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Evolution of the Ferric Enterobactin Receptor in Gram-Negative Bacteria

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Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis of iron-deficient and replete cell envelopes, ^{59}Fe-siderophore uptake studies, and Western immunoblots and cytofluorimetric analyses with monoclonal antibodies (MAbs), we surveyed a panel of gram-negative bacteria to identify outer membrane proteins that are structurally related to the *Escherichia coli* K-12 ferric enterobactin receptor, FepA. Antibodies within the panel identified FepA epitopes that are conserved among the majority of the bacteria tested, as well as epitopes present in only a few of the strains. In general, epitopes of FepA that are buried in the outer membrane bilayer were more conserved among gram-negative bacteria than epitopes that are exposed on the bacterial cell surface. The surface topology and tertiary structure of FepA are quite similar in *E. coli* and *Shigella flexneri* but differ in *Salmonella typhimurium*. Of the 18 different genera tested, 94% of the bacteria transported ferric enterobactin, including members of the previously unrecognized genera *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Haemophilus*, *Hafnia*, *Morganella*, *Neisseria*, *Proteus*, *Providencia*, *Serratia*, and *Yersinia*. The ferric enterobactin receptor contains at least one buried epitope, recognized by MAb 2 (C. K. Murphy, V. I. Kalve, and P. E. Klebba, J. Bacteriol. 172:2736–2746, 1990), that is conserved within the structure of an iron-regulated cell envelope protein in all the bacteria that we have surveyed. With MAb 2, we identified and determined the M_r of cell envelope antigens that are immunologically related to *E. coli* FepA in all the gram-negative bacteria tested. Collectively, the library of anti-FepA MAbs showed unique patterns of reactivity with the different bacteria, allowing identification and discrimination of species within the following gram-negative genera: *Aeromonas*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Escherichia*, *Haemophilus*, *Hafnia*, *Klebsiella*, *Morganella*, *Neisseria*, *Proteus*, *Providencia*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Vibrio*, and *Yersinia*.

Eucaryotic proteins with high affinities for iron, such as transferrin and lactoferrin, render procaryotes iron deficient in vivo (53). This competition for iron between host and microbe selects for efficient bacterial iron chelators (siderophores) that can capture iron from mammalian proteins and for specific bacterial outer membrane (OM) receptor proteins that recognize ferric siderophore complexes and transport them into the microbial cell. Both pathogenic and commensal bacteria produce and utilize siderophores in response to iron stress; certain siderophore uptake systems may confer virulence or invasiveness to bacteria that contain them (15, 24, 40, 74).

The native iron acquisition system of *Escherichia coli* contains enzymes for the synthesis and secretion of the siderophore enterobactin; an OM receptor protein, FepA, that specifically adsorbs ferric enterobactin; and a variety of other cell envelope proteins that act in the transport and deferration of ferric enterobactin (26, 53, 56, 59, 70). The FepA structural gene has been cloned and sequenced (44); mature FepA contains 723 amino acids and has a molecular mass of 81 kDa (48). In *E. coli* the ferric enterobactin receptor also acts as the cognate OM receptor for colicins B and D (25, 72). Related members of the family *Enterobacteriaceae*, including *Klebsiella*, *Salmonella*, and *Shigella* spp., both produce and transport enterobactin (15, 42, 53); the structural gene for the ferric enterobactin receptor protein has been cloned from the latter two bacteria (64a, 67a). The effects of iron stress have been studied in many gram-negative bacteria, including *Escherichia* (12, 14, 36, 48, 53), *Haemophilus* (49, 50), *Klebsiella* (33, 42), *Neisseria* (4, 58, 73), *Pasteurella* (16, 31, 55, 66), *Pseudomonas* (60), *Proteus* (18), *Salmonella* (17, 20, 75), *Serratia* (46), *Shigella* (57), *Vibrio* (2, 15, 37, 69), and *Yersinia* (8, 9, 61) spp. These bacteria synthesize numerous iron-regulated membrane proteins (IRMP) whose biochemical functions are for the most part uncharacterized.

Pathogenesis of gram-negative bacteria in mammals elicits a humoral immune response to the external surface structures of the bacterial cell (7, 19, 20, 27, 38, 39, 45, 67, 71). More significantly, antibodies raised against OM proteins or lipopolysaccharide (LPS), the major surface antigens of gram-negative bacteria, can protect against infection by enteric bacteria (19, 32, 38, 39, 76).

The immunochemical identification of external surface epitopes (henceforth referred to as surface epitopes) of *E. coli* OM proteins has led to predictions about their secondary and tertiary structures, the number and composition of their surface-exposed residues, their functional domains, and their associations with LPS (11, 21, 35, 51). *E. coli* FepA contains surface epitopes in at least five different regions of its primary structure, bounded by residues 27 to 37, 204 to 227, 258 to 290, 290 to 339, and 382 to 400 (3, 51). The *E. coli* K-12 LPS core and O-antigen sugars obscure most of these surface epitopes of FepA, but the receptor's ferric enterobactin and colicin-binding domains in region 290 to 339 are

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free of LPS steric hindrance. FepA contains other epitopes in regions of its tertiary structure that are normally buried in the OM bilayer (28, 51) (henceforth referred to as buried epitopes).

The families of gram-negative bacteria encompass an assortment of species that are evolutionarily related to *E. coli*. For any specific protein that is conserved among them, these different bacteria constitute a repository of mutants that have been naturally selected for their biochemical fitness over the millennia. The analysis of structural changes that have occurred during such evolution may lead to the identification of important domains within a protein of interest, as well as insights about the dynamics of its conformation. With anti-FepA monoclonal antibodies (MAbs) of known specificities, we evaluated the conservation of surface and buried epitopes of the *E. coli* ferric enterobactin receptor in both closely and distantly related gram-negative bacteria. Each of the bacteria we surveyed contained an IRMP that is antigenically related to *E. coli* FepA; the immunochemical structure of these homologs of *E. coli* FepA has diverged more in the surface domains of the receptor than in its buried, transmembrane polypeptides.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Thirty-nine different strains of gram-negative bacteria representing 18 different genera were tested (see Table 1). The enteric bacteria were grown at 37°C in Luria broth to mid-log phase, subcultured (1%) in Trypticase soy broth (Difco), and rendered iron deficient by the addition of 0.1 mM apoferrichrome A (36). For *Edwardsiella tarda*, α,α-dipyridyl (0.2 mM) was used to elicit iron stress, since apoferrichrome A was ineffective. *Haemophilus* spp. were cultured in brain heart infusion medium plus Fildes enrichment (1%) (Difco), aerated with 10% CO_2, and subjected to iron stress by subculture into brain heart infusion medium containing 0.1 mM apoferrichrome A. *Neisseria* strains were grown in GC medium (10), aerated with 10% CO_2, and rendered iron deficient by growth in chemically defined medium (10, 73). Iron-deficient cultures were shaken with vigorous aeration at 37°C for 6 h and harvested by centrifugation. The cells were washed in Tris-HCl buffer (pH 7.2), suspended in the same buffer at 5×10^9/ml, and stored at −70°C.

Tn*phoA* insertions into *fepA*, and FepA::PhoA fusion proteins. *fepA* was mutagenized with Tn*phoA*, and the sites of *phoA* gene fusion were sequenced by the dideoxy chain termination method (52).

Cell lysates, envelopes, and OMs. Bacteria (10^8 cells) were lysed by boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (35). Cell envelopes were prepared by passing a concentrated bacterial cell suspension through the French press at 14,000 lb/in^2 and pelleting the cell envelopes by centrifugation (65). OMs were produced by extracting the cell envelopes with 0.5% Sarkosyl (48) in Tris-buffered saline (TBS) for 30 min at 25°C and collecting the Sarkosyl-resistant pellet after centrifugation at 30,000 × *g* for 45 min.

SDS-PAGE and Western blots. SDS-PAGE was performed and analyzed by ammoniacal silver staining as described previously (35, 51). Cell envelopes and OM fractions from wild isolates of *Salmonella*, *Citrobacter*, *Serratia*, and *Enterobacter* resolved poorly in SDS-PAGE, probably because of the association of OM proteins with LPS (54, 62) and capsule. The incubation of OM or cell envelopes with lysozyme (1 mg/ml) for 30 min at 0°C and the addition 0.03 M EDTA and 0.2 M NaCl to the SDS-PAGE sample buffer improved electrophoretic separation. For silver-stained gels, 5 μg of each sample was boiled for 5 min and spun in the microcentrifuge for 1 min, and the supernatant was loaded into the slab.

For Western immunoblots (35), 10^8 bacteria or 20 μg of cell envelopes or OM was suspended in SDS-PAGE sample buffer (with the modifications described above), boiled for 5 min, and electrophoresed on 11% polyacrylamide slabs (43). Prestained molecular weight markers (Bio-Rad) were included in one lane. Proteins were electrophoretically transferred to nitrocellulose (NC) at 10 V for 12 h. The NC was incubated in TBS containing 1% gelatin and 0.2% NaN_3 (TBSG) for 30 min, and a 2-in. (5-cm) strip containing proteins in the molecular mass range of 50 to 150 kDa was cut from the paper and incubated overnight in diluted monoclonal ascitic fluid at ambient temperature. Western blots were developed with goat anti-mouse immunoglobulin-alkaline phosphatase and nitroblue tetrazolium-bromochloroindoyl phosphate (5). The reaction was stopped after 30 min by washing the strips in distilled water.

Anti-FepA MAbs. Ascitic fluids from murine anti-FepA hybridomas were titered by enzyme-linked immunosorbent assay and Western blot against purified denatured *E. coli* FepA and were appropriately diluted such that equivalent reaction intensities were obtained for each MAb against *E. coli* FepA. The adjusted ascitic fluids were used in Western blots and flow cytometry. Conventional normal mouse serum, germfree antigen-free normal mouse serum, and ascitic fluid from the cell fusion partner, P3-X63-Ag8.653 (34), and from a nonspecific immunoglobulin G1 murine MAb were prepared in TBSG at 1% as negative controls.

Flow cytometry. *E. coli* (51) KDL118 (*rfa*$^+$) and KDF29 (*rfaD*), containing plasmids expressing either *E. coli* K-12 (pITS449 [3]) or *Shigella flexneri* (pMPS13 [64a]) *fepA*, and *Salmonella typhimurium*, *Salmonella paratyphi* B, *Salmonella typhi*, and *Klebsiella pneumoniae rfa* strains were grown to mid-log phase in LB broth with appropriate antibiotic selection, rendered iron deficient by subculture (1%) into T medium (48), and grown to the late mid-log phase (approximately 6 h). The bacteria were stained with anti-FepA MAb and fluorescein-conjugated goat anti-mouse immunoglobulin (Sigma) and analyzed cytofluorimetrically (51).

Ferric enterobactin uptake measurements. Gram-negative bacteria were subjected to iron stress as described above, and their ability to accumulate chromatographically purified [^{59}Fe]enterobactin (74) and [^{59}Fe]ferrichrome was determined (41, 51). Results were evaluated in comparison with ^{59}Fe-siderophore uptake by the *E. coli* K-12 strains BN1071 (*fepA*$^+$ *tonA*$^+$) (36), UT2300 (*fepA*) (48), and AN193 (*tonA*) (36).

RESULTS

Location of epitopes within FepA primary structure. The majority of anti-FepA MAbs used in this study were characterized previously (51). Utilizing a collection of *fepA*::*phoA* gene fusions which produce FepA::PhoA fusion proteins that are in essence C-terminal truncations of FepA (52), each of the epitopes recognized by anti-FepA MAbs was localized within the primary structure of the receptor (51). During the completion of this study, however, the isolation of FepA::PhoA fusions at residues 142, 307, 314, 444, 474, 482, 566, and 609 in the sequence of mature FepA facilitated a more precise mapping of the epitopes recognized by

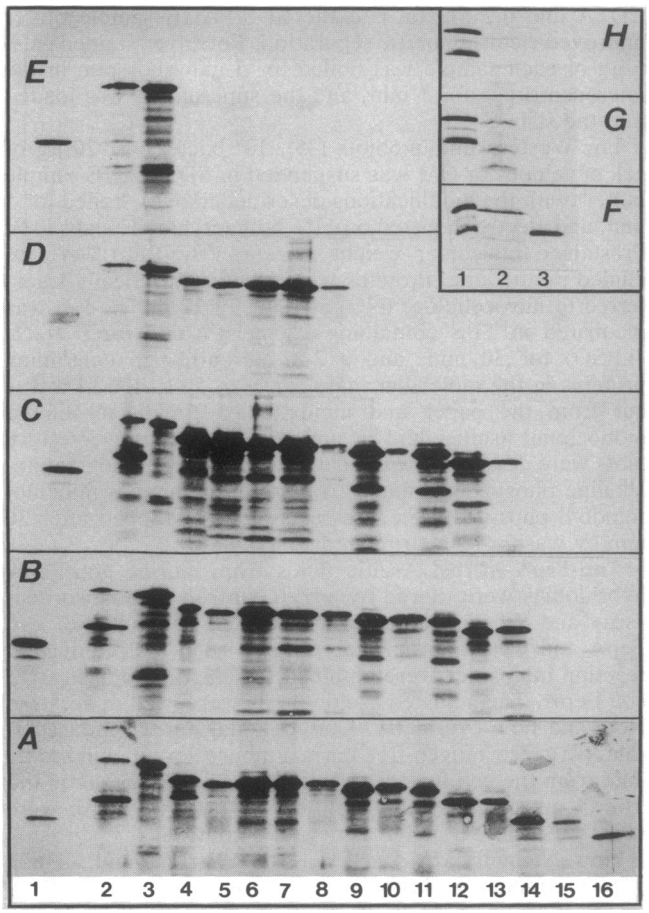

FIG. 1. Localization of epitopes within FepA by Western blots with FepA::PhoA fusion proteins. *E. coli* BN1071 (lane 1, panels A to E) or KDL118 containing *fepA::phoA* fusion plasmids (lanes 2 to 16, respectively, in panels A to E) pFP609, -566, -495, -483, -480, -475, -474, -444, -429, -400, -382, -352, -314, -307, and -290 or (lanes 1 to 3, respectively, in panels F to H) pFP178, -142, and -100 were grown in T medium containing appropriate antibiotics (52), collected by centrifugation, lysed by boiling in SDS-PAGE sample buffer (10^8 cells), and electrophoresed on 11% polyacrylamide slabs. The cell lysates were transferred electrophoretically to NC and immunoblotted with anti-FepA MAbs 29 (A and F), 44 (B), 31 (C), 57 (D), 64 (E), 11 (G), and 2 (H).

several of the antibodies. One of the results that was enabled by these new gene fusions was the differentiation of antibodies that bind within region 100 to 178 (Fig. 1). All the antibodies in this group recognized FepA within region 100 to 142 (MAbs 2, 3, 4, 5, 7, 11, 27, 38, 39, and 41), but MAbs 2 and 3 showed reduced reactivity with pFP142, indicating that they bound at a distinct site near the FepA::PhoA fusion junction at amino acid 142. Furthermore, the epitope recognized by MAb 44, which was originally localized to region 290 to 339 of *E. coli* FepA (51), mapped differently from all other epitopes that reside within that region. The weak positive reaction of MAb 44 with pFP290 (52) and pFP307 and pFP314 (Fig. 1) suggests that its epitope lies immediately upstream of residue 290. Finally, two novel anti-FepA MAbs were isolated that bound sites downstream from any previously known epitopes (Fig. 1), within regions 444 to 474 (MAb 57) and 495 to 566 (MAb 64).

All anti-FepA MAbs were evaluated for their uniqueness in the antibody panel and classified as recognizing either surface or buried epitopes. Comparisons of their reactivity with the collection of gram-negative bacteria established the similarity or singularity of MAbs that recognized epitopes within the same region of FepA sequence (see below). Confidence in the assignment of surface epitopes was high, because these were unambiguously defined by their FepA-dependent reactivity with intact bacteria. Buried epitopes were assigned by two criteria: nonreactivity with intact cells, and localization within a region of FepA sequence containing a high hydrophobic moment, capable of spanning the OM bilayer as an amphiphilic β-strand. In total, the current anti-FepA MAb panel identified 10 distinct buried epitopes in five different regions of FepA sequence and 11 unique surface epitopes in five different regions of primary structure (Table 1).

Reactivity of anti-FepA MAbs with IRMP of gram-negative bacteria. SDS-denatured whole-cell lysates of 39 gram-negative bacterial species from 18 different genera were screened by Western immunoblot with 28 anti-FepA MAbs. Collectively, the anti-FepA MAb panel recognized one or two OM proteins in each of the gram-negative bacteria. These bands were approximately 80 kDa and were regulated by iron availability in the media (Fig. 2). Western immunoblot recognition of iron-regulated doublets by individual anti-FepA MAbs suggested that the lower band was analogous to 81K*, the OmpT-generated degradation product of *E. coli* FepA that lacks the receptor's N-terminal 37 amino acids (30). In several cases (*Salmonella typhimurium*, *Citrobacter freundii*, *Yersinia enterocolitica*, *Haemophilus parainfluenzae*), MAbs that reacted with *E. coli* FepA near its N terminus bound the upper but not the lower band of the doublet, substantiating this inference. An exception to the noted specificity was MAb 2, which reacted with *E. coli* and *S. typhimurium* CirA (with less avidity, Fig. 2) in addition to its recognition of FepA and 81K*. MAb 2 was also unique in that it sometimes showed faint reactivity with other IRMP in the gram-negative bacteria. Although we observed differences in the intensity of the anti-FepA reactions with these homologs of *E. coli* FepA, the distinction between positive and negative was clear (Fig. 2). Such variations in reaction intensity with anti-FepA MAbs may arise both from differences in the level of IRMP induction among the bacterial species in response to iron stress (Fig. 2) and from sequence divergence of the FepA analogs from the immunogen FepA protein of *E. coli*. Immunoblot reactions were therefore evaluated as strongly positive, weakly positive, or negative (Fig. 2 and Table 1). In summary, because the immunoreactive proteins were localized in the cell envelope, iron regulated, similar in molecular weight to FepA, reactive with anti-FepA MAb, and, in numerous cases, proteolytically modified like FepA, we infer that they are structural homologs of the *E. coli* K-12 ferric enterobactin receptor.

The anti-FepA antibodies showed a spectrum of reactivities with gram-negative bacteria (Table 1). The 28 independently isolated MAbs ultimately resolved into 24 unique reactivities. All the antibodies reacted with FepA in seven different laboratory strains and wild isolates of *E. coli* and *Shigella boydii*. *S. paratyphi* B, *K. pneumoniae*, and *Enterobacter aerogenes* IRMP, each recognized by 16 anti-FepA MAbs, were next most closely related to *E. coli* FepA. MAb reactivity with FepA homologs in gram-negative bacteria paralleled the relatedness of these organisms (63), but exceptions to the established phylogenetic relationships were observed. For example, *E. coli* FepA surface epitopes were more conserved in *Enterobacter* than in *Salmonella* spp.

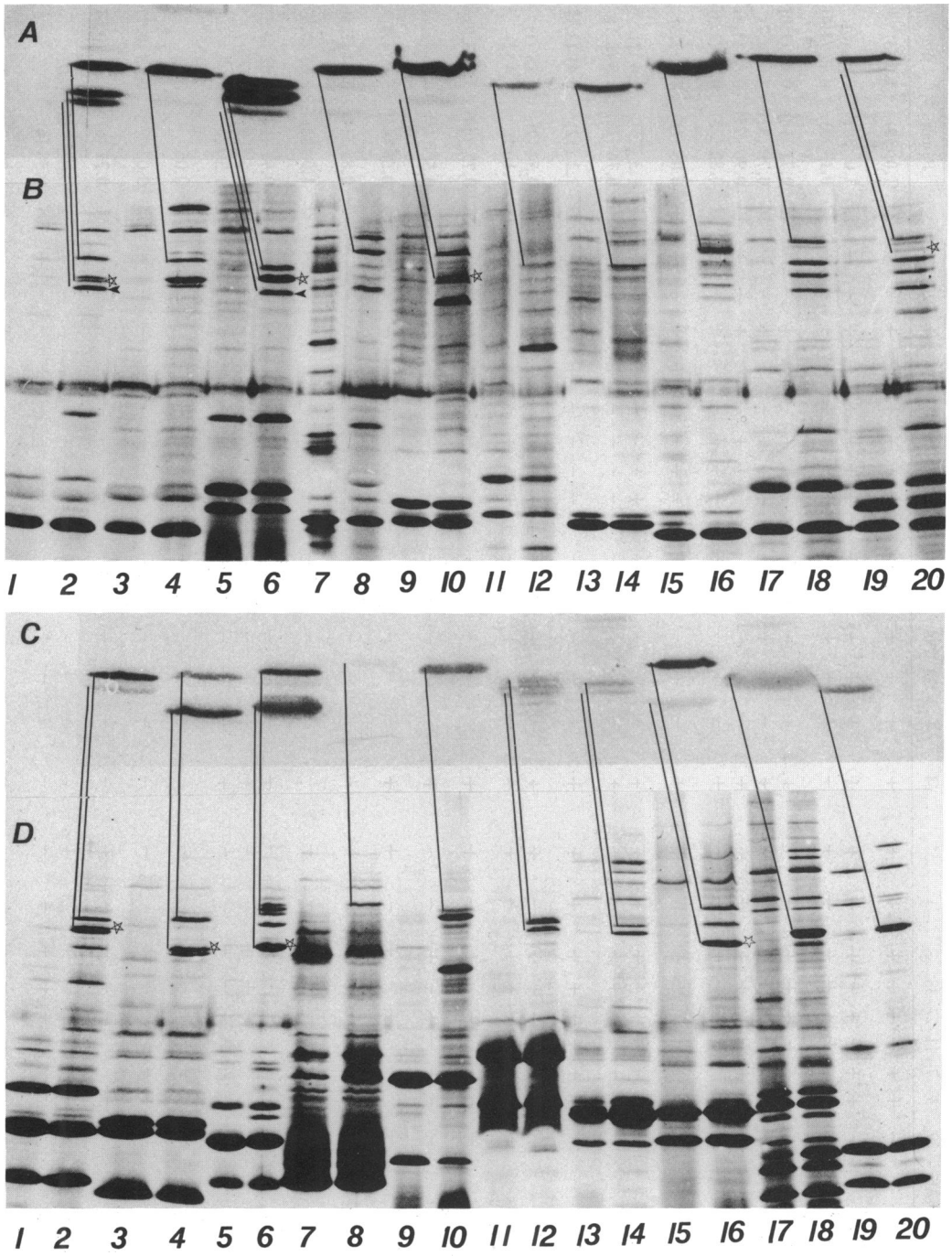

FIG. 2. SDS-PAGE resolution of Sarkosyl-extracted OM proteins and identification of OM proteins immunologically related to *E. coli* FepA in gram-negative bacteria subjected to iron stress. Bacteria were grown in either LB broth (iron sufficient) or Trypticase soy broth containing 100 μM apoferrichrome A (36) and lysed in a French pressure cell, and the cell envelope pellet was washed with 0.5% sodium sarcosinate. Whole-cell envelopes were also analyzed for comparison (data not shown). OMs were suspended in SDS-PAGE sample buffer (35) containing 0.03 M EDTA and 0.2 M NaCl and boiled for 5 min. Five and 20 μg, respectively, were loaded onto gels that were stained with ammoniacal silver (B and D) or transferred to NC and immunoblotted anti-FepA MAb 2 (A and C). Odd-numbered lanes contain iron-replete OM; even-numbered lanes contain iron-stressed OM. Proteins that reacted with MAb 2 on immunoblots are identified on the corresponding stained gels. The bacteria in panels A and B are *E. coli* (lanes 1 and 2), *S. boydii* (lanes 3 and 4), *S. typhimurium* (lanes 5 and 6), *K. pneumoniae* (lanes 7 and 8), *Citrobacter freundii* (lanes 9 and 10), *Serratia marcescens* (lanes 11 and 12), *E. aerogenes* (lanes 13 and 14), *E. tarda* (lanes 15 and 16), *Hafnia alvei* (lanes 17 and 18), and *Yersinia enterocolitica* (lanes 19 and 20). Panels C and D contain *Providencia stuartii* (lanes 1 and 2), *Morganella morganii* (lanes 3 and 4), *Providencia vulgaris* (lanes 5 and 6), *P. aeruginosa* (lanes 7 and 8), *V. cholerae* (lanes 9 and 10), *Aeromonas hydrophila* (lanes 11 and 12), *H. influenzae* (lanes 13 and 14), *H. parainfluenzae* (lanes 15 and 16), *Neisseria meningitidis* (lanes 17 and 18), and *Neisseria gonorrhoeae* (lanes 19 and 20). Starred bands (★) are analogous to 81K*, the OmpT degradation product of FepA (see text). Arrowheads (◀) mark the position of CirA in *E. coli* and *S. typhimurium*.

TABLE 1. Reactivity[a] of anti-*E. coli* FepA MAb with and siderophore uptake by gram-negative bacteria

Species	FepA residues:	Buried epitopes[b]										Surface epitopes[c]											Unknown[d]			Total	Siderophore uptake[e]	
		1–24	100–142a					100–142b		444–475	495–566	27–37	200–227		258–290		314–339				382–400		27–37					
	MAb:	29	5	7	11	27	38	2	3	57	64	6	33	34	16	44	31	35	37	45	23	24	1	20	26		Fe-ent	Fc
Escherichia coli[f]		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	24	**1.0 (39)**	**1.0 (3)**
Shigella boydii[g]		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	24	**1.0 (30)**	**0.66 (6)**
Shigella flexneri[h]		+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	+	22	**0.13 (3)**	**0.47 (1.4)**
Salmonella typhimurium[i]		+	−	+	−	+	+	+	−	+	+	−	+	+	−	−	−	−	+	−	−	+	+	−	+	13	**0.71 (18)**	**0.44 (1.0)**
Salmonella paratyphi B[j]		+	−	+	−	+	+	+	−	+	+	−	+	+	+	−	+	+	+	+	−	−	+	−	+	16	**0.79 (32)**	**2.0 (4.7)**
Salmonella typhi[k]		+	−	+	−	+	+	+	+	+	+	−	−	−	+	−	−	−	+	+	−	−	+	−	+	13	**0.96 (25)**	**0.86 (3.5)**
Salmonella panama[h]		+	−	−	−	+	+	+	−	+	+	−	+	−	−	−	−	+	+	−	−	−	+	−	+	11	**0.80 (25)**	**0.98 (1.8)**
Klebsiella pneumoniae[h]		+	−	+	−	+	+	+	+	+	+	−	+	−	−	+	−	−	+	−	+	+	+	+	+	16	**0.85 (16)**	**3.0 (2.5)**
Citrobacter freundii[h]		+	+	−	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	−	−	−	+	−	+	15	**1.3 (26)**	**1.4 (2)**
Citrobacter amatonaticus[g]		−	−	+	−	+	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	+	8	**0.76 (28)**	**2.5 (2.1)**
Serratia marcescens[g]		−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	+	+	−	−	13	**1.2 (12)**	**0.41 (2.1)**
Enterobacter aerogenes[g]		+	−	−	−	+	+	+	−	+	+	−	+	+	−	−	+	+	+	+	+	+	+	−	+	16	**0.54 (7)**	**0.75 (1)**
Enterobacter cloaceae[h]		+	−	−	−	+	+	+	−	+	+	−	+	+	−	−	+	+	+	+	+	+	−	−	+	15	**1.1 (9)**	**1.4 (2)**
Edwardsiella tarda[h]		−	−	−	−	+	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	+	6	**0.77 (44)**	**3.5 (3.9)**
Hafnia alvei[g]		−	−	+	−	+	+	+	−	−	+	−	−	−	+	−	−	−	−	−	−	+	+	−	−	8	**0.58 (43)**	**0.36 (2.4)**
Yersinia enterocolitica[g]		−	−	−	−	+	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−	+	−	+	+	6	**0.56 (43)**	**5.5 (4.4)**
Providencia stuartii[h]		−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	2	**0.89 (14)**	**0.34 (1.4)**
Morganella morganii[h]		−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	2	**1.0 (32)**	**0.65 (0.89)**
Proteus vulgaris[g]		−	+	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	2	**0.91 (14)**	**0.82 (0.52)**
Proteus rettgeri[h]		−	−	+	−	−	+	+	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	5	**1.5 (29)**	**10 (28)**
Proteus mirabilis[h]		−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	2	**1.3 (20)**	0.04
Pseudomonas aeruginosa[l]		−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	2	0.05	0.13
Vibrio cholerae[m]		+	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	4	0.02	**0.53 (0.77)**
Aeromonas hydrophila[g]		−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	1	**0.21 (6)**	**0.27 (0.61)**
Haemophilus influenzae[n]		−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	2	0.02	0.01
Haemophilus parainfluenzae[n]		−	−	−	−	+	+	+	−	−	−	−	−	+	−	−	−	+	−	−	+	+	−	−	+	8	**0.58 (6)**	0
Neisseria lactamica[n]		−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	2	**0.28 (2)**	0.12
Neisseria meningitidis[n]		−	−	−	−	−	−	+	+	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	−	5	**0.24 (6)**	0.01
Neisseria gonorrhoeae[n]		−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	3	**0.25 (5)**	0
Total		10	7	10	4	16	14	27	7	14	19	6	9	6	6	3	4	8	10	5	5	11	11	6	13			

[a] Iron-deficient bacterial cell lysates were suspended in SDS-PAGE sample buffer, boiled, electrophoresed in sequential lanes of 11% slab gels, and transferred to NC. Pieces of NC paper containing approximately 20 different gram-negative bacterial cell lysates were blocked with TBSG and incubated overnight with anti-FepA MAb, washed, incubated overnight with goat anti-mouse immunoglobulin-alkaline phosphatase, washed, and developed with nitroblue tetrazolium-bromochloroindoyl phosphate. Reactions were scored as negative (−), weakly positive (+), or strongly positive (+) in comparison with the intensity of reaction with *E. coli* BN1071 (Fig. 1) and negative controls. For totals, averages, and statistical comparisons of epitope conservation among gram-negative bacteria (see Results), the two *Shigella* strains and the two *Enterobacter* strains were considered identical, hence the number of distinct gram-negative species in the analysis was 27.

[b] Epitopes with a high probability of localization in the OM bilayer (see text).

[c] Epitopes previously demonstrated as cell surface exposed by cytofluorimetry (51).

[d] Epitopes currently unclassified with regard to their location on native FepA.

[e] The uptake of [^{59}Fe]enterobactin (Fe-ent) and [^{59}Fe]ferrichrome (Fc) was normalized to that observed for *E. coli* BN1071; parenthetic values are the fold induction of siderophore uptake caused by iron stress. Boldface type indicates uptake at a level that was more than twice the uptake by controls.

[f] Seven different strains of *E. coli* were assayed, including BN1071 (36), serotypes O1K$^-$, O26, O55, O75K$^-$ (29), and strains 0000 and 065, provided by M. Spengler.

[g] Provided by J. Dankert (29).

[h] Provided by Ethel Oldham.

[i] Five strains of *S. typhimurium*, including LT2, SL1292, and SL1420, provided by B. A. D. Stocker; BH10, provided by Ethel Oldham; and Vi260, provided by J. Dankert.

[j] Strain SL5205, provided by B. A. D. Stocker.

[k] Strain 205aTy, provided by B. A. D. Stocker.

[l] Strain PAO1, provided by D. Frank.

[m] Strain JBK-70, provided by J. Kaper.

[n] Provided by L. W. C. Cashdollar.

Noteworthy in this regard was *H. parainfluenzae*, which contained an IRMP that reacted strongly with eight anti-FepA MAbs.

Individual species within a genus were usually equivalent to one another in their reactivity with the MAb test panel (*Shigella*, *Enterobacter*, *Salmonella* spp.), but again, exceptions were observed (*Citrobacter*, *Proteus*, *Haemophilus* spp.). Such discrepancies were not caused by differences in the level of IRMP expression between different strains (which was monitored by SDS-PAGE) and were verified by repeated trials. The disparate strains were not misidentified, because the cell envelope protein profiles of species within a genus were identical to one another, yet distinct from all other genera tested (Fig. 2). Hence, in these instances significant variation in OM protein structure occurred in different species within the same genus. The level of IRMP expression sometimes varied within a genus (the best example was reduced expression of FepA in *S. flexneri* relative to *S. boydii*), which was reflected on immunoblots as a difference in the degree of positivity rather than an absence of reactivity (Table 1).

Discrimination of FepA epitopes. The differential reactivity of anti-FepA MAbs with the collection of gram-negative bacteria usually discriminated antibodies that recognize the same general region of FepA sequence. Each of the 24 unique anti-FepA MAbs produced a distinctive pattern of reactivity with the gram-negative bacteria that was consistent with other previously reported characterizations (51). For example, MAbs 23 and 24, which both bind FepA in region 382 to 400 but differ from one another in their abilities to inhibit ligand binding, showed different patterns of reactivity with the collection of bacteria.

The most cross-reactive MAbs bound epitopes of FepA that are ostensibly buried within the OM bilayer (51). The determinant recognized by MAb 2 in region 100 to 142 of *E. coli* FepA was conserved in all the organisms. Several other buried epitopes appeared in many of the bacteria (MAb 26, 13 species; MAb 27, 16; MAb 38, 14; MAb 57, 13; MAb 64, 19). The mean conservation of the buried determinants of *E. coli* FepA ($\bar{x}$ = 12.6) differed from the mean conservation of its surface epitopes ($\bar{x}$ = 6.6) at a level of significance of 0.02 ($t_{0.02(2),19}$ = 2.76). This comparison was skewed by a few highly preserved buried polypeptides, but if the two most conserved buried epitopes, identified by MAbs 2 and 64, were excluded from statistical calculations on the assumption that they may function in ferric enterobactin uptake and hence be structurally unique, the mean conservation of the remaining buried epitopes ($\bar{x}$ = 10.13) still differed from the mean conservation of surface epitopes, at a level of significance of 0.05 ($t_{0.05(2),17}$ = 2.31). These data imply that although structural evolution has occurred in the buried polypeptides of FepA (MAbs 3, 5, and 11 in Table 1), overall buried epitopes are more conserved among gram-negative bacteria than surface epitopes, and specific buried sequences may be universally conserved.

Ferric enterobactin transport by gram-negative bacteria. In light of the extensive conservation of FepA epitopes throughout the families of gram-negative bacteria, it was of interest to determine the ability of individual species to transport ferric enterobactin. Bacteria were cultured in iron-replete broth and subjected to iron stress by subculture into analogous iron-deficient media. The uptake of [^{59}Fe]enterobactin was measured for both iron-replete and iron-deficient cells of each strain; transport of [^{59}Fe]ferrichrome was also determined to provide a basis for comparison. As suggested by Western blots with anti-FepA MAbs and

SDS-PAGE of iron-replete and iron-deficient outer membranes, (i) most of the bacteria within the collection transported ferric enterobactin, and (ii) iron stress augmented the rate of enterobactin accumulation 20- to 40-fold (Table 1). Among bacteria closely related to *E. coli*, the only exception to this rule was *S. flexneri*, which transported ferric enterobactin at low levels even when subjected to iron stress. A few distantly related bacteria did not accumulate ferric enterobactin after growth in iron-deficient media, including *Pseudomonas aeruginosa* (see below), *Vibrio cholerae*, and *Haemophilus influenzae*, despite the fact that these strains contained an iron-regulated OM protein that was recognized by anti-FepA MAbs. The growth of *P. aeruginosa* in medium supplemented with ferric enterobactin, however, did significantly augment the uptake of [^{59}Fe]enterobactin by this organism (Table 1), in agreement with a recent report (60).

Flow cytometric analysis of MAb binding to external surface epitopes of *S. typhimurium* and *S. flexneri* FepA. *E. coli* LPS O-antigen and core sugars restrict the reactivity of antibodies with surface epitopes of FepA and other OM proteins (5, 51). Surface-reactive anti-*E. coli* FepA MAbs did not bind to the surface of *S. flexneri* or *S. boydii*, even though almost complete conservation of *E. coli* FepA epitopes was observed in the *Shigella* strains on immunoblots (Table 2). As expected, though, expression of *S. flexneri fepA* in *E. coli* K-12 *rfa*$^+$ or *rfaD* strains resulted in FepA-dependent MAb binding to intact bacteria. The influence of LPS on MAb binding to *S. flexneri* FepA in *E. coli* paralleled that observed previously for *E. coli* FepA (51), indicating that in these two bacteria the surface epitopes of the ferric enterobactin receptor are virtually identical in composition and topology. The exceptions to this conclusion were surface determinants in regions 27 to 37 (MAb 6) and 200 to 227 (MAb 34). MAb 6 reacted only weakly with *S. flexneri* FepA on immunoblots; flow cytometric experiments may lack the sensitivity to demonstrate the adsorption of MAb 6 to *S. flexneri* in vivo. MAb 34 did not bind to *rfaD* bacteria expressing *S. flexneri* FepA, although it reacted strongly with the protein on Western blots. The epitope recognized by MAb 34 is apparently not surface localized in *S. flexneri* FepA. MAb 33, which also binds an epitope in region 200 to 227, did react with a surface epitope of *S. flexneri* FepA in deep rough *E. coli*, indicating that part of this region of the *Shigella* receptor remains localized on the cell surface.

Smooth and rough variants of *S. paratyphi* B, *S. typhi*, and *K. pneumoniae*, subjected to iron stress, were also tested for cytofluorimetric reactivity with the anti-FepA MAb panel. No binding was observed to either *rfa*$^+$ or *rfa* strains of these three species (data not shown), despite the fact that strong binding of certain surface-reactive anti-*E. coli* FepA MAbs to these proteins was observed in Western blots. However, the exact nature of the *rfa* mutations carried by rough strains (LPS-specific bacteriophage-resistant mutants) of these species was unknown, raising the possibility that their core structures are fully or partially intact. In *S. typhimurium*, on the other hand, the availability of well-defined *rfa* mutations permitted the analysis of its FepA surface epitopes. No significant anti-FepA reactivity was seen with *rfa*$^+$ (SL3840), *rfaF* (*Rd*$_2$ chemotype; SL3789), or *rfaE* (*R*$_e$ chemotype; SL1102) derivatives of *S. typhimurium* LT2, even though MAbs 24, 33, 34, and 37 recognized *S. typhimurium* FepA on immunoblots. These data suggest a significant change in the surface topology of the *S. typhimurium* ferric enterobactin receptor, relative to the comparable proteins of *E. coli* and *S. flexneri*.

TABLE 2. Cell surface-exposed epitopes of *S. flexneri* and *S. typhimurium* FepA[a]

Strain	FepA residues:	Buried epitopes										Surface epitopes												Unknown	
		1–24	100–142a				100–142b		444–475	495–566	27–37	200–227		258–290		314–339			382–400		27–37			
	MAb:	29	5	7	11	27	38	2	3	57	64	6	33	34	16	44	31	35	37	45	23	24	1	20	26
E. coli																									
KDL118(pITS449)		–	–	–	–	–	–	–	–	–	–	–	–	–	**58**	**58**	**52**	**58**	**61**	45	–	–	–	–	–
KDF29(pITS449)		–	–	–	–	–	–	–	–	–	**72**	**80**	**57**	46	**90**	**110**	**83**	**92**	**96**	**84**	**71**	–	–	–	–
KDL118(pMPS13)		–	–	–	–	–	–	–	–	–	–	–	–	–	**51**	**57**	**50**	**57**	**60**	–	–	–	–	–	–
KDF29(pMPS13)		–	–	–	–	–	–	–	–	–	–	**64**	–	–	**88**	**113**	**96**	**103**	**81**	**87**	**56**	–	–	–	–
S. typhimurium																									
SL3840		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SL3789		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SL1102		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] *E. coli* KDL118 (*fepA rfa*$^+$) and KDF29 (*fepA rfaD*) containing pITS449 (*E. coli fepA*$^+$) or pMPS13 (*S. flexneri fepA*$^+$) and *S. typhimurium* SL3840 (*fep*$^+$ *rfa*$^+$), SL3789 (*fep*$^+$ *rfaD1*), and SL1102 (*fepA*$^+$ *rfaE*) were grown in T medium to mid-log phase, incubated with anti-FepA MAbs, stained with fluoresceinated goat anti-mouse immunoglobulin, and analyzed in the flow cytometer. Buried and surface epitopes were designated as in Table 1. For positive samples, the mean peak fluorescence intensity of the cell population is given; bold print indicates that the mean peak fluorescence intensity is greater than twice the level established by negative control bacterial (KDL118 and KDF29), which averaged 24; other MAbs gave mean peak fluorescence intensities indistinguishable from background levels (–).

DISCUSSION

Because the ferric enterobactin receptor is an integral OM protein that interacts with its ligands (and can be bound by antibodies) in the environment, it contains both membrane bilayer-spanning domains (51) and external cell surface domains. In addition, if FepA physically interacts with periplasmic proteins that are necessary for ferric enterobactin uptake, then it also contains residues that are localized at the periplasmic face of the OM bilayer. The peptides in these dissimilar regions of FepA structure are subject to thermodynamic and physiological constraints that restrict their amino acid composition. Surface polypeptides must possess an overall hydrophilicity that facilitates their interaction with the aqueous interfaces of the outer membrane. For external cell surface residues, associations with the core and O-antigen sugars of LPS may exert further requirements for hydrophilicity. Polypeptides that traverse the OM bilayer, on the other hand, associate with phospholipid and LPS fatty acids (54, 62) and must therefore include residues of sufficient hydrophobicity to stabilize this interaction. Enteric bacterial porins, for example, contain transmembrane β-strands that possess a hydrophobic face that interacts with the OM lipids on the porin exterior and a hydrophilic face that lines the surface of the water-filled channel in the porin interior (11, 35, 68). FepA contains a similar series of amphiphilic β-strands, proposed to face the bilayer lipids on one side and a hydrophilic region of the FepA interior on the other (51). Superimposed upon these underlying thermodynamic restrictions, certain FepA residues bind ferric enterobactin and/or colicins or interact with other cell envelope proteins (TonB, ExbB, FepB). These factors imply that evolutionary changes will progress more rapidly in the membrane surface domains of FepA (with the predicted exception of ligand-binding sites) than in its transmembrane polypeptides, because a greater variety of substitutions can be accommodated in surface regions without disruption of receptor protein structure. For example, mutations that substitute a hydrophobic residue for a hydrophilic one will likely be tolerated in FepA's external surface polypeptides because of the inability of a single residue to alter the immense mean hydrophilicity of such regions. The fact that an assortment of hydrophobic amino acids occurs throughout the known external surface epitopes of FepA supports this contention. Conversely, natural selection will act against antithetical substitutions in bilayer lipid-contiguous residues of a transmembrane β-strand, because such mutations may disrupt ferric enterobactin transport, as a result of perturbation of receptor structure, increased susceptibility to OM proteases, or improper localization within the cell envelope. The data reported herein on the immunochemical structure of FepA in gram-negative bacteria support these intuitive arguments; the surface epitopes of FepA have in fact diverged more than its buried epitopes.

Antibodies to continuous epitopes (64) are sensitive indicators of residue substitution in proteins because they recognize short polypeptide sequences (22, 23), and single-residue substitutions may completely abolish recognition by a particular antibody combining site (1). The primary source of uncertainty in the immunochemical analysis of FepA epitopes is the accuracy of the designation of buried epitopes. Cytofluorimetry unambiguously identifies surface epitopes, but no such methodology exists to measure antibody binding to buried epitopes. Hence, buried epitopes must be identified by inference. That is, transmembrane residues of OM proteins are inaccessible to antibody binding on either the external or periplasmic surfaces of the OM bilayer; putative buried epitopes of FepA were assigned in this way (51). However, globular surface domains of a membrane protein may shield its other surface epitopes from MAb binding, and hence the failure of an antibody to bind the periplasmic and external surface residues of FepA does not conclusively identify its epitope as buried. For many putative buried epitopes, however, knowledge of amino acid sequence composition mitigates uncertainty about their localization. In the FepA epitope within region 1 to 24, for example, the first 12 residues of this sequence are significantly hydrophilic and incapable of insertion into a membrane bilayer. The fact that the remaining 14 residues contain a predicted β-strand surface of mean hydrophobicity 0.51 (51) and that MAb 29, which recognizes this determinant, does not bind to the surface of either intact bacteria or inverted OM vesicles implies that the epitope is in fact buried. Similar arguments can be made for epitopes in regions 142 to 178, 444 to 475, and 495 to 566. So although presently we cannot assign buried epitopes with complete certainty, these assignments have a high probability of correctness. The conclusion that surface and buried epitopes evolve differently in gram-negative bacteria supports the idea that the constituent peptides of these two epitope categories are structurally and functionally dissimilar, as predicted by the model of ferric enterobactin receptor secondary structure (51).

The extensive conservation of buried epitopes within regions 100 to 142 (MAb 2) and 495 to 566 (MAb 64) suggests that polypeptides in these regions are crucial to receptor structure or the passage of ferric enterobactin through the OM bilayer. Because of their conservation, buried epitopes may act to broadly immunize animals against infection by gram-negative bacteria (47). IRMPs are immunogenic and protective in vivo (6, 7, 13, 20, 28, 58, 71), but the relative contributions of surface and buried epitopes of OM proteins to such protection are unknown. The function of antisera to OM proteins in defense against bacterial infection is paradoxical, because their surface epitopes are masked by LPS and capsule and their buried epitopes are protected by the structural integrity of the OM. Yet serum samples from convalescent individuals infected with gram-negative bacteria show reactivity with OM proteins of the pathogen (7, 20, 39, 71). Such humoral immunity may act during infection to clear bacterial debris from circulation (19, 76) rather than in the initial recognition and opsonization of the pathogen.

Antibody binding to certain surface epitopes of *E. coli* FepA inhibits the interaction of the receptor with its ligands, ferric enterobactin and colicins B and D, but none of these epitopes, recognized by MAbs 23, 31, 34, 35, 37, 44, and 45, are generally conserved among the gram-negative bacteria that utilize ferric enterobactin. Several surface epitopes, recognized by MAbs 24, 33, 35, and 37, were present in closely related bacteria, but it seems unlikely that any of these determinants contain the microdomains involved in ferric enterobactin recognition, because numerous bacteria that utilize the siderophore do not possess these epitopes. These data suggest that MAb inhibition of ligand interactions within regions 200 to 227, 290 to 339, and 382 to 400 occurs by antibody binding to FepA residues that are in close proximity to, but distinct from, its ferric enterobactin-binding sites.

An unexpected finding of this study was the deviation of surface topology and tertiary structure in *S. typhimurium* FepA, relative to the *E. coli* ferric enterobactin receptor. The *S. typhimurium* protein was more heavily proteolyzed,

recognized significantly less in Western blots by the MAb panel, and unreactive with MAbs to *E. coli* FepA surface epitopes. Although the inability of anti-FepA MAbs to bind *S. typhimurium* could be caused by steric hindrance from capsule or some other currently unidentified OM component, this seems unlikely because deep rough strains were utilized, which do adsorb anti-OmpF MAbs (5). It is therefore likely that extensive evolution of surface topology has occurred in *S. typhimurium* FepA relative to the *E. coli* ferric enterobactin receptor. This finding is perhaps not surprising, given the deviations in primary structure between the two proteins that are evident from Western blots with the MAb panel.

The ferric enterobactin receptor, or some significant structural remnant of it, appears conserved in all the bacteria we have studied. Although we cannot conclude with certainty that each of these FepA homologs is an authentic ferric enterobactin receptor protein, the uptake of [^{59}Fe]enterobactin by such organisms is evidence for this relationship. Reactivity with the library of anti-FepA MAbs diminished precipitously for bacteria less related to *E. coli* than *Enterobacter* spp., but ferric enterobactin transport was observed in several distant organisms, including *Proteus* spp., *H. parainfluenzae*, and *Neisseria* spp. Although *Neisseria* spp. accumulated ferric enterobactin only 25% as efficiently as *E. coli*, we believe that these data are biologically relevant. Preincubation of the gonococci with KCN eliminated [^{59}Fe]enterobactin uptake, and ferric enterobactin stimulated the growth of iron-deficient *Neisseria* spp. (data not shown). The finding that distantly related bacteria contain an OM protein that transports the native *E. coli* siderophore but possesses only slight structural homology to *E. coli* FepA indicates that extensive alteration of ferric enterobactin receptor structure can occur without disrupting the protein's stability in the OM or its ability to recognize and transport ferric enterobactin.

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