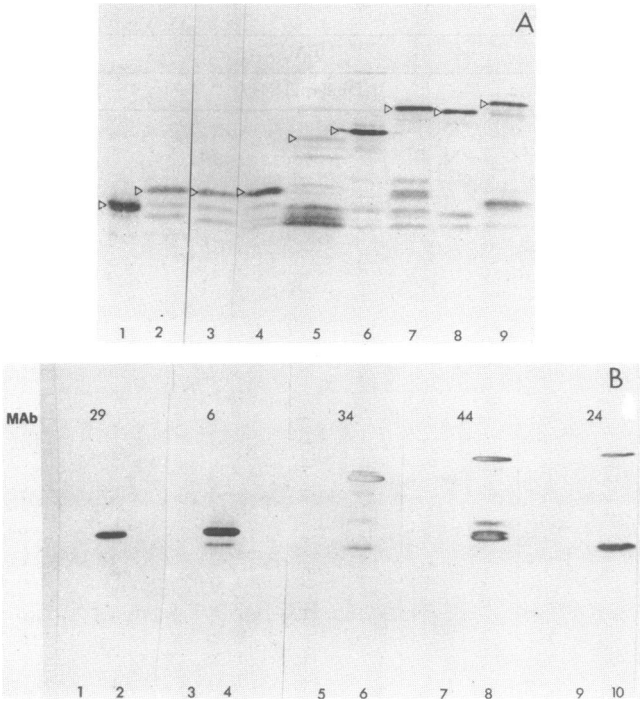


FIG. 1. (A) Quantitation of FepA::PhoA fusion proteins by immunoblot. KDL118 cells containing pFP fusion plasmids (26) were run on SDS-PAGE and immunoblotted, using rabbit anti-AP sera to estimate the amount of full-length FepA::PhoA fusion protein in each sample (indicated by arrowheads). FepA::PhoA fusion proteins from plasmids pFP2 (lane 1), pFP24 (lane 2), pFP27 (lane 3), pFP56 (lane 4), pFP258 (lane 5), pFP290 (lane 6), pFP352 (lane 7), pFP382 (lane 8), and pFP400 (lane 9) were analyzed. (B) Immunoblots of FepA::PhoA fusion proteins with anti-FepA MAb. Whole cells ($\sim 5 \times 10^7$) containing fusion proteins were subjected to SDS-PAGE and then immunoblotted with different MAb, as shown. *E. coli* KDL118 contained plasmids pFP2 (lane 1), pFP24 (lane 2), pFP27 (lane 3), pFP56 (lane 4), pFP258 (lane 5), pFP290 (lanes 6 and 7), pFP352 (lane 8), pFP382 (lane 9), and pFP400 (lane 10).

Additional cleavage at 147, 172, or 680 is inconsistent with the observed molecular mass of 81K* (74 kilodaltons [kDa]).

Other hybridomas were isolated which produced antibodies that reacted with Cir (MAb 9), TonA (MAb 32 and 36) OmpA (MAb 19), and LPS (MAb 46 and 53) (Table 2); these were used as controls in the analysis of anti-FepA MAb surface reactivity and ligand-binding inhibition (see below).

Surface-reactive MAb. To identify MAb that recognized surface-exposed determinants of FepA, the test panel was assayed cytofluorimetrically for reactivity with intact bacteria. Cells of the *rfa*$^+$ strain SA8, the *rfaD* strain KDF29, or their OM protein-deficient or plasmid-containing derivatives (Table 1) were incubated with MAb, stained with FITC-GaMIg, and analyzed for green fluorescence by flow cytometry. FepA expression in these bacteria was maximized by the *fur* mutation of SA8 and growth of KDF29(pITS449) in iron-deficient minimal media. The overexpression of FepA in these strains was confirmed by immunoblot (data not shown).

MAb 31, 35, 44, and 45 bound to SA8 (*fepA*$^+$) cells but not to RWB18-60 (*fepA*), indicating their specificity for outer surface-exposed epitopes of FepA (Table 2; Fig. 2A). These antibodies also exhibited FepA-dependent adsorption to KDF29(pITS449). MAb 37 was similar but distinct in that its binding to FepA in SA8 was only weakly positive and greatly

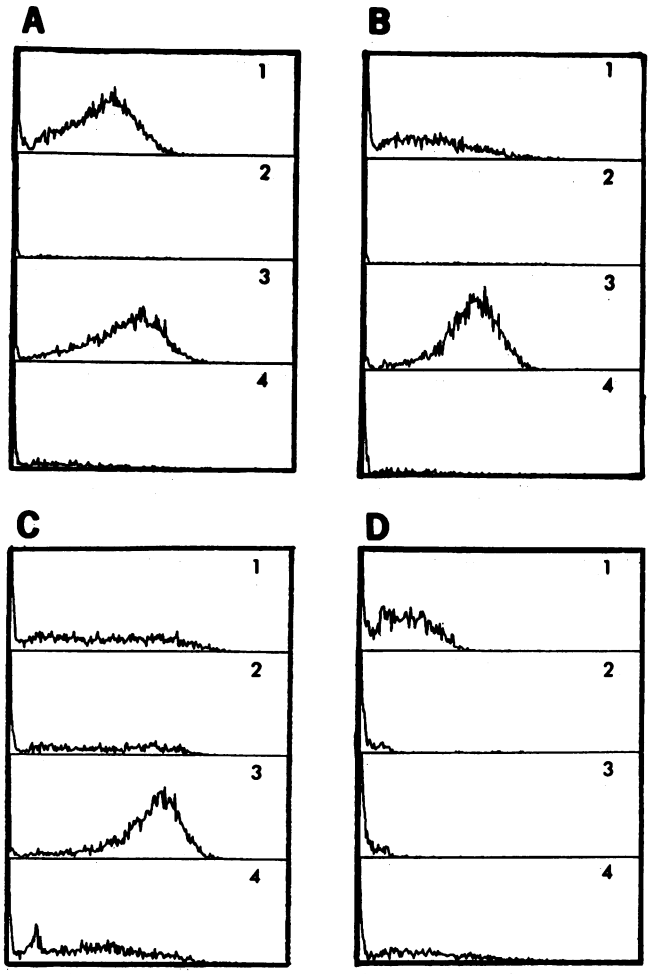


FIG. 2. Fluorescence histograms of cells or vesicles stained with anti-FepA MAb and FITC-GaMIg. (A) Whole cells of SA8 (panel 1), RWB18-60 (panel 2), KDF29(pITS449) (panel 3), or KDF29 (panel 4) were reacted with MAb 44 and GaMIg-FITC and analyzed for green fluorescence on the flow cytometer. (B) Panels are the same as for part A, but MAb 37 was used instead of MAb 44. (C) Panels are the same as in parts A and B, but MAb 6 was used to stain the bacteria. (D) FPOM from SA8 (panel 1) and KDF101 (panel 2) and whole cells of SA8 (panel 3) or KDF29 (panel 4) were reacted with MAb 19 and stained with GaMIg-FITC.

enhanced in the *rfaD* strain (Fig. 2B). MAb 31, 35, 37, 44, and 45 all reacted with FepA within region 290 to 339. Another category of antibodies was found, including MAb 6, 23, 24, 33, and 34, which showed FepA-dependent binding to whole cells of KDF29(pITS449) but not SA8 (Fig. 2C). MAb within this group recognized FepA eiptopes that were shielded from antibody binding in SA8 by the LPS core and became accessible to antibody binding only in its absence. It is relevant that all of the antibodies in this category bound FepA epitopes that were distinct from the residues within region 290 to 339 (Table 2).

The Cir-specific MAb 9 reacted cytofluorimetrically with *cir*$^+$ strains KDF29 and RWB18-60 but not the *cir* strain SA8 (Table 2), indicating that it binds an outer surface epitope of the colicin I receptor. Numerous antibodies were found that did not recognize OM proteins in immunoblots but reacted

TABLE 3. Inhibition of colicin killing and siderophore uptake by anti-FepA MAb

MAb[a]	Inhibition[b]													
	BN1071[c]							CL29[d]						
	FC[e]	Siderophore		Colicin				FC	Siderophore		Colicin			
		E	F	B	D	Ia	M		E	F	B	D	Ia	M
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	+	−	−	−	−	−	−
9	+	−	−	−	−	+++	−	+	−	−	−	−	+++	−
23	−	−	−	−	−	−	−	+	++	−	+++	+	−	−
24	−	−	−	−	−	−	−	+	−	−	−	−	−	−
31	+	++	−	++	+++	−	−	+	++	−	+++	+++	−	−
33	−	−	−	−	−	−	−	+	−	−	−	−	−	−
34	−	−	−	−	−	−	−	+	+	−	+	+	−	−
35	+	++	−	+++	+	−	−	+	+++	−	+++	+++	−	−
37	±	−	−	−	+	−	−	+	−	−	++	+++	−	−
44	+	+	−	++	+	−	−	+	++	−	+++	+++	−	−
45	+	++	−	+++	++	−	−	+	++	−	+++	+++	−	−
46	+	−	−	+	++	+++	++	−	++	++	+	−	−	−
53	−	−	−	+	−	−	−	+	−	−	+++	+++	+++	++
F14[f]	+	−	−	−	−	−	−	+	−	−	+	−	−	−

[a] Hybridomas were grown as ascitic tumors, and ascitic fluid was used at appropriate dilutions (see text).
[b] Inhibition of [^{59}Fe]enterobactin (E) and [^{59}Fe]ferrichrome (F) uptake and inhibition of killing by colicins B, D, Ia, and M were scored as follows: 0 to 25%, −; 26 to 50%, +; 51 to 75%, ++; 75 to 100%, +++.
[c] *E. coli* BN1071 was grown in L broth for colicin assays and in L broth plus 100 μM deferriferrichrome A for siderophore uptake studies; SA8, which is a *fur cir* derivative of BN1071, was used for flow cytometry.
[d] *E. coli* CL29, a deep rough strain, was grown in L broth for colicin assays and in L broth plus 100 μM deferriferrichrome A for siderophore uptake studies.
[e] Flow cytometric determination of MAb binding to cell surface epitopes of FepA.
[f] Recognizes a surface epitope of OmpF porin (1).

with purified LPS by ELISA and Western blot; two (MAb 46 and 53) were deemed prototypic and characterized further. MAb 46 showed strong flow cytometric reactivity with *E. coli* K-12 cells containing a complete core but failed to bind to deep rough strains (data not shown). MAb 53, on the other hand, reacted only weakly with *rfa*$^+$ bacteria but adsorbed well to *rfaD* strains. MAb 46 and 53 therefore appeared to recognize distal and proximal residues, respectively, in the *E. coli* K-12 LPS core.

FPOM vesicles were analyzed by flow cytometry to identify MAb in the test panel specific for periplasmic surface determinants of FepA. No antibodies that recognized periplasmic surface epitopes of FepA were observed. MAb 19, however, showed OmpA-dependent adsorption to OM vesicles but no binding to either *rfa*$^+$ or *rfaD* whole cells. The OmpA epitope recognized by MAb 19 was delineated by examining the reactivity of the antibody with trypsin degradation products of OmpA (41). The periplasmically located C terminus of OmpA is susceptible to trypsin degradation, whereas its membrane-spanning N-terminal portion is trypsin resistant. Hence, French pressure cell-generated, OmpA-containing FPOM was subjected to trypsin proteolysis and assayed by Western blot with MAb 19. Conversion of OmpA to its 24-kDa degradation product by trypsin was assessed in immunoblots using anti-OmpA polyclonal serum. Although the 24-kDa degradation product was detected with polyclonal serum, MAb19 reacted only with full-length OmpA, localizing the epitope it recognizes within the C-terminal portion of OmpA (data not shown). These data indicate that the epitope recognized by MAb 19 resides within the periplasmically exposed C terminus of OmpA. MAb 19 was therefore used in all flow cytometric analyses of whole cells as an indicator of OM structural integrity.

Since FepA::PhoA fusion proteins containing 227 or more residues of mature FepA at the N terminus are successfully exported to the OM such that the FepA moiety of the hybrid protein is inserted into and spans the OM bilayer (26), it was of interest to determine whether these mutant FepA polypeptides would be recognized by MAb that react with native FepA surface epitopes. Although several OM-localized FepA::PhoA fusion protein-containing strains, including pFP495 and pFP710 (26), were assayed cytofluorimetrically, none adsorbed MAb 31, 44, or 45. These data indicate that one of the major cell surface determinants of FepA, between residues 290 and 339, is physically disrupted in OM-localized FepA::PhoA fusion proteins, even if the fusion junction lies downstream in the FepA polypeptide.

MAb inhibition of ferric enterobactin uptake and colicin killing. Siderophore uptake in the presence of MAb was measured by both siderophore nutrition assays (48) and ^{59}Fe siderophore transport studies (23). The results of these two methods were always consistent with each other. For inhibition of colicin killing, the method of Guterman (12, 13) was modified to include MAb.

Four anti-FepA MAb that recognized the cell surface-exposed region 290 to 339 (MAb 31, 35, 44, and 45) inhibited killing by colicins B and D and the uptake of ferric enterobactin (Table 3). These same antibodies did not block the interaction of colicin Ia with Cir or the interaction of colicin M or ferrichrome with TonA. Furthermore, neither surface-reactive anti-Cir MAb (which prevented colicin Ia killing) nor surface-reactive anti-OmpF MAb inhibited the interaction of FepA with colicins B and D or ferric enterobactin (Table 3). These data indicate that anti-FepA MAb 31, 35, 44, and 45 specifically block the interaction of the ferric enterobactin receptor with its ligands. They also refute the idea that antibodies which bind to surface epitopes of other *E. coli* OM proteins may sterically hinder the ferric enterobactin receptor. Some differential blocking of colicins B and D was observed among MAb 31, 35, 44, and 45. For instance, MAb 31 inhibited colicin D killing to a greater extent

than colicin B killing, whereas MAb 35, 44, and 45 blocked colicin B more efficiently than colicin D. This phenomenon was also observed for MAb 37, which did not significantly inhibit colicin B killing of BN1071 but did weakly retard killing of this strain by colicin D.

The biochemical significance of antibody binding to peptides within region 290 to 339 was emphasized by the inability of several other surface-reactive anti-FepA MAb to block the interaction of colicins and ferric enterobactin with the receptor. Antibodies that bound surface determinants within regions 27 to 37 (MAb 6), 204 to 227 (MAb 33), 258 to 290 (MAb 34), and 382 to 400 (MAb 23 and 24) were analyzed in a deep rough, *rfaD* background [KDF29(pITS449)] because, as described above, an intact *E. coli* K-12 LPS core prevented their adsorption to FepA epitopes. When evaluated cytofluorimetrically, these antibodies exhibited FepA-dependent cell surface binding to KDF29(pITS449), but three of the five (MAb 6, 33, and 24) showed no inhibition of ligand interaction with FepA in this strain. Only MAb 23 and 34 blocked ferric enterobactin uptake into and colicin B and D killing of KDF29(pITS449); inhibition by MAb 34 was quantitatively less than that caused by MAb 23 for all three ligands. MAb 31, 35, 44, and 45 showed greater ability to protect the *rfaD* strain against colicin killing and inhibit ferric enterobactin transport than did the *rfa*$^+$ strain BN1071 (Table 2). MAb 37, which recognized an epitope within region 290 to 339 but did not significantly inhibit colicin B killing or ferric enterobactin uptake in the *rfa*$^+$ strain, strongly blocked both colicins and siderophore in KDF29(pITS449) (Table 3). This inhibitory effect of the intact LPS core on the reactivity of MAb 37 differentiates it from the other antibodies that bind epitopes within region 290 to 339.

Unlike the anti-FepA- or anti-Cir-mediated inhibition of killing by colicin B or Ia, respectively, anti-LPS MAb 46 and 53 reacted with residues in the LPS core (see above) to prevent the killing by colicins B, D, Ia, and M. This phenomenon is illustrated by the fact that MAb 46, which apparently recognizes residues in the distal portion of the LPS core (see above), inhibited colicin killing of *rfa*$^+$ strains, whereas MAb 53, which reacts with LPS either in the 2-keto-3-deoxyoctulosonic acid region of core or in the lipid A moiety, significantly inhibited colicin killing only in deep rough, *rfaD* strains (Table 3). These data indicate that antibody binding to LPS may generally inhibit the killing activity of group B colicins, ostensibly through steric hindrance at the stage of adsorption to the OM.

Native FepA structure. FepA and Cir migrated with characteristic mobilities in SDS-PAGE after denaturation by heating in solutions of SDS to R_f values of 0.34 (81 kDa) and 0.37 (74 kDa), respectively (Fig. 3). The availability of anti-FepA and anti-Cir MAb allowed us to determine the mobilities of these OM proteins in SDS-PAGE without heat denaturation and, in the process, identify electrophoretic forms of FepA and Cir that were previously unrecognized. If solubilized in SDS without heating and subjected to SDS-PAGE, both proteins exhibited multiple electrophoretic forms with increased mobilities relative to those of the SDS-denatured proteins (Fig. 3). When such gels were subjected to Western immunoblotting, FepA showed a minor band of R_f 0.45 (61 kDa) and a major band of R_f 0.47 (56 kDa). The latter band could be resolved as a triplet on extended electrophoresis (data not shown). Cir had two major nondenatured forms (Fig. 3) with R_fs of 0.53 and 0.59 (46 and 37 kDa, respectively) and a triplet of minor bands with an approximate R_f of 0.56 (43 kDa).

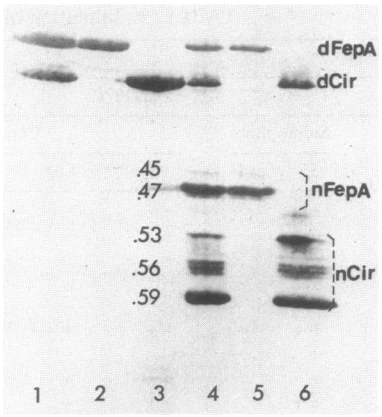


FIG. 3. Western immunoblot of boiled (lanes 1 to 3) and unboiled (lanes 4 to 6) *E. coli* K-12 strain BN1071 FPOM, reacted with anti-FepA MAb 29 (lanes 1, 2, 4, and 5) or anti-Cir MAb 9 (lanes 1, 3, 4, and 6). Note that lanes 1 and 4 contain both MAb 29 and MAb 9. The positions of denatured (d) and nondenatured (n) FepA and Cir, as well as the R_fs of the nondenatured bands, are indicated.

DISCUSSION

One of the unusual characteristics of enteric bacterial OM proteins is their ability to interact with several structurally different nutrilites, toxins, or bacteriophage (30, 31). Although such multifunctionality is a common trait among OM proteins, the molecular mechanisms of these receptor-ligand interactions are not understood. Our immunochemical analysis of FepA addresses the nature of its ligand-binding sites: do ferric enterobactin, colicin B, and colicin D all interact with FepA within a single structural domain of the protein, or does the receptor contain multiple ligand-binding regions? The results we report suggest that all three ligands bind FepA within a relatively short, surface-exposed sequence of the receptor that is bounded by residues 290 and 339. Antibody recognition of a surface-exposed epitope in this region inhibits both ferric enterobactin uptake into the cell and killing by either colicin. By themselves, these data do not definitively implicate the epitope within region 290 to 339 as a ligand-binding domain; other explanations are possible. For example, residues 290 to 339 may be physically distinct from the ligand-binding site but in such close proximity that antibody binding to this region inhibits receptor-ligand contact. Alternatively, residues 290 to 339 may be distant from the siderophore- and colicin-binding domain, but antibody binding to these residues may prevent ligand recognition or transport by inducing or retarding conformational changes in FepA structure. Other results, nevertheless, support the idea that amino acids within region 290 to 339 participate directly in ligand binding. Residues within this region are accessible to MAb binding in the presence of an *rfa*$^+$ LPS core, whereas other surface epitopes of FepA (within residues 27 to 37, 204 to 227, 258 to 290, and 382 to 400) are obscured by *rfa*$^+$ LPS. These data argue that colicins B and D, which approximate the molecular dimensions of antibodies (58 and 92 kDa, respectively [33, 44]), are likewise able to bind region 290 to 339 but are sterically inhibited by LPS from contact with regions 27 to 37, 204 to 227, 258 to 290, and 382 to 400.

The LPS core may not constitute a barrier to ferric enterobactin, which is physically much smaller (700 Da) than colicins or antibodies and may diffuse through the core sugars to interact with other FepA domains. Hypothetically,

ferric enterobactin may bind to FepA regions that are ensheathed in LPS. The inhibition of ferric enterobactin uptake by MAb 31, 35, 44, and 45 is inconsistent with this idea, however, because their binding to the exposed epitope in region 290 to 339 is not expected to sterically hinder other FepA domains that are buried within the LPS core. Furthermore, region 290 to 339 is extremely hydrophilic (<h> = −0.22; Fig. 4) and contains five basic residues that may participate in electrostatic bonds with the acidic [$3^{(-)}$] ferric enterobactin. We conclude that either residues 290 to 339 are directly involved ferric enterobactin binding or MAb recognition of this region allosterically inhibits interaction with the siderophore at another site.

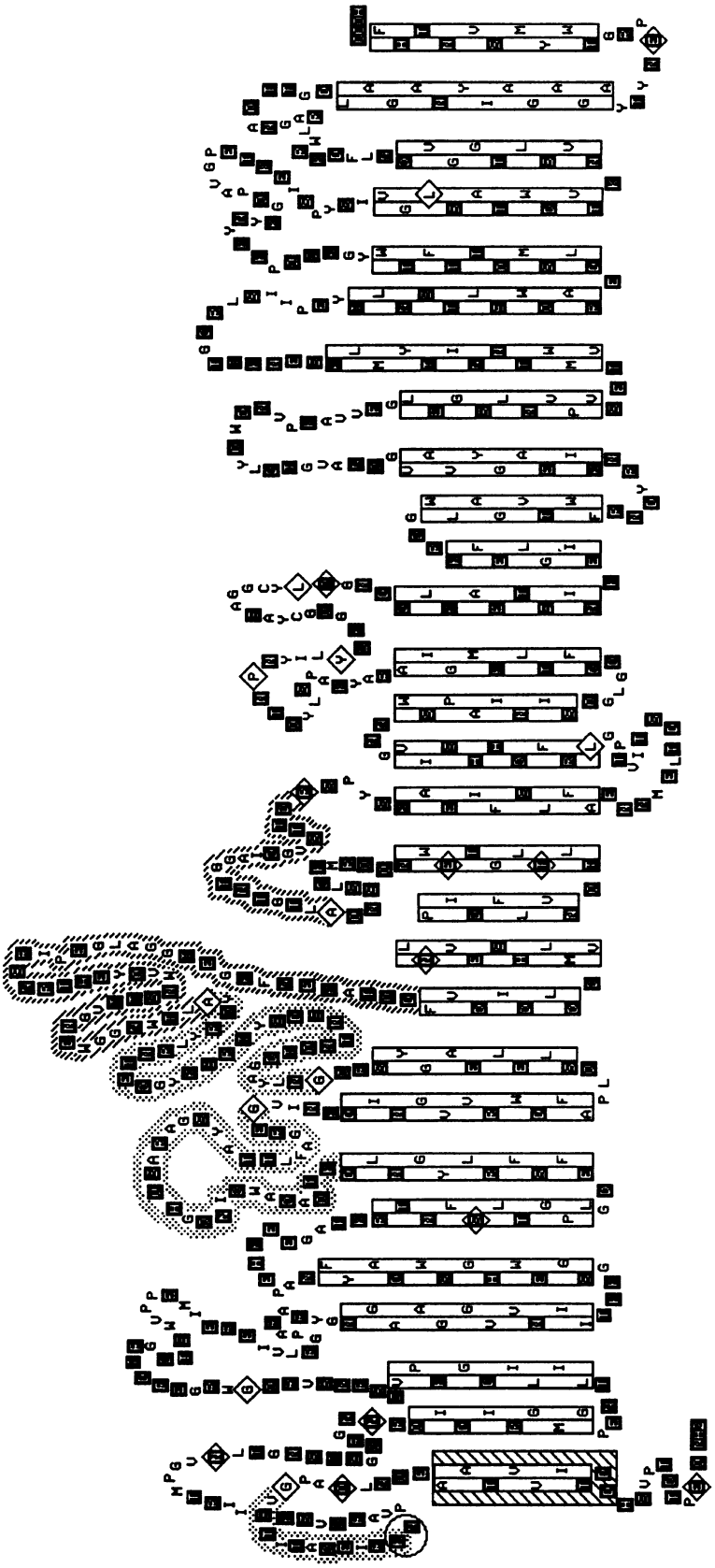
The possibility of antibody-mediated inhibition of allostery within the ferric enterobactin receptor appears unlikely. Antibody binding to certain surface FepA epitopes (within regions 27 to 37, 204 to 227, and 382 to 400) had no effect on either ferric enterobactin transport or colicin killing. If conformational changes occur during physiological function of FepA, they are localized and unaffected by antibody binding within these sites. A final category of surface-reactive anti-FepA MAb (MAb 23 and 34) adsorbed only to deep rough strains and blocked ligand binding (although the inhibition by MAb 34 was weak). This reactivity suggests that residues within regions 258 to 290 and 382 to 400 either participate in or are in close proximity to the siderophore- and colicin-binding site in region 290 to 339. The model of FepA structure that emerges from these immunochemical analyses contains at least five distinct polypeptide domains that are located at the exterior surface of the OM and recognized by MAb. Four of these are sterically masked by the LPS core, but antibody recognition of the remaining epitope, between residues 290 and 339, is independent of core structure. It is likely that FepA ligands physically bind to the receptor within this domain. Although the molecular structure of region 290 to 339 cannot be deduced from the available data, the fact that surface-reactive antibodies which recognize this region show quantitative differences in the extent to which they inhibit the receptor-ligand interaction suggests that colicins B and D and ferric enterobactin may recognize distinct microdomains within this region. This assumption agrees with the genetic studies of McIntosh et al. (23).

All of the anti-FepA MAb characterized in this study, including those that bind surface determinants and block ligand function, ostensibly recognize sequential determinants (42). For example, MAb 31, 35, 37, 44, and 45 bind a site that is entirely contained within region 290 to 339, because they recognize their epitope even when the FepA polypeptide has been denatured by boiling in SDS. Their affinity for denatured FepA is not enhanced by the presence of residues downstream from 290 to 339 and is completely eliminated by truncations that delete residues 290 to 339. Hence, these antibodies react specifically and exclusively with residues within region 290 to 339. These arguments apply equally well to all of the anti-FepA MAb that we have isolated and therefore distinguish them from antibodies raised to *E. coli* porin trimers. For OmpF, MAb have been raised that specifically bind conformational determinants in the cell surface-exposed regions of the trimer; they are unreactive with denatured OmpF monomer (1). No such conformation-dependent anti-FepA MAb were found in the test panel. These differences in the immunochemistry of porin and FepA probably arise from intrinsic differences in the stability of their tertiary structures. Porin trimers are resistant to both heat denaturation and protease degradation, whereas FepA is both sensitive to denaturation and susceptible to proteolysis. Hence, during the process of antigen recognition by the immune system, FepA may present sequential determinants, while OmpF may present strictly conformation epitopes. This interpretation is supported by the fact that antibodies which recognize sequential OmpF determinants can be raised by immunizing mice with SDS-denatured OmpF monomer (1; P. E. Klebba, S. A. Benson, S. Bala, T. Abdullah, J. Reid, S. P. Singh, and H. Nikaido, J. Biol. Chem., in press).

We attempted, unsuccessfully, to isolate MAb that recognized periplasmic surface determinants of FepA. Our failure to find hybridomas specific for periplasmic epitopes of FepA may indicate their lack of immunogenicity. Alternatively, antibodies specific for periplasm-exposed domains of FepA may exist in the current panel, but FepA may be situated in the OM such that these regions are inaccessible to antibody. Periplasmic epitopes of FepA could also be conformational and reactive only with MAb to the native protein. Finally, the panel of anti-FepA MAb that we have compiled is not considered exhaustive; periplasmic FepA epitopes may be identified upon further study.

Numerous *E. coli* membrane proteins exhibit atypical mobility in SDS-PAGE that depends on sample preparation conditions (25, 28, 38, 40). Such proteins contain extensive β structure that imparts a compactness to their form, increasing their electrophoretic mobility (40). Heating in SDS converts them to more diffuse, unfolded structures that migrate with consistent R_fs characteristic of their true molecular weights. FepA and Cir also have SDS-stable, compact forms that are denatured by heating. The multiple native FepA and Cir electrophoretic forms may be caused by association of the receptors with LPS or may represent partially unfolded conformations of the native OM protein that are produced by the in vitro conditions.

Using the accumulated data on the surface epitopes and putative ligand-binding domains of FepA, as well as the predicted hydrophobicity (6) and hydrophobic moment (7) of the receptor, we have constructed a theoretical model of its structure in the bacterial OM. We base this hypothesis on the following facts. (i) FepA contains cell surface-exposed epitopes in regions 27 to 37, 204 to 227, 258 to 290, 290 to 339, and 382 to 400. (ii) The predominant secondary structure of FepA is probably β-sheet. Several other enteric bacterial OM proteins, including OmpA, OmpF, LamB, and PhoE, consist almost exclusively of β structure (3, 10, 17, 37). Although spectrophotometric data on the purified native receptor are not available, the heat modifiability of FepA in SDS-PAGE (Fig. 3) is consistent with a compact native structure dominated by β-strands (15). (iii) The FepA polypeptide is not particularly hydrophobic (6), and in fact contains numerous strongly hydrophilic domains interspersed throughout its sequence. Examination of FepA hydrophobic moment (7), however, shows that the protein contains many amphiphilic stretches of sufficient length to span the OM lipid bilayer in β structure. The mean hydrophobicities of the opposing faces of these putative β-strands are consistent with the hypothesis that they interact with a hydrophobic surface on one side and a hydrophilic surface on the other (Fig. 4; 36). The hydrophobic faces of these amphiphilic transmembrane strands are expected to interact with the OM lipid bilayer; the hydrophilic faces may circumscribe a water-filled pore (Klebba et al., in press) or may be juxtaposed against one another. The model was created by identifying the first potential transmembrane β-strand in the sequence of the mature protein. Since a surface epitope was



strand	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
residues	13-19	71-78	83-91	135-45	150-61	174-83	186-96	232-42	246-54	337-43	346-53	354-60	362-70	405-13	429-37	431-38	444-52	500-08	510-16	520-27	534-42	566-78	577-88	606-15	617-26	656-65	667-76	694-705	714-23
<h>	.11	.42	.06	.06	.38	.58	.67	.51	.48	.29	.38	.28	.42	.07	.33	.54	.22	.67	.33	.55	.62	.41	.46	.62	.28	.24	.46	.16	.29
interface	I|M	A|M	A|M	A|M	A|M	A|M	A|M	A|M	A|M	A|M	A|M	A|M	A|M	A|I	A|M	A|M	A|M	M|A	M|A	A|M	A|M	A|M	A|M	A|M	I|I	A|M

FIG. 4. Predicted structural and topological features of the ferric enterobactin receptor. Transmembrane β-strands (□) were identified by the method of Eisenberg et al. (6, 7). The sites of FepA::PhoA fusion (◇); significantly hydrophilic residues D, E, N Q, R, K, H, T, and S (■); cleavage point of OmpT (○); predicted TonB consensus region (▨); surface epitopes that do not participate in ligand binding (▩); and surface epitopes that are involved in ligand binding (▧) are shown. Region 258 to 290 is not designated as involved in ligand interactions because inhibition of siderophore uptake and colicin killing by MAb 34 was relatively weak. The average residue hydrophobicity (<h>) of opposing sides of predicted transmembrane β-strands is shown below each strand, as is the expected interface (A, aqueous; M, membrane; I, protein interior [36]) with which each face of the strand interacts.

found immediately downstream from this site (amino acids 27 to 37), residues preceding the transmembrane domain were assumed to reside on the periplasmic face of the OM, and those immediately downstream (to the site of the next transmembrane (β-strand) were considered cell surface exposed. Succeeding downstream regions, separated by transmembrane β-structures, were assumed to alternate between the periplasmic and outer surfaces of the OM. Although we searched for potential transmembrane or surface-seeking α-helical domains in FepA, none were found. Turns in the polypeptide were assigned according to the algorithm of Wilmot and Thornton (49). This approach to the modeling of FepA predicts several features that are consistent with empirical data. Foremost, each of the immunochemically defined cell surface-exposed regions of FepA is predicted by the method to exist on the exterior surface of the OM. Second, for the complete mature FepA sequence, the mean hydrophobicity of residues proposed to associate with the OM bilayer lipids is 0.38, whereas that for residues proposed to associate with an aqueous interface is −0.17. These values agree with the average residue hydrophobicities of bilayer-exposed and aqueous-exposed residues of other membrane proteins (36). Other points of interest are the localization of the FepA proposed TonB consensus region (20) embedded within the OM bilayer at the periplasmic surface and its OmpT cleavage site on the bacterial cell surface. Finally, the predicted locations of the sites of PhoA fusion into FepA (26) are dispersed throughout the exterior surface, transmembrane strands, and periplasmic surface domains of the receptor. More significantly, the immunochemically defined surface epitopes of FepA, presented herein, include sites of PhoA fusion (pFP178, -227, -258, -290, -382, and -400). These data establish that even though the PhoA moiety of FepA::PhoA fusion proteins remains periplasmic (26), the sites of PhoA fusion need not be periplasmic surface domains of FepA. This conclusion is supported by our finding that the immunochemical structure of the FepA portion of FepA::PhoA fusion proteins is distorted relative to the native ferric enterobactin receptor.

ACKNOWLEDGMENTS

We thank David Eisenberg and Carrie Wilmot for their algorithms and insights on protein structure prediction and Mark McIntosh, Sandy Armstrong, Ulf Henning, David Brunner, and Bill Reznikoff for bacterial strains and phage.

This work was supported by Public Health Service grant AI22608 from the National Institutes of Health.

LITERATURE CITED

1. **Bentley, A. T., and P. E. Klebba.** 1988. Effect of lipopolysaccharide structure on the reactivity of anti-porin monoclonal antibodies with the bacterial cell surface. J. Bacteriol. **170:** 1063–1068.
2. **Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich.** 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. Anal. Biochem. **1l36:**175–179.
3. **Charbit, A., J. M. Clement, and M. Hofnung.** 1984. Further sequence analysis of the phage lambda receptor site: possible implications for the organization of the LamB protein in *Escherichia coli* K12. J. Mol. Biol. **175:**395–401.
4. **Coleman, W. G., Jr., and L. Leive.** 1979. Two mutations which affect the barrier function of *Escherichia coli* K-12 outer membrane. J. Bacteriol. **139:**899–910.
5. **Csonka, L., and A. J. Clark.** 1980. Construction of an Hfr strain useful for transferring *recA* mutations between *Escherichia coli* strains. J. Bacteriol. **143:**529–530.
6. **Eisenberg, D.** 1984. Three-dimensional structure of membrane and surface proteins. Annu. Rev. Biochem. **53:**595–623.
7. **Eisenberg, D., R. M. Weiss, and T. C. Terwilliger.** 1984. The hydrophobic moment detects periodicity in protein hydrophobicity. Proc. Natl. Acad. Sci. USA **81:**140–144.
8. **Fiss, E. H., P. Stanley-Samuelson, and J. B. Neilands.** 1982. Properties and proteolysis of the ferric enterobactin outer membrane receptor in *Escherichia coli* K-12. Biochemistry **21:**4517–4522.
9. **Gabay, J., S. Benson, and M. Schwartz.** 1983. Genetic mapping of antigenic determinants on a membrane protein. J. Biol. Chem. **258:**2410–2414.
10. **Garavito, R. M., J. Jenkins, J. N. Jansonius, R. Karlsson, and J. P. Rosenbusch.** 1983. X-ray diffraction analysis of matrix porin, an integral membrane protein from *Escherichia coli* outer membranes. J. Mol. Biol. **164:**313–327.
11. **Garnier, J., D. J. Osguthorpe, and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120:**97.
12. **Guterman, S.** 1971. Inhibition of colicin B by enterochelin. Biochem. Biophys. Res. Commun. **44:**1149–1155.
13. **Guterman, S. K.** 1973. Colicin B: mode of action and inhibition by enterochelin. J. Bacteriol. **114:**1225–1230.
14. **Guterman, S. K., and L. Dann.** 1973. Excretion of enterochelin by *exbA* and *exbB* mutants of *Escherichia coli*. J. Bacteriol. **114:**1225–1230.
15. **Heller, K. B.** 1978. Apparent molecular weights of a heat-modifiable protein from the outer membrane of *Escherichia coli* in gels with different acrylamide concentrations. J. Bacteriol. **134:**1181–1183.
16. **Hollifield, W. C., E. H. Fiss, and J. B. Neilands.** 1978. Modification of a ferric enterobactin receptor protein from the outer membrane of *Escherichia coli*. Biochem. Biophys. Res. Commun. **83:**739–746.
17. **Jap, B. K.** 1988. High-resolution electron diffraction of reconstituted PhoE porin. J. Mol. Biol. **199:**229–231.
18. **Klebba, P. E., M. A. McIntosh, and J. B. Neilands.** 1982. Kinetics of biosynthesis of iron-regulated membrane proteins in *Escherichia coli*. J. Bacteriol. **149:**880–888.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
20. **Lundrigan, M. D., and R. J. Kadner.** 1986. Nucleotide sequence of the gene for the ferrienterochelin receptor FepA in *Escherichia coli*: homology among outer membrane receptors that interact with TonB. J. Biol. Chem. **261:**10797–10801.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Manning, P. A., A. Puspurs, and P. Reeves.** 1976. Outer membrane of *Escherichia coli* K-12: isolation of mutants with altered protein 3A by using host range mutants of bacteriophage K3. J. Bacteriol. **127:**1080–1084.
23. **McIntosh, M. A., S. S. Chenault, and C. F. Earhart.** 1979. Genetic and physiological studies on the relationship between colicin B resistance and ferrienterochelin uptake in *Escherichia coli* K-12. J. Bacteriol. **137:**653–657.
24. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Mizushima, S.** 1974. Effect of sodium dodecylsulfate and heating on protein conformation in outer and cytoplasmic membranes from *Escherichia coli*. Biochem. Biophys. Res. Commun. **61:** 1221–1226.
26. **Murphy, C. K., and P. E. Klebba.** 1989. Export of FepA::PhoA fusion proteins to the outer membrane of *Escherichia coli* K-12. J. Bacteriol. **171:**5894–5900.
27. **Nabedryk, E., R. M. Garavito, and J. Breton.** 1988. The orientation of B-sheets in porin. A polarized Fourier transform infrared spectroscopic investigation. Biophys. J. **53:**671–676.
28. **Nakamura, K., and S. Mizushima.** 1976. Effects of heating in dodecyl sulfate solution on the conformation and electrophoretic mobility of isolated major outer membrane proteins from *Escherichia coli* K-12. J. Biochem. **80:**1411–1422.

29. **Neilands, J. B.** 1966. Naturally occurring non-porphyrin iron compounds, p. 59–108. *In* C. K. Jorgenson, J. B. Neilands, R. S. Nyholm, D. Reinin, and R. J. P. Williams (ed.), Structure and bonding. Springer-Verlag, New York.
30. **Neilands, J. B., K. Konopka, B. Schwyn, M. Coy, R. T. Francis, B. A. Paw, and A. Bagg.** 1987. Comparative biochemistry of microbial iron assimilation, p. 3–33. *In* G. Winkelman, D. van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants, and animals. VCH Verlagsgesellschaft, Weinheim, Federal Republic of Germany.
31. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of outer membrane permeability. Microbiol. Rev. **49**:1–32.
32. **Oi, V. T., and L. A. Herzenberg.** 1980. Immunoglobulin-producing hybrid cell lines, p. 351–372. *In* B. B. Mishell and S. M. Shiigi (ed.), Selected methods in cellular immunology. W. H. Freeman & Co., San Francisco.
33. **Olschlager, T., E. Schramm, and V. Braun.** 1984. Cloning and expression of the activity and immunity genes of colicins B and M on ColBM plasmids. Mol. Gen. Genet. **196**:482–487.
34. **Pugsley, A. P., and P. Reeves.** 1976. Characterization of group B colicin-resistant mutants of *Escherichia coli* K12: colicin resistance and the role of enterochelin. J. Bacteriol. **127**:218–228.
35. **Pugsley, A. P., and P. Reeves.** 1977. Comparison of colicins B-K260 and D-CA23: purification and characterization of the colicins and examination of colicin immunity in the producing strains. Antimicrob. Agents Chemother. **11**:345–358.
36. **Rees, D. C., L. DeAntonio, and D. Eisenberg.** 1989. Hydrophobic organization of membrane proteins. Science **245**:510–513.
37. **Reithmeier, R. A. F., and P. D. Bragg.** 1977. Molecular characterization of a heat-modifiable protein from the outer membrane of *Escherichia coli*. Arch. Biochem. Biophys. **178**:527–534.
38. **Rosenbusch, J. P.** 1974. Characterization of the major envelope protein from *Escherichia coli*. Regular arrangement on the peptidoglycan and unusual dodecyl sulfate binding. J. Biol. Chem. **249**:8019–8029.
39. **Schenkman, S., E. Couture, and M. Schwartz.** 1983. Monoclonal antibodies reveal LamB antigenic determinants on both faces of the *Escherichia coli* outer membrane. J. Bacteriol. **155**:1382–1392.
40. **Schnaitman, C. A.** 1973. Outer membrane proteins of *Escherichia coli*. I. Effect of preparative conditions on the migration of protein in polyacrylamide gels. Arch. Biochem. Biophys. **157**:541–552.
41. **Schweizer, M., I. Hindennach, W. Garten, and U. Henning.** 1978. Major proteins of *Escherichia coli* outer cell envelope membrane. Interaction of protein II* with lipopolysaccharide. Eur. J. Biochem. **82**:211–217.
42. **Shinnick, M. T., J. G. Sutcliffe, N. Green, and R. Lerner.** 1983. Synthetic peptide immunogens as vaccines. Annu. Rev. Microbiol. **37**:425–446.
43. **Sugimura, K., and N. Higashi.** 1988. A novel outer-membrane-associated protease in *Escherichia coli*. J. Bacteriol. **170**:3650–3654.
44. **Timmis, K.** 1972. Purification and characterization of colicin D. J. Bacteriol. **109**:12–20.
45. **Tommassen, J., and B. Lugtenberg.** 1984. Amino terminus of outer membrane protein PhoE protein: localization by use of a *bla-phoE* hybrid gene. J. Bacteriol. **157**:327–329.
46. **Vogel, H., and F. Jahnig.** 1986. Models for the structure of outer-membrane proteins of *Escherichia coli* derived from Raman spectroscopy and prediction methods. J. Mol. Biol. **190**: 191–199.
47. **Wang, C. C., and A. Newton.** 1971. An additional step in the transport of iron defined by the *tonB* locus of *Escherichia coli*. J. Biol. Chem. **246**:2147–2151.
48. **Wayne, R., K. Frick, and J. B. Neilands.** 1976. Siderophore protection against colicins M, B, V, and Ia in *Escherichia coli*. J. Bacteriol. **126**:7–12.
49. **Wilmot, C. M., and J. M. Thornton.** 1988. Analysis and prediction of the different types of β-turn in proteins. J. Mol. Biol. **203**:221–232.