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Permeability properties of a large gated channel within the ferric enterobactin receptor, FepA

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ABSTRACT **FepA is an *Escherichia coli* outer membrane receptor protein for the siderophore ferric enterobactin. Prior studies conducted *in vivo* suggested that FepA and other TonB-dependent outer membrane proteins transport ligands by a gated-channel mechanism. To corroborate and extend these findings we have determined the permeability properties of the FepA channel *in vitro*, by measuring the diffusion rates of hydrophilic nonelectrolytes through the FepA channel in liposome swelling experiments. Like porins, the FepA deletion mutant ΔRV showed a size-dependent permeability to oligosaccharides, indicating that it forms a nonspecific, hydrophilic pore. Unlike OmpF and other *E. coli* porins, however, ΔRV proteoliposomes transported stachyose (666 Da) and ferrichrome (740 Da). These data, and other uptake results with a series of maltodextrins of increasing size, confirm the existence of a channel domain within FepA that is considerably larger than OmpF-type pores. These results represent a reconstitution of the channel function of a TonB-dependent receptor protein and establish that FepA contains the largest channel that has been characterized in the *E. coli* outer membrane.**

Nutrients traverse the Gram-negative outer membrane through several different pathways. Small hydrophilic solutes enter through porins such as OmpF, which form rigid, nonspecific, water-filled channels across the bilayer (1–3). Certain molecules that are too large to pass through such archetypal porins enter through specialized porins that contain solute-binding domains within their transmembrane channels (1, 4). Another class of large nutrilites, metal chelates, penetrate the outer membrane through a third, ostensibly energy-dependent uptake pathway (5–7). These molecules, which include siderophores (greek, "iron bearer") and vitamin B_{12}, pose a nutritional paradox: they are required for metabolism but are present in the environment in very low concentrations. Furthermore, their relatively large size (600–1200 Da) exceeds the diffusion limit of known *Escherichia coli* porin channels. Enteric bacteria cope with this dilemma by inserting high-affinity receptor proteins for metal chelates into the outer membrane (5, 6). The prototypic siderophore receptor is FepA, an outer membrane protein that specifically binds and transports the indigenous *E. coli* siderophore ferric enterobactin (8). Uptake of ferric enterobactin through the outer membrane requires the function of another cell envelope protein, TonB (7), and additional proteins (ExbB, ExbD) that may stabilize TonB (6, 9). Neither the transport mechanism of TonB-dependent receptor proteins nor the participation of TonB itself in this reaction is fully understood. However, Rutz *et al.* (10) showed that internal deletions within FepA converted the high-affinity receptor into a nonspecific, TonB-independent, diffusion channel. These data suggested that TonB-dependent receptor proteins are gated porins that bind metal chelates within a cell surface domain and open in response to interaction with TonB to release the ligand into an underlying membrane channel.

The gated-porin model of FepA transport originated from *in vivo* experiments, but in this report we have incorporated either purified FepA or a FepA deletion mutant protein into artificial liposomes. *In vitro* swelling experiments with these vesicles demonstrated the existence of a large hydrophilic channel within FepA, at least twice the diameter of the pore formed by OmpF. These data establish that TonB-dependent outer membrane proteins are gated porins: their tertiary structure contains a cell surface ligand-binding domain that selectively controls transport through a large underlying channel.

MATERIALS AND METHODS

Bacterial Strains. All bacteria were *E. coli* K-12 strains. KDF541 (10) is *fepA, tonA*, and *cir*. In some experiments a strain that is deficient in the expression of the OmpF and OmpC porins was utilized; KDF781 is a *fepA, cir* derivative of HN705 (*ompF, ompC*; obtained from Hiroshi Nikaido, University of California, Berkeley). KDF810 is a *fepA, cir* derivative of the LamB-deficient strain MCR106 (11). KDF781 and KDF810 were made FepA-deficient and Cir-deficient by sequential selection for spontaneous resistance to colicins B and Ia, respectively.

Reagents. Saccharides were purchased from Sigma, maltodextrins from Boehringer Mannheim, and Detran T-40 from Pharmacia. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and egg L-α-phosphatidyl-DL-glycerol (EPG) were purchased from Avanti Polar Lipids. Ferrichrome was purified from culture supernatants of *Ustilago sphaerogena* (12).

Outer Membranes, Protein Purification, and Liposome Preparation. For preparation of outer membranes bacteria were grown in Trypticase soy broth containing 100 μM apoferrichrome A (12). Bacteria were collected and lysed in a French pressure cell, and outer membrane fragments were purified by sucrose gradient centrifugation (13).

For protein purification, bacteria were grown to late exponential phase in Mops-buffered medium with appropriate antibiotics (10). To minimize contamination with porins during purification, KDF781 (*ompF, ompC*) was used as host for plasmids carrying *fepA*$^+$ or *fepAΔRV* alleles. After cell lysis the envelope fractions were examined by SDS/PAGE and Western immunoblot for the presence of OmpF, OmpC, and NmpC with anti-OmpF/C monoclonal antibody 35 (recognizes OmpF, OmpC, and NmpC; ref. 13). Although neither OmpF nor OmpC porins were found, in some instances a 36-kDa outer membrane protein was observed, which was most likely an Nmp porin (1). These membranes were discarded and new cultures were initiated from isolated single

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Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; EPG, egg L-α-phosphatidyl-DL-glycerol.

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colonies. This procedure was repeated until no Nmp porin was seen in the cell envelope. OmpF was purified as described previously (13). FepA and ΔRV were purified by differential extraction of iron-deficient outer membranes with Triton X-100, followed by successive ion-exchange and gel-filtration chromatography (14–16). Protein concentrations were determined by the Lowry procedure (17), with 2% SDS present for samples containing Triton X-100. FepA, ΔRV, and OmpF were >95% pure as estimated by densitometric analysis (AMBIS Systems) of Coomassie blue-stained gels (Fig. 1).

For liposomes, DOPC (9 μmol) and EPG (1 μmol) in chloroform were dried under a soft stream of N_2 to form a thin film on the bottom of a test tube, which was kept at 25°C *in vacuo* for 10 hr. Outer membrane fragments (20 μg of protein) or purified proteins (1–40 μg) in 100 μl of distilled water were added to the tube, which was then vortexed, dried under vacuum, and incubated for 20 min *in vacuo*. Dextran T-40 (0.6 ml of a 15% solution in 5 mM Tris·HCl, pH 7.2) was added to the phospholipids, incubated at 37°C for 45 min, and shaken vigorously by hand 30 times while turning the tube to suspend the vesicles. The tube was returned to 37°C for 20 min, and aliquots of the vesicle solution (usually 10 μl) were diluted into 300 μl of test sugars at the appropriate concentration (usually ≈35 mM). Control vesicles without protein were formed similarly and used in each experiment to confirm that the observed swelling was caused by the incorporated outer membrane proteins.

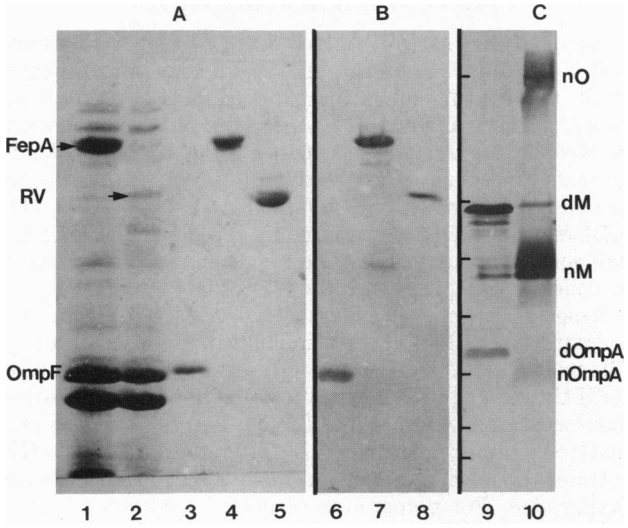

FIG. 1. Purification of FepA, ΔRV, and OmpF. (*A*) Outer membranes from KDF541/pITS449 (lane 1) and KDF541/pΔRV (lane 2), or purified OmpF, FepA, or ΔRV proteins (lanes 3–5, respectively), were subjected to denaturing SDS/PAGE and stained with Coomassie blue. (*B*) Purified OmpF (37 kDa), FepA (81 kDa), and ΔRV (64.5 kDa) (lanes 6–8, respectively) were subjected to denaturing SDS/PAGE, electrophoretically transferred to nitrocellulose, and immunoblotted with a mixture of anti-FepA monoclonal antibody 29 (12) and anti-OmpF/C monoclonal antibody 35 (13). (*C*) Native FepA oligomers. Outer membranes from *E. coli* strain KDF541/pITS449, grown in Mops medium, were solubilized with 1% lithium dodecyl sulfate/30 mM EDTA/0.1% lysozyme at 0°C, either boiled (lane 9) or not boiled (lane 10), and subjected to PAGE at 2°C (12). After electrophoresis, the slab gel was heated to 100°C for 5 min and the proteins were electrophoretically transferred to nitrocellulose and immunoblotted with a mixture of anti-FepA monoclonal antibody 29 and anti-OmpA monoclonal antibody 19 (12). The positions of native FepA oligomer (nO), native FepA monomer (nM), and denatured FepA monomer (dM) are indicated. Native (n) and denatured (d) OmpA are also indicated for reference. Horizontal bars in *C* mark the position of molecular size markers (Bio-Rad): top to bottom, myosin (200 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (68 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa).

Liposome Swelling Assays. The relative rates of sugar permeation into liposomes were calculated from the initial rates of vesicle swelling upon dilution into isotonic solutions of solutes (18, 19). For saccharides, changes in turbidity were monitored at 450 nm with a Beckman DU model 64 spectrophotometer. For ferrichrome, changes in turbidity were measured at 650 nm, because its peak of visible absorption (λ_{max} 425 nm) obscured measurements at 450 nm.

RESULTS

Permeability of Liposomes Containing ΔRV Outer Membranes. Using swelling assays we evaluated the ability of stachyose to permeate into DOPC liposomes containing ΔRV outer membrane fragments. It was difficult to prepare outer membranes that were completely porin-free, due to the tendency of KDF781 to revert to porin expression ($NmpC^+$). However, the tetrasaccharide stachyose is too large to penetrate the OmpF, OmpC, PhoE, and NmpC porins (18, 19). Since the putative FepA channel was found to be larger than that of OmpF (10), we expected outer membranes from bacteria expressing ΔRV to confer permeability to stachyose. Outer membranes from KDF541/pΔRV transported stachyose into liposomes, while outer membranes containing OmpF or FepA did not (Fig. 2*A*). These data show the existence of a nonspecific hydrophilic channel within ΔRV.

Permeability of Liposomes Containing Purified FepA and ΔRV. The molecular dimensions of porin channels have been accurately estimated from the dependence of solute diffusion rates on solute molecular size (1, 18). Since stachyose diffused passively through ΔRV, we performed experiments to estimate the dimensions of the ΔRV pore. In these studies liposome swelling was dependent on the presence of ΔRV in the vesicles (Fig. 2*B*); 1 μg of purified ΔRV conferred permeability to arabinose, and increasing concentrations of ΔRV increased the swelling rate in nearly linear fashion. In both isotonic (Fig. 2 *C* and *D*) and hypertonic (ref. 20; data not shown) sugar solutions, purified ΔRV showed a size-dependent permeability to saccharides that demonstrated the presence of a large channel with an exclusion limit approximately twice that of OmpF (18, 19) and comparable to that of the OprF channel (20). Ten micrograms of ΔRV was used for these measurements. The rate of swelling conferred by ΔRV was less than that for OmpF, but unlike OmpF, FepA and ΔRV are susceptible to thermal denaturation at room temperature (P.E.K., unpublished data). It was not possible to measure the concentration of native ΔRV in liposomes, but we suspect that a significant fraction of the ΔRV was denatured during purification, which may explain its low specific activity relative to OmpF. If liposomes without protein or containing denatured ΔRV (by boiling in SDS and purification by acetone precipitation) were prepared, then no swelling was observed, confirming that native ΔRV conferred permeability on the vesicles. Since prior experiments showed that ferrichrome can pass through ΔRV *in vivo*, we tested the permeability of ΔRV-containing vesicles to ferrichrome (740 Da; the molecular mass of ferric enterobactin is 716 Da). As anticipated, ferrichrome passed through the ΔRV channel into liposomes (Fig. 2*D*). Vesicles containing FepA, either purified or as a component of outer membranes, were generally impermeable to saccharides but showed some slight uptake of the smallest sugar tested, arabinose (data not shown).

Structure of Native FepA. Since FepA contains a channel domain that is presumed comparable in structure (12), and demonstrated comparable in function, to the channels of other porins, we investigated the possibility that FepA exists as a trimer *in vivo*. Outer membranes from bacteria express-

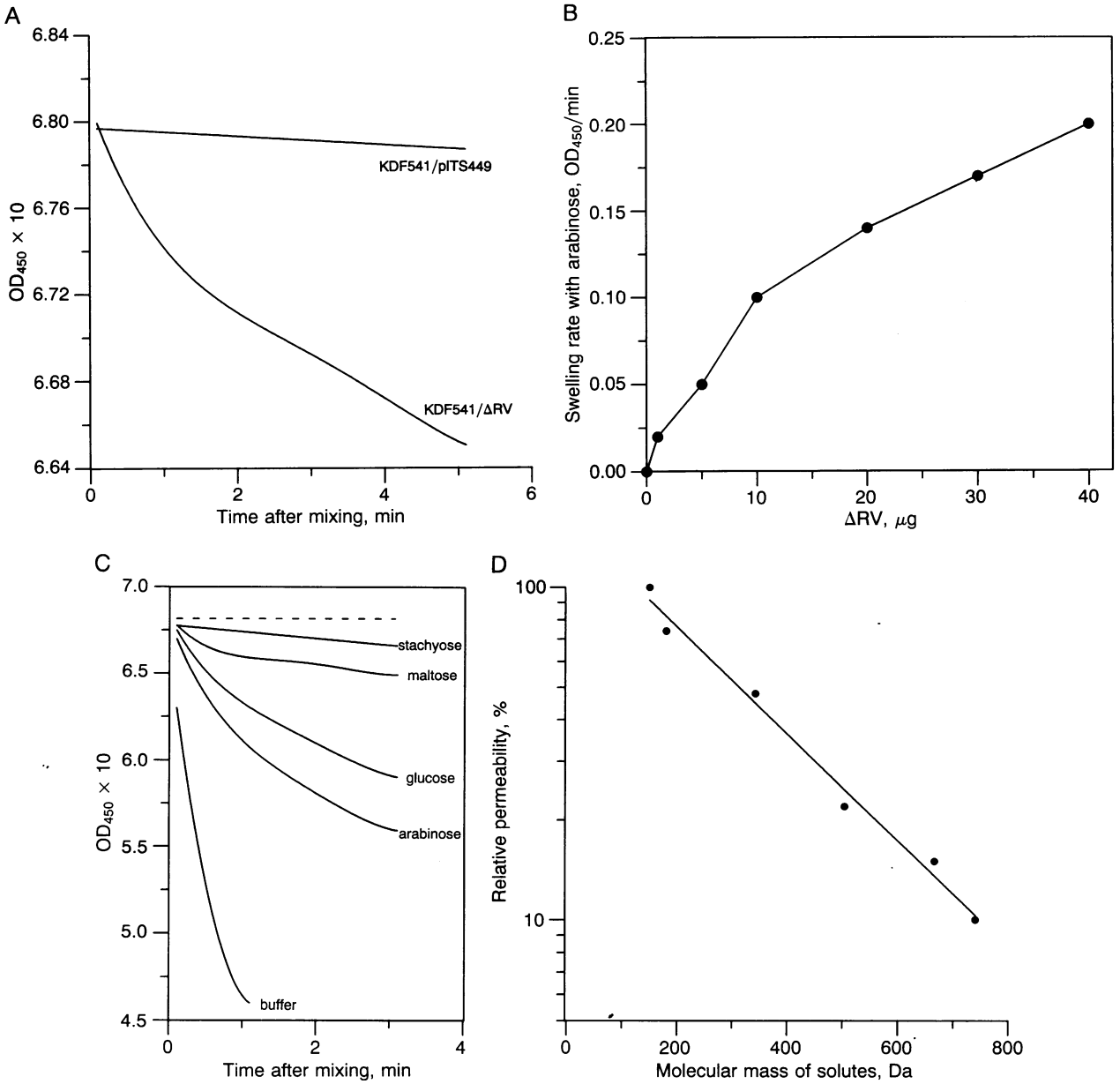

FIG. 2. (*A*) Swelling experiments with proteoliposomes containing outer membrane fragments. Outer membranes from KDF541/pITS449 and KDF541/pΔRV were prepared and incorporated into liposomes, as described in *Materials and Methods*. The proteoliposomes were suspended in isotonic suspensions of stachyose and changes in the turbidity of the solution were monitored at 450 nm. (*B*) Dependence of swelling rate of proteoliposomes on the amount of ΔRV. Various amounts of ΔRV (0–40 μg) were reconstituted with 10 μmol of DOPC/EPG, 9:1 (mol/mol). Aliquots of the proteoliposome suspensions were diluted into isoosmotic arabinose, and the initial rates of change in OD_{450} were measured. Data points are the average of five experiments. (*C*) Swelling experiments with proteoliposomes containing purified ΔRV. Multilamellar vesicles containing 10 μg of ΔRV and 10 μmol of DOPC/EPG, 9:1, were prepared and suspended in isotonic solutions of stachyose, maltose, glucose, or arabinose. Changes in turbidity were measured at 450 nm. Vesicles without protein were also prepared (dashed line) and did not show significant swelling in the sugar solutions used. (*D*) Relative rates of solute penetration into proteoliposomes. The initial rates of sugar and siderophore uptake into ΔRV-liposomes were measured as in *A* and plotted relative to the rate of uptake of the smallest sugar tested, arabinose, as described by Luckey and Nikaido (4). The compounds tested were: arabinose (150 Da), glucose (180 Da), maltose (342 Da), raffinose (504 Da), stachyose (666 Da), and ferrichrome (740 Da). Each data point represents the average of three experiments.

ing FepA were subjected to nondenaturing lithium dodecyl sulfate/PAGE (12) and Western immunoblot with an anti-FepA monoclonal antibody. Two native forms of FepA were identified by the antibody (Fig. 1): a compact, presumably native monomer (apparent molecular mass equal to that of a protein of 63 kDa) and a high molecular mass oligomer. The relative mobility of the oligomer was consistent with that of a trimer of the native monomer—a protein of ≈200 kDa. Native structures were also seen for ΔRV, but at lower levels, suggesting that it is more sensitive to denaturation by dodecyl sulfate (data not shown).

Estimation of Pore Dimensions *in Vivo*. To substantiate the *in vitro* estimation of pore diameters, plasmids containing mutant *fepA* alleles were transformed into a *lamB* genetic background, and the transformants were tested for their ability to transport a series of linear maltodextrins, from maltose to maltohexose, through the outer membrane. Because maltodextrins larger than maltotriose cannot traverse OmpF or OmpC channels, LamB-deficient *E. coli* cannot grow on maltodextrins larger than maltotriose. Benson *et al.* (11) have used this approach to determine the exclusion limits of deletions in the transverse peptide loop of OmpF. When

Table 1. Growth of bacteria expressing FepA deletion mutants on linear maltodextrins

Strain	Plasmid	Growth on maltodextrins					
		1	2	3	4	5	6
MC4100 (*ompF*$^+$, *lamB*$^+$, *fepA*$^+$)	None	+	+	+	−	−	−
MCR106 (*ompF*$^+$, *lamBΔ106*, *fepA*$^+$)	None	+	+	−	−	−	−
OC115 (*ompFΔ115*, *lamBΔ106*, *fepA*$^+$)	None	+	+	+	+	+	+
KDF810 (*ompF*$^+$, *lamBΔ106*, *ΔfepA*)	None	+	+	−	−	−	−
	pfepA$^+$	+	+	−	−	−	−
	pΔMC	+	+	+	+	+	+
	pΔRV	+	+	+	+	+	+
	pΔH261	+	+	−	−	−	−

E. coli K-12 strains MC4100, MCR106, or OC115 (11) or KDF810 carrying pUC18 derivatives with *fepA*$^+$, *fepAΔMC*, *fepAΔRV*, or *fepAΔH261* (10) were plated on M63 minimal medium containing glucose, maltose, maltotriose, maltotetraose, maltopentose, or maltohexose (columns numbered 1–6, respectively) at 0.6%. Colony formation was evaluated after 48 hr.

this assay was applied to the collection of FepA mutants, only ΔRV and ΔMC (10) supported growth on maltotetraose, -pentose, or -hexose (Table 1). These data correlated well with liposome swelling experiments utilizing various oligosaccharides and ferrichrome.

DISCUSSION

Proteoliposome swelling experiments *in vitro* with stachyose and ferrichrome show that the *fepAΔRV* allele encodes a channel-forming protein, and that this pore is considerably larger than that of OmpF. Since neither stachyose nor ferrichrome can traverse the OmpF channel, their diffusion into ΔRV-containing liposomes excludes the possibility that swelling arises from contamination of our protein preparations with OmpF or related porins. This is a critical point, because the OmpF- and OmpC-deficient strain from which FepA and ΔRV were purified mutates to express Nmp porins at a measurable rate (H. Nikaido, personal communication; P.E.K., unpublished data). Even though we monitored the bacteria for this transmutation and discarded cultures that showed Nmp expression, since our protein preparations were not homogeneous we could not exclude the possibility of low-level contamination with other porins. However, the demonstration of channels much larger than those of any other known *E. coli* porin makes it unlikely that Nmp or other contaminating porins were responsible for the observed swelling. The dependence of swelling rate on solute size indicates that the ΔRV pore is approximately twice the size of the OmpF channel and slightly larger than the OprF channel (20), which implies a channel diameter of about 20 Å. Growth of bacteria expressing ΔRV or ΔMC on linear maltodextrins confirms this estimate and shows further that the ΔRV pore has dimensions similar to those of mutant OmpF porins that have lost or altered their transverse loop polypeptide (11). The actual FepA channel diameter may be even larger than 20 Å, because the predicted structure of FepA contains numerous other surface peptide loops besides those deleted in ΔRV and ΔMC, which may still obstruct the pore. Given the size of this opening, which is to our knowledge the largest channel that has been described in enteric bacteria, it is apparent why the pore is normally closed *in vivo*. Bacteria expressing ΔRV or ΔMC show dramatically increased susceptibility to antibiotics and SDS (10); if the FepA pore were always open, bacteria carrying it would surely perish in the gut from exposure to bile salts.

Our results with ΔRV confirm previous studies conducted *in vivo* (10) and provide proof for the existence of channel domains in TonB-dependent receptor proteins. A conceivable objection to the work of Rutz *et al.* (10) is the possibility that deletion mutations *fepAΔRV* and *fepAΔMC* alter outer membrane permeability in some unknown way that is independent of FepA structure. Liposome swelling with purified ΔRV conclusively refutes this idea, and establishes the bifunctional domain structure of TonB-dependent outer membrane proteins. These receptors contain two distinct structural features: surface polypeptides that bind ligands with high affinity and underlying channels that are large enough to allow flux of molecules as large as 1500 Da (10). We have shown that FepA may exist as a homotrimer in the outer membrane; these data further suggest its similarity to archetypal porins.

Purified FepA was impermeable to all of the solutes tested, except arabinose. This result was unexpected but is not irreconcilable. These data may account for the survival of porin-deficient (*ompF, ompC*) bacteria in rich medium (e.g., Luria broth), with little discernible loss of fitness. In such an environment nutrients may enter TonB-dependent channels by conformational flexibility or random thermal motion that partially opens the surface polypeptides of these receptors. On the other hand, this *in vitro* result may not be relevant to *in vivo* physiology, where direct or indirect interactions of receptor proteins with TonB may tightly control the opening and closing of their channels.

The finding that FepA functions as a gated channel unites several distinct theories of enteric bacterial outer membrane transport. The porins OmpF, OmpC, and PhoE have been recognized as open, hydrophilic transmembrane channels (1). LamB and Tsx most likely represent evolutionary advances that modify but do not alter this basic design. They are also open channels, but their ability to specifically bind the substrates that pass through them increases the efficiency of the uptake process. TonB-dependent outer membrane proteins constitute a further evolutionary transition: closed channels that recognize and transport large substrates, while excluding other molecules of similar size. Recent advances in knowledge of porin structure suggest a molecular mechanism for this adaptation. OmpF and related porin trimers contain loops of surface polypeptides that form an expansive vestibule domain exterior to their actual channels through the bilayer (3). These "petals" of the porin trimer increase the cell surface area in which collisions with solutes will lead to uptake. We speculate that this aspect of OmpF structure may explain the trimeric nature of porins, since the surface area enclosed by the vestibule of the trimer is greater than that of the individual monomers. OmpF-type porins also contain transverse loops of polypeptide that restrict the size of their channel opening. Closure of such pores during the evolution of TonB-dependent receptor proteins may have emanated from an infolding of the vestibule polypeptides or from an increase in the number or size of transverse loops that occlude the channel. We favor the latter explanation, because (*i*) it retains the possibility of vestibule polypeptides encircling FepA, increasing its collection efficiency, and (*ii*) deletions that open the FepA channel are phenotypically strikingly similar to deletions that alter the transverse loop of

OmpF. In conjunction with the findings reported herein, the discovery (21) that OmpA also forms static channels across the outer membrane raises the possibility that most outer membrane proteins are porins of some kind.

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