



Article

Internal Ion Pairs Control Transport Through TonB-Dependent Siderophore Receptors

Salete M. Newton and Phillip E. Klebba *

Department of Biochemistry & Molecular Biophysics, Kansas State University, Manhattan, KS 66506, USA; sallyn@ksu.edu

* Correspondence: peklebba@ksu.edu

Abstract

The TonB-dependent receptors (TBDRs) FepA and FhuA transport the siderophores ferric enterobactin (FeEnt) and ferrichrome (Fc), respectively, through the Gram-negative bacterial outer membrane. Their uptake mechanism involves conformational change in an ~150 residue N-terminal luminal domain (NTLD), located within their C-terminal β -barrel (CT β B) channels. We identified four internal sites (1–4) in TBDR that form a conserved network of ion pairs encircling the NTLD-CT β B interface. We tested the mechanistic importance of these electrostatic interactions by engineering systematic Ala substitutions in FepA and FhuA for the acidic or basic side chains that comprise them. Siderophore nutrition assays, colicin susceptibility tests and fluorescence spectroscopic uptake measurements of the mutants showed the importance of site-2, that adheres the base of NL1/N β 3 and N β 5 of the NTLD to β 14 and β 17 on the interior of the CT β B. Disruption of electrostatic bonds at site-2 reduced or eliminated ferric siderophore uptake and severely curtailed colicin susceptibility. Despite these reductions in ligand transport, fluorescent spectroscopic binding measurements showed that the site-2 mutations did not alter the affinity of FepA for FeEnt, nor FhuA for Fc. Elimination of ionic interactions at the three other locations in FepA (sites-1, -3, -4) did not reduce FeEnt uptake. Lastly, in FepA the disruption of ionic bonding at site-2 rendered it more susceptible to proteolysis, in part by OmpT, suggesting that ablation of ionic interactions in site-2 destabilized the NTLD within the CT β B. Overall, the experiments demonstrated that the ion pairs at site-2 in FepA and FhuA, that are evolutionarily conserved in the TBDR superfamily, are essential to the movement of ferric siderophores through the CT β B into the periplasm.

Keywords: siderophore; outer membrane; TonB-dependent transport; mutagenesis



Academic Editor: Yoshinori Marunaka

Received: 19 October 2025

Revised: 15 February 2026

Accepted: 16 February 2026

Published: 20 February 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

1. Introduction

Microbiological siderophores [1,2] solubilize precipitated, otherwise unavailable iron in the environment. Over 500 different siderophores are known and characterized [3], that may chelate iron with unparalleled high affinity [4,5]. Iron uptake, via chelates like the catecholate siderophore ferric enterobactin (FeEnt [6]) and the hydroxamate siderophore, ferrichrome (Fc [1,7]), is essential to the proliferation of biological communities, because ferric siderophores are an entry point for iron into prokaryotic metabolism. More than 80 enzymes require either iron or iron-containing cofactors, like aconitase and succinate dehydrogenase in the Krebs cycle, proton-pumping oxido-reductases in electron transport, class Ia ribonucleotide reductases in DNA synthesis, monooxygenases like cytochrome P450, and catalases, and superoxide dismutases that detoxify reactive oxygen species [8–10].

Iron's central role in both pro- and eukaryotic metabolism underscores the importance of understanding its biochemical uptake pathways.

In Gram (−) bacteria, outer membrane (OM) TonB-dependent [11] receptors (TBDRs) recognize and transport ferric siderophores. These high-affinity (e.g., $K_D \sim 10^{-10}$ M [12,13]) receptors bind iron complexes [14–17], vitamins [12,18–20] or nutrients [21–23]. When activated by TonB [11,24] they import ligands through the OM to the periplasm. TBDRs are omnipresent in Gram (−) bacteria to varying degrees of representation. Members of Enterobacterales encode many (7–20) that act in iron or other metal uptake, but other families of Proteobacteria contain many more (Pseudomonadaceae: 35–38; Caulobacteriaceae: 63; Xanthamonadaceae: 42–70 [25]), often with non-metal substrates. Hence, a multitude of TBDRs actively transport compounds into Gram (−) bacteria, but none are biochemically well enough defined to reveal the detailed steps of ligand internalization. Knowledge of the transport mechanism relates to research on bacterial susceptibility to siderophore antibiotics, among which the catecholate compound cefidericol is a paradigm of FDA-licensed Trojan Horse compounds [26].

The architecture of TBDRs constitutes a distinct structural class. They contain 22-stranded transmembrane β -barrels, so they are porins [27]. But unlike general and specific porins [28], TBDRs do not contain open channels: an N-terminal luminal domain (NTLD) blocks their channels [15–17,29–31]. Secondly, their active transport reactions in the OM are enigmatic, because thousands of 10 Å (general porin) channels [32,33] preclude the formation of an electrochemical ion gradient across the OM. Next, metal uptake through closed TBDR channels entails structural rearrangements in the receptor protein: (i) ligand binding in cell surface loops provokes movements in the NTLD that enable interactions with TonB in the periplasm [34,35]. Then, TonB exploits proton-motive force [13,18] (by an uncertain mechanism) to (ii) open the channel and trigger passage of the metal complex into the periplasm [13] (also by an unknown mechanism). Hence, although the transport of a bound ligand involves signal transduction, conformational dynamics and protein–protein interactions in the bacterial cell envelope, the molecular details of the internalization reaction remain obscure.

The crystal structures of FepA [15], FhuA [17,36] and FecA [16] revealed contiguous pairs of basic and acidic residue side chains in the TBDR interiors, that presumably hold the NTLD to the interior surface of the CT β B [37–39] (Figure 1). Bioinformatic analyses of 79 TBDR orthologs and paralogs [40] found that the basic and acidic residues comprising one of these electrostatic interactions (site-2) are highly conserved (>95% identical). The residues in site-2 are Arg on the NTLD, juxtaposed to Glu on the CT β B, close enough together to form salt bridges and hydrogen bonds that adhere the NTLD to the channel wall. In FepA the pairs are R75 and R126, associated with E511 and E567 (site-2: cyan bridges in Figures 1 and S2). The positive charge center of the R75 guanidino group is within a few angstroms of the E511 and E567 carboxylates, close enough for ionic and H-bonds to both acidic side chains; the R126 guanidino is similarly close to the E567 carboxylate, to allow both ionic and H-bonding [15]. Identical acidic–basic ion pairs exist in site-2 of other ferric catecholate (EcoFiu [30]: Figure 1D, EcoCir [29]) and ferric hydroxamate receptors (EcoFhuA [17]; Figure 1C,F). Clustal Ω sequence alignments identified the site-2 pairs as the most conserved residues in TBDRs, but numerous (eight or more) other potential NTLD–CT β B electrostatic bonds exist around the interior perimeter of TBDR channels (Figures 1 and S1–S4), providing more extensive ionic and H-bond stabilization of the NTLD. In some cases the other ion pairs are evolutionarily covariant: an acidic side chain on the NTLD approaches a basic side chain on the β -barrel interior. Given that PMF underlies TonB-dependent OM transport, and that ionic bonds are susceptible to neutralization by protonation, these residues were postulated as mechanistically relevant

to ligand internalization [39,40]. On the other hand, these configurations also imply that without conditions that neutralize ionic bonds (e.g., local decreases in pH), the internal salt bridges will restrict NTLD dynamics to localized movements of minimal amplitude. This notion agrees with experiments and simulations, which inferred that small movements of the NTLD, in situ within the CT β B, may open a pathway for ferric siderophore entry to the periplasm [41–43]. Evidence [44–49] suggests that PMF promotes OM transport by driving rotation of the TonB–ExbBD complex in the inner membrane [50], which transfers energy to TBDR by protein–protein interactions with their TonB-box motif [11,14].

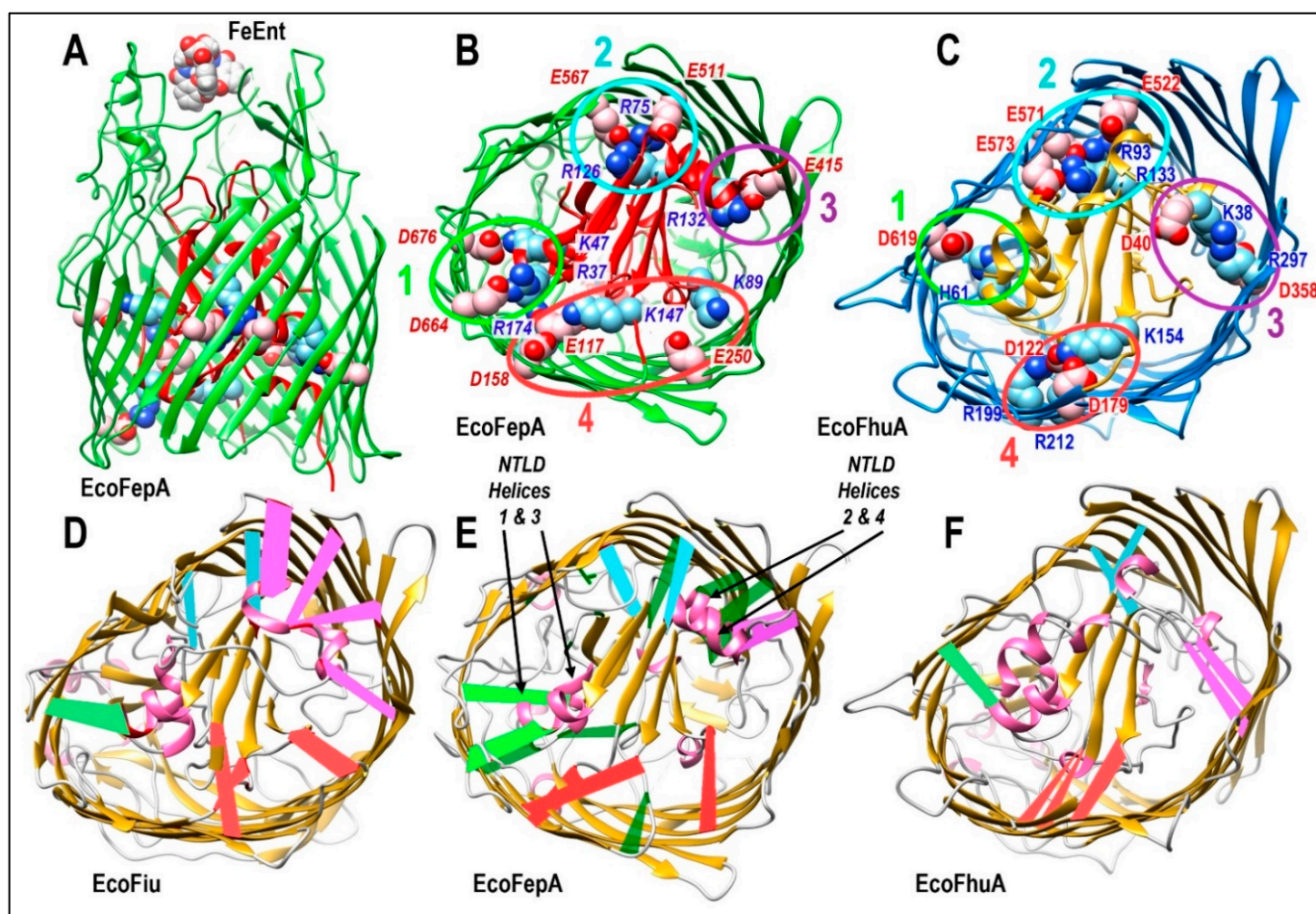


Figure 1. Internal ion pairs in *E. coli* TBDRs. (A) Side view of FepA (1FEP) showing putative salt bridges formed by basic residues (sky blue) in the NTLD (red ribbon) and acidic residues (pink) on the CT β B (green ribbon). (B) 90° rotation on the x-axis gives a periplasmic view of the internal ring of basic side chains on the NTLD, juxtaposed to an outer ring of acidic side chains on the CT β B. Four conserved, comparably located bridge sites exist in all of the *E. coli* TBDRs (Figures S1–S4), that are circled and labeled as sites-1 to -4 in the ferric catecholate transporter FepA (panel B) and the ferric hydroxamate transporter FhuA (panel C; NTLD is gold; CT β B is Dodger Blue). Sites-1 to -4 are circled with spring green, cyan, purple, and red, respectively. (Panels D–F) Ribbon diagrams of Fiu, FepA and FhuA depict salt bridges, with the same colors as in panels B and C. In D–F, α -helices are magenta; β -strands are gold; loops are light gray. In (E), the FepA model shows the location of internal disulfide bonds that do not prevent FeEnt transport [42] (dark green).

In this report we systematically substituted Ala for residues involved in the noted ion pairs, to determine how electrostatic and H-bond stabilizations of NTLD inside the CT β B of TBDRs affect the internalization of ferric siderophores. By single or multiple Ala substitution mutagenesis, we sequentially eliminated the internal ion pairs and determined

the effects on ligand uptake by *E. coli* FepA and FhuA. The results show that the basic–acidic residue juxtaposition at site-2 (R75, R126, E511, E567 in FepA; R93, R133, E522, E571 in FhuA) must remain intact for ferric siderophore and colicin uptake to occur. conversely, elimination of internal ion pairs at sites-1, -3 and -4 did not impair uptake.

2. Results

2.1. TBDR Ion Pair Networks

Crystallographic data (Figure 1), sequence alignments and bioinformatics [40] (Figures S1–S4) revealed an electrostatic network that may facilitate, restrict or immobilize NTLD structure within the CT β B. We aligned the structurally solved *E. coli* TBDR [15–17,29–31,36,51], and that of the ferric yersiniabactin receptor of *Y. pestis* [52], by CLUSTAL Ω [53]. This group of eight prototypes included three ferric catecholate receptors (Fiu, FepA, Cir), three ferric hydroxamate receptors (FhuE, FhuA, FyuA), the ferric citrate (FecA) and the vitamin B12 (BtuB) receptors. In each protein, 7–10 basic residue side chains pair with acidic residue side chains at four sites around the interior of the NTLD/CT β B interface (sites-1 to -4 in Figure 1). These basic/acidic pairs presumably adhere the NTLD to the β -barrel channel with ionic and/or hydrogen bonds (Figures 1 and S1–S4). Besides revealing identical/homologous residues among the proteins, the CLUSTAL Ω analysis pinpointed sites-1 to -4 in NTLD primary structure, and illustrated the different attributes of the ion pairs at these positions.

Site-1: basic or acidic residues in helices N α 2 and N α 3, associated with CT β B strands 17, 19, 20, 21, or 22;

Site-2: invariant Arg at the ends of N β 3 and N β 5 paired with invariant Glu in CT β B strands 14 and 17;

Site-3: basic or acidic residues in helices N α 1, N α 4 or N α 5, paired with CT β B strands 7, 8, 9, 10 or 11;

Site-4: basic or acidic residues at the peripheries of helices N β 2, N β 3 and N β 4, associated with CT β B strands 1, 2, 3, 4 or 5.

Site-2. The invariance of site-2 was most striking. The residues composing site-2 were always identical: Arg on the N-domain surface associated with Glu on the C-domain interior (cyan connections in Figure S2), and always at the same relative position in the sequence alignment (in FepA, R75-E511 and R126-E567). These facts distinguished site-2 from all other salt bridge locations, and this invariance was more noteworthy in light of the variability in composition and position of the ion pairs at sites-1, -3 and -4. Further, site-2 always links the base of NL1 and distal portion of N β 3 (in EcoFepA, at residue R75) and N β 5 (R126) to the interior wall of the β -barrel (E511 in β 14 and E567 in β 17, respectively). Thus, the site-2 ion pairs, and by implication their electrostatic interactions, were exactly comparable in every TBDR that we considered.

Sites-1, -3 and -4. Unlike the invariant Arg and Glu in site-2, sites-1, -3 and -4 sometimes had Lys or His at different positions in the NTLD, sometimes paired to Asp in the CT β B. For example, site-1 bridges (green lines in Figure S1) were variable in compositions and locations, originating in N α 2 or N α 3 and connecting to multiple β -strands: 17, 19, 20, 21, 22, or L10. Two of these site-1 pairs also showed evolutionary covariance (an indicator of mechanistic importance [54]) among the TBDRs we studied. That is, besides basic residues on the NTLD and acidic residues on the CT β B, in site-1 we also found acidic side chains on the NTLD paired to basic side chains on the CT β B. Site-3 (purple) connections were similarly variable and one was covariant, originating at different locations in the N-domain and connecting to β -strands 7–11 of the CT β B (Figure S3). Site-4 bridges (red lines in Figure S3) involved N β 2, N β 3 or N β 4, but the individual residues within them manifested more diversity of locations and composition. For example, in N β 2 a nearly conserved Asp

(D117 in FepA) linked to either K or R at various positions in (β 1– β 5) of the CT β B. Other site-4 acidic and basic residues in N β 3 or N β 4, respectively, associated with oppositely charged side chains positions in β 1 or β 2 of the CT β B; two positions were evolutionarily covariant. Despite the different components, positions and attributes of ion pairs in sites-1 to -4, their conservation in TBDR interiors intimated stabilization of the NTLD within the CT β B, which presumably plays a mechanistic role in transport.

2.2. Site-Directed Mutagenesis of Ion Pair Residues in *E. coli* FepA and FhuA

In light of the apparent electrostatic interactions between the NTLD and the CT β B interior, we used alanine scanning mutagenesis [43,55] to investigate the function of sites-1 to -4 in FeEnt transport by FepA. We first eliminated ionic bonding in the sites (Figure 1) by individually changing the predominantly acidic β -barrel residues to Ala. In FepA, three of the eight target β -barrel amino acids are Asp, four are Glu, and the eighth is Arg. Besides introducing these individual Ala substitution mutations in the FepA β -barrel, we combined them as double or triple mutations to fully eliminate any ionic bonding at the individual sites-1 to -4 (Table 1). For example, at site-1 we made the single substitutions E664A and E676A, as well as the double substitution E664A_E676A. At site-4 we made the single substitutions E117A, R178A and E250A, as well as their double and triple combinations. Because our initial findings with the ferric catecholate transporter FepA showed the importance of site-2, we made the equivalent Ala substitutions in site-2 of the Fc transporter, FhuA, with single mutations E522A and E571A, and their double combination. The Ala scanning approach systematically replaced the individual acidic or basic residues with a similarly sized, non-ionizable side chain that precluded the formation of electrostatic or H-bonds. Lastly, besides substituting Ala for Asp or Glu on the CT β B, we further substituted Ala for ion-paired Arg residues on the NTLD: R75A, R126A, R75A_R126A in FepA; R93A, R133A, R93A_R133A in FhuA.

Table 1. Ala substitutions for residues composing NTLD-CT β B salt bridges in FepA and FhuA. Colors designate 4 sites containing ionic pairs that presumably link the NTLD to the CT β B (depicted in panels B–F of Figure 1). We created single, double or triple Ala substitutions for the enumerated residues.

FepA		FhuA	
Bridge Site	Ala Substitution	Bridge Site	Ala Substitution
1a(R37-D664)	D664A	1 (H61-D19)	ND
1b(K47-D676)	D676A	-	ND
1a, 1b	D664A_D676A	-	ND
2a(R75-E511)	R75A, E511A	2a(R93-E522)	R93A, E522A
2b(R126-E567)	R126A, E567A	2b(R133-E571)	R133A, E571A
2a, 2b	R75A_R126A E511A_E567A	2a, 2b	R93A_R133A E522A_E571A
3a(R132-E415)	E415A	3a(K38-D358)	ND
3b	-	3b(D40-R297)	ND
4a(E117-R174)	R174A	4a(D122-R199,-R212)	ND
4b(K89-E250)	E250A	4b(K154-D179)	ND
4c(K147-D158)	D158A		
4a, 4b	R174A_E250A		
4a, 4b, 4c	R174A_E250A_D158A		

2.3. Expression of FepA and FhuA Mutants

Immunoblots of bacterial cell lysates (Figure 2) evaluated the expression of wild-type FepA and its mutants. The site-1 to -4 charge ablation mutations did not reduce expression of the FepA proteins: *E. coli* produced FepA and the Ala substitution mutants at about the same level (Figure 2). However, upon cell lysis FepA is susceptible to proteolysis by OmpT near its N-terminus [56–59], and some of the FepA site-2 mutants (R75A, E567A and their combinations) were more degraded in the immunoblots (Figure 2A,B). OmpT is an OM-resident protease that cleaves host factors, including cationic peptides and complement [60–63], and adventitiously cleaves other OM proteins when released into solution by cell lysis or detergent solubilization [57,58,64–66]. To minimize this degradation we engineered an OmpT-deficient strain of *E. coli* (OKN2359: *entA*, Δfli , $\Delta fepA$, Δcir , $\Delta ompT$) as the host for pITS23 (*fepA*+) and its derivatives. Some proteolysis of FepA_R75A, FepA_E567A and their combinations still occurred in the $\Delta ompT$ strain, but the extent was reduced (Figure 2C). The enhanced protease susceptibility of site-2 mutants was noteworthy (see Discussion). Most important regarding the phenotypes of the FepA site-2 Ala mutants, a large amount of each full-length, unproteolyzed FepA polypeptide remained intact in the $\Delta ompT$ host. Further, with one exception, the site-2 Ala substitution mutants of FhuA were not proteolytically degraded (Figure 2). The exception, FhuA_E522A_E571A, was partially proteolyzed in SDS-PAGE gels of FM-labeled cell lysates.

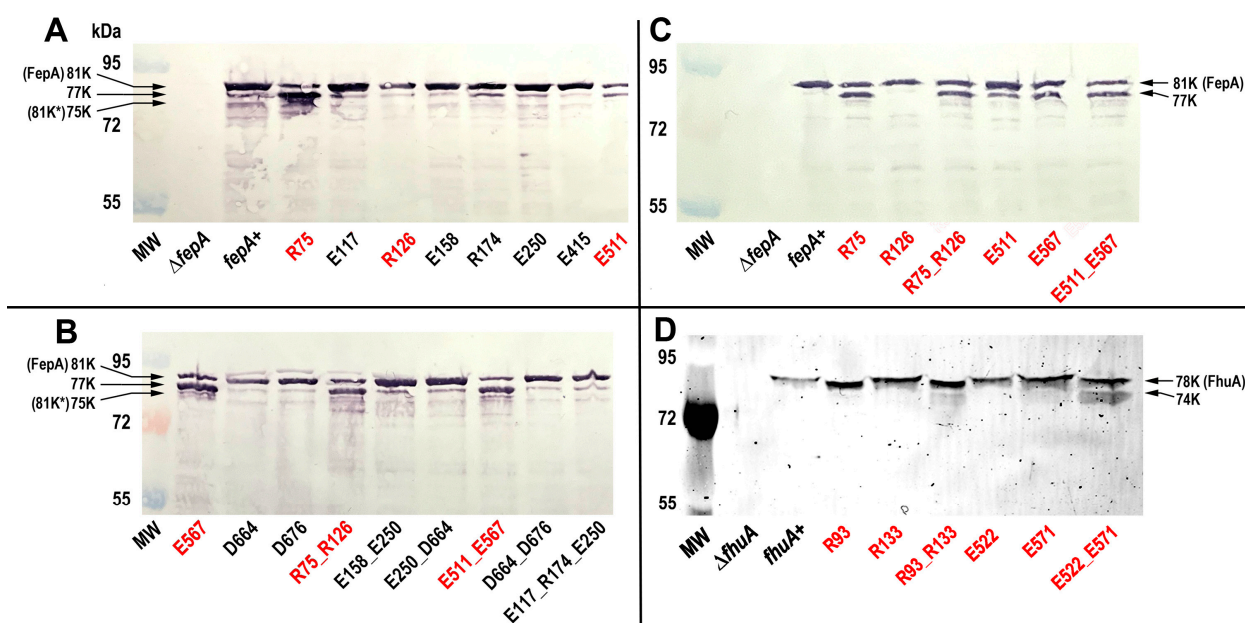


Figure 2. Expression of FepA and FhuA Ala substitution mutants. (Panels A,B) *E. coli* OKN359 (Δ*fli*, Δ*fepA*, Δ*cir*)/pHSG575, carrying *fepAA698C* or its Ala substitution mutants, were grown in MOPS media, collected by centrifugation and boiled in sample buffer. We loaded the lysates (5×10^7 cells) in an SDS –PAGE gel, and after electrophoresis we transferred the proteins in the gels to nitrocellulose and developed it with α-FepA mAb 44 [67] and GαMlg-alkaline phosphatase. Band 81K* designates the known degradation product of FepA by OmpT [56–58]. Mutant FepA proteins at site-2 (red text) were more susceptible to proteolysis by OmpT. (C) We repeated the procedure in OKN2359 (Δ*fli*, Δ*fepA*, Δ*cir*, Δ*ompT*). Deletion of *ompT* reduced the proteolysis: wild-type FepA was not degraded, and the site-2 Ala mutants were less degraded ($\geq 50\%$ of the full-length mutant polypeptides remained). (D) We grew *E. coli* OKN17 (Δ*tonB*, Δ*fhuA*)/pHSG575 carrying *fhuAS396C*, or its Ala mutant derivatives, in MOPS media, labeled them with FM [1], lysed them by boiling in sample buffer and loaded 5×10^7 cells into the lanes of an SDS–PAGE gel. After electrophoresis we used a UV transilluminator to visualize the fluorescently labeled FhuA proteins.

2.4. Siderophore Nutrition and Colicin Susceptibility

FepA is the OM receptor for FeEnt and colicin B (ColB). Using microbiological siderophore nutrition assays [68,69], we first evaluated the FeEnt uptake activities of eight individual and four double Ala substitutions for Glu or Asp at sites-1–4 on the CT β B interior of FepA. Only the double mutation at site-2 (E511A_E567A) fully blocked FeEnt uptake (Figure 3; Table 2): no growth was seen around a disk of 50 μ M FeEnt on plates of the FepA_E511A_E567A mutant, illustrating the importance of the site-2 salt bridge interactions. On the other hand, Ala substitutions for Glu or Asp at sites-1, -3 and -4, including their double-mutant combinations, did not reduce FeEnt uptake in nutrition tests (Figure 3).



Figure 3. Siderophore nutrition tests of FepA and FhuA salt bridge mutants. After substitution of Ala for Asp or Glu residues in sites-1 to -4, we expressed the *fepA* proteins in *E. coli* OKN359 (*entA*, $\Delta fhuA$, $\Delta fepA$, Δcir), and the mutant *fhuA* alleles in *E. coli* OKN7 ($\Delta fhuA$). Siderophore nutrition tests assessed the uptake 50 μ M FeEnt or Fc in the FepA and FhuA mutants, respectively.

The strong impact of E511A and E567A in FepA on FeEnt uptake led us to construct Ala substitutions for their electrostatic partners, R75 and R126. In this case, the individual Ala substitutions R75A and R126A in FepA changed the morphology of the FeEnt nutrition halos (larger or smaller, respectively; Figure 3, Table 2), suggesting a reduction in rate or extent of FeEnt uptake [70]. Furthermore, like E511A_E567A, the double mutant R75A_R126A eliminated the growth halo around 50 nM FeEnt. So, replacement of either the acidic or basic salt bridge partners with Ala at site-2 blocked FeEnt transport by FepA. The FeEnt nutrition assays gave the same results in *ompT*⁺ or $\Delta ompT$ backgrounds (Table 2).

FhuA is the cognate TBDR for Fc and ColM [69]. Because ablation of ionic interactions at site-2 disrupted FeEnt uptake by FepA, we evaluated the equivalent site-2 residues in FhuA during Fc uptake. As seen for FepA_R75A, the single substitutions at site-2 in FhuA (R93A, R133A, E522A, E571A) resulted in larger nutrition halos around 50 μ M Fc (Figure 3), indicating an impaired rate of Fc uptake. As in FepA, the double FhuA substitution mutants at site-2 (R93A_R133A, E522A_E571A) abolished Fc transport (Figure 3; Table 2).

Bacteriocin susceptibility followed nearly the same patterns for both receptors. Single Ala substitutions for Glu or Arg in site-2 of FepA decreased susceptibility to ColB 5–20-fold, and the double mutants R75A_R126A and E511A_E567A were 100–500-fold less sensitive to ColB (Figures 4 and S5, Table 2). The ColB killing assays gave the same results in *ompT*⁺

or $\Delta ompT$ backgrounds (Table 2). Analogous reductions in sensitivity to ColM occurred in cells expressing FhuA and its mutants (Figure 4, Table 2): the site-2 single mutants R93A and R133A reduced ColM susceptibility 2–5-fold, and double mutants FhuA_R93A_R133A, and FhuA_E522A_E571A decreased killing 100–500-fold (Table 2). Unlike their behavior in siderophore nutrition tests, the double Ala substitutions at sites-1, -3 and -4 in FepA also reduced sensitivity to ColB, although less (2–10-fold) than at site-2 (100–500-fold, Figure 4). In summary, nutrition tests implicated charge interactions at site-2 during iron uptake through both FepA and FhuA, and bacteriocin titrations reiterated the importance of site-2 in uptake of ColB and ColM by FepA and FhuA, respectively. ColB killing was affected by Ala substitution at sites-1, -3 and -4 in FepA. Both siderophore nutrition and colicin susceptibility test results were unaffected by the presence or absence of OmpT in the plasmid host strains (Table 2).

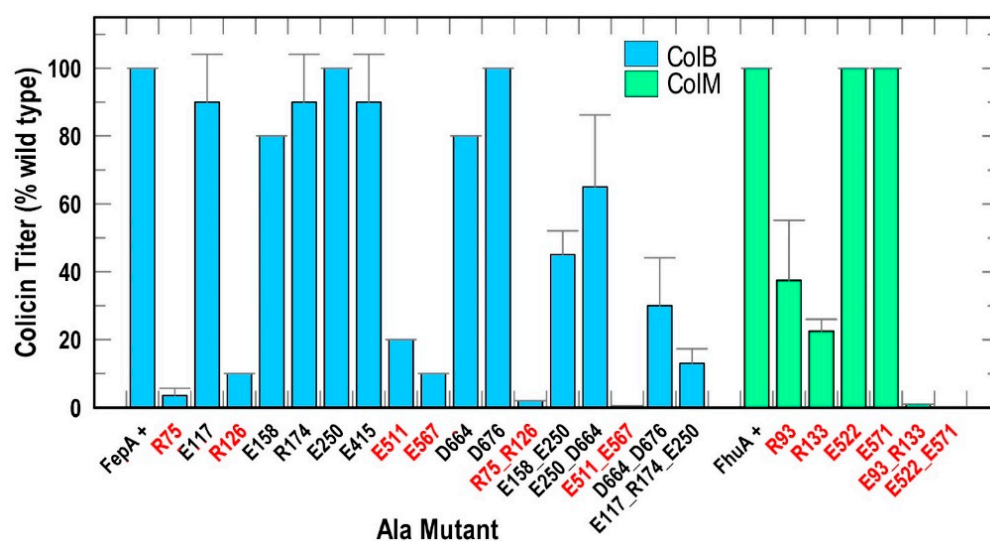


Figure 4. Sensitivity of Ala mutants to colicins. We deposited dilutions of ColB and ColM on lawns of bacteria expressing FepA and FhuA (respectively) or their Ala mutants (Figure S5). The graph plots colicin titer (Table 2) on the Ala mutants relative to wild-type FepA (100%). Ala substitutions at site-2 (red text) produced the largest reductions in colicin susceptibility.

2.5. Fluorescent Spectroscopic Measurements of FeEnt Binding and Transport

We used fluorescent sensor assays [71] to measure the binding and transport of FeEnt or Fc by site-2 Ala substitution mutants in FepA and FhuA. These assays revealed that the uptake deficiencies in site-2 mutants did not originate from impaired FeEnt or Fc binding. The (nanomolar) binding affinities of FepA for FeEnt and FhuA for Fc were not altered by the site-2 Ala substitutions, either singly or in combinations (Figures S6 and S7). The apparent affinity of the mutant FepA receptor proteins for FeEnt was statistically indistinguishable from that of wild-type FepA ($K_D \approx 1$ nM; Figure S6; see Discussion). In some cases (R75A, R75A_R126A; E567A, E511_E567A) the overall extent of fluorescent quenching by saturating concentrations of FeEnt was less, but this reduction did not alter the K_D value. We found comparable results for site-2 mutants in FhuA (Figure S7): the apparent affinities of all the FhuA proteins were the same ($K_D \approx 1$ nM). The normal affinities of site-2 Ala mutants of FepA and FhuA for FeEnt and Fc, respectively, implied that their transport defects were restricted to the ligand internalization reaction.

Spectroscopic FeEnt and Fc uptake studies confirmed the results of siderophore nutrition assays (Figure 5; Table 2). In FepA, R126A reduced the rate of FeEnt uptake (63% of wild-type level), while the single mutation R75A and the double Ala substitution R75A_R126A completely blocked FeEnt acquisition. Likewise, both E567A and

E511A_E567A eliminated FeEnt uptake in these tests. Analogously at site-2 in FhuA, both R93A and the double Ala substitution R93A_R133A eliminated Fc uptake (Figure 5; Table 2). E522A reduced the rate of Fc uptake (to 55% of wild-type level), and E522A_E571A further decreased the Fc uptake rate (to 27% of wild-type levels; Figure 5; Table 2). Overall, the siderophore nutrition assays and spectroscopic uptake experiments consistently showed transport defects in Ala substitution mutants at site-2.

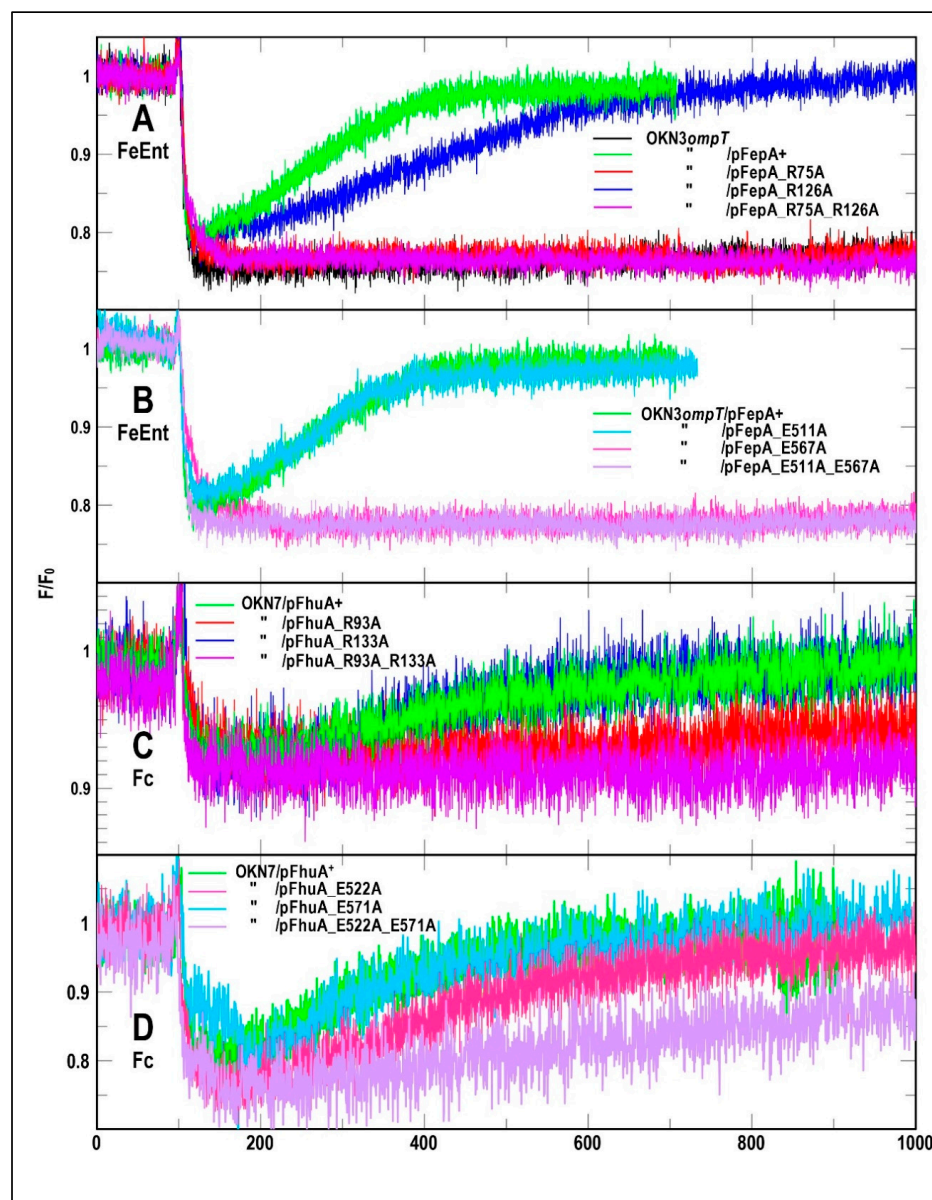


Figure 5. FeEnt and Fc uptake by FepA and FhuA site-2 Ala mutants. (A,B) FeEnt uptake. We grew OKN2359 ($\Delta fhuA$, $\Delta fepA$, Δcir , $\Delta ompT$)/pITS23 with *fepA*⁺ or its site-2 Ala mutants in iron-deficient MOPS media and measured FeEnt uptake with sensor OKN1359 ($\Delta tonB$, $\Delta fhuA$, $\Delta fepA$, Δcir)/pFepA_A698C-FM [1]. (A) Relative to FepA⁺, FepA_R126A had a reduced uptake rate; FepA_R75A and FepA_R75A_R126A did not transport FeEnt. (B) FepA_E511A acquired FeEnt like FepA⁺, but FepA_E567A and FepA_E511A_E567A did not acquire it. (C,D) Fc uptake. We grew OKN7 ($\Delta fhuA$)/pITS11 with *fhuA*⁺ or its site-2 Ala substitutions in iron-deficient MOPS media and measured Fc uptake with sensor OKN17 ($\Delta tonB$, $\Delta fhuA$)/pFhuA_S3968C-FM. (C) FhuA_R133A transported Fc like FhuA⁺, but FhuA_R93A had a much lower Fc uptake rate and FhuA_R93A_R133A did not transport Fc. (D) FhuA_E571 transported Fc like FhuA⁺, but FhuA_E522A had reduced uptake, and FhuA_E522A_E571A was more impaired.

Table 2. Phenotypic attributes of Ala substitutions for site-2 salt bridge residues in *E. coli* FepA and FhuA. ^a For spectroscopic binding experiments we expressed the *fepA* alleles on pITS23 in OKN1359 (Δ tonB, Δ fepA, Δ cir, Δ fiu) (Figure S6); for FeEnt uptake and ColB susceptibility assays, we expressed them in host strain OKN359 (Δ fepA, Δ cir, Δ fiu) or OKN2359 (Δ fepA, Δ cir, Δ fiu, Δ ompT) (Figure 5). ^b Apparent K_D (nM) derived from fluorescence quenching of FepA or FhuA Ala mutants in titrations with increasing amounts of FeEnt or Fc, respectively (Figures S6 and S7). ^c Diameter (mm) of growth around discs containing 50 μ M FeEnt or Fc on iron-deficient NB plates seeded with *E. coli* expressing wild-type FepA or FhuA, respectively, or their Ala substitution mutants. ^d Rate of FeEnt or Fc uptake (pmol/min/ 10^9 cells), calculated from rate measurements in fluorescence sensor assays. ^e Titer (reciprocal of the limiting dilution of ColB or ColM stock that gave clearing of the agar in plate tests (Figure S5)) on *E. coli* expressing FepA or FhuA Ala mutant proteins, relative to titer on wild-type FepA or FhuA (arbitrarily set to 100). ^f We expressed the *fhuA* alleles on pITS11 in host strain OKN17 (Δ tonB, Δ fhuA) for spectroscopic binding experiments, and in host strain OKN7 (Δ fhuA) for Fc uptake and ColM susceptibility assays.

<i>fepA</i> allele ^a	Binding ^b	FeEnt Transport				Colicin B ^e	
		Nutrition ^c		Uptake ^d			
		<i>ompT</i> +	Δ <i>ompT</i>	<i>ompT</i> +	Δ <i>ompT</i>	<i>ompT</i> +	Δ <i>ompT</i>
<i>fepA</i> ⁺	0.4 ± 0.05	15	14	40	50	100	100
<i>R75A</i>	0.7 ± 0.1	16	15	0	0	2	0.1
<i>R126A</i>	0.4 ± 0.1	17	16	17	32	10	10
<i>R75A_R126A</i>	0.4 ± 0.15	0	0	0	0	4	5
<i>E511A</i>	1.0 ± 0.1	15	15	50	50	40	50
<i>E567A</i>	0.4 ± 0.1	18	17	24	0	10	20
<i>E511A_E567A</i>	0.6 ± 0.1	0	0	0	0	0.2	2

<i>fhuA</i> allele ^f	Binding ^b	Fc Transport		ColM ^e
		Nutr ^c	Uptake ^d	
<i>fhuA</i> ⁺	0.4 ± 0.1	13	12	100
<i>R93A</i>	1 ± 0.2	15	0	37
<i>R133A</i>	0.7 ± 0.2	15	6	25
<i>R93A_R133A</i>	1.3 ± 0.1	0	0	0
<i>fhuA</i> ⁺	0.4 ± 0.1	13	15	100
<i>E522A</i>	0.5 ± 0.1	15	9.2	100
<i>E571A</i>	0.7 ± 0.3	16	15	100
<i>E522A_E571A</i>	1.1 ± 0.1	0	4	0

The crystal structures of FepA (PDB 1Fep) and FhuA (PDB 1by3 and 1by5), in the latter case complexed with Fc, do not fully explain the individual electrostatic interactions at site-2. In both proteins the four residue side chains associate together at the NTLD-CT β B interface, directly above the TonB-box (Figure S10) and across the channel from the transition point of NTLD to CT β B (residue 150 in FepA, 160 in FhuA). The ferric siderophore uptake measurements suggest that R75 and E567 are most functionally important in FepA, and that R133 and E522 are most functionally important in FhuA. The strong (95%) [40] evolutionary conservation of these residues suggests the overarching mechanistic importance of site-2 in all TBDRs.

3. Discussion

Maurice Hofnung, who studied maltodextrin transport through the *E. coli* OM protein LamB (malto porin; [72,73]) and the IM complex MalEFGK [74–76], once stated that he sought to find single critical residues that were indispensable to the biochemical activities of these transporters. Our findings identified crucial interactions in FepA, FhuA, and conceivably all TBDRs that influence both uptake of ferric siderophores and susceptibility to bacteriocins. In the crystal structures of FepA [15], FhuA [17] and other TBDRs, this electrostatic association at site-2 presumably restricts NTLD motion directly above the TonB-box by bonding the distal part of N β 3 (beneath NL1) and the distal part of N β 5 to the CT β B (Figure S10). This adherence is apparently necessary for the biochemical activities of TBDRs: unlike other interactions at sites-1, -3 and -4, Ala substitutions at site-2 severely impaired or abrogated FepA- and FhuA-mediated iron transport, as well as their susceptibility to ColB and ColM, respectively. These findings imply that adherence of the NTLD to the β -barrel at site-2 is a necessary condition of ligand uptake. On the other hand, adherence at sites-1, -3 and -4 was not necessary: Ala substitutions that compromised those salt bridges did not prevent FeEnt uptake.

The previously noted ion pairs in FepA [15,37,39,40] raised the possibility of electrostatic channel gating during ligand uptake, for example between R75 and R126 on the NTLD, and E511 and E567 on β 14 and β 17 of the CT β B. Protonation of E511 and E567 will neutralize any ionic bonding, releasing the NTLD to either move out of the channel, or to undergo conformational motion within the channel. Either conformational dynamic may open a pathway for ligand transport. Mutagenesis indicates that the ion pairs at site-2 are requisite for ligand uptake to occur. However, the interpretations of this result are not necessarily straightforward. It is conceivable that in native TBDRs the neutralization of the site-2 salt bridges unlocks their NTLD to gross movements that facilitate ligand internalization. Yet, the numerous other stabilizing ion pairs in the NTLD-CT β B interface, and the lack of any known pathway of proton flow into TBDR interiors, undercut such a potential mechanism. It is especially noteworthy that disulfide bonds that hold the NTLD within the CT β B do not interfere with FeEnt uptake [42]. Additionally, the electrostatic interactions of ion pairs at sites-1 to -4 may purposefully stabilize the position and conformation of the NTLD inside the transmembrane β -barrel, preserving an extant pathway of ligand passage through the receptors [43]. Ionic and hydrogen bonds are the strongest non-covalent interactions in proteins; their presence between the NTLD and the CT β B opposes NTLD movement, either out of or within the channel. Site-2 involves the distal ends of N β 3 and N β 5, that associate with β 14 and β 17 of the CT β B. We suggest that adherence at this location creates a molecular anchor that prevents motion in certain regions of NTLD structure (e.g., above N β 5), but allows conformational change in other regions, like the TonB-box and connected structural elements (N α 1, N β 3, N α 2). For example, N α 2, that was implicated in the gating of ferric catecholate transport through Fiu [43] is directly connected to site-2 by N α 1 (Figure S10). If iron complexes move through TBDR interiors to an equilibrium position above a portal to the periplasm that is blocked by N α 2, then rotational motion of TonB [44], bound to the TonB-box, may relocate N α 1- N α 2, and thereby open a pathway for the ligand to the periplasm. This postulate concurs with the conclusion that part of the NTLD is more susceptible to conformational motions as a result of interactions with the TonB C-terminus [77]. Other studies also concluded that the NTLD of FepA undergoes conformational rearrangements during FeEnt transport [42,78].

Ala substitutions increased susceptibility of *E. coli* K-12 FepA to proteolysis. One known degradation was by OmpT, a 10-stranded OM β -barrel, aspartyl protease in the OmpT family [79–81], that naturally defends against host antimicrobial peptides. Its active site resides on the bacterial cell surface (Figure S10) [82], and preferentially cuts between

dibasic residues (RK KR, RR, KK), but the residue following the scissile bond may vary (e.g., Arg, Lys, Ile, Gly, Val) [83–87]. OmpT is not readily inactivated by denaturing conditions (boiling, SDS, urea) [83–87], and it does not cleave other OM proteins *in vivo*, but degrades them when it is released into solution by cell lysis or detergent solubilization [57,58,64–66]. When the OM is solubilized by detergent, OmpT cuts near the N-terminus of FepA [56–59]. The chromosomal deletion of *ompT* that we generated eliminated degradation of wild-type FepA and reduced the degradation of its site-2 Ala substitution mutants, but cleavage at one site persisted in cell lysates (Figure 2), even in the OmpT-deficient background (OKN2359: Δfli , $\Delta fepA$, Δcir , $\Delta ompT$). Additional experiments are needed to identify the source of this remaining proteolysis. Nevertheless, various findings indicate that the degradation by OmpT seen in immunoblots did not occur *in vivo*, but rather occurred during cell lysis, as is consistent with the known activity of OmpT. These and other data, summarized below, signify that the abrogated uptake activities of the site-2 mutants resulted from the effects of the mutations themselves on the transport process.

- (i). FeEnt nutrition tests, ColB killing assays, and spectroscopic FeEnt uptake studies of FepA gave the same results whether the plasmid host strain was OKN359 (*ompT*+) or OKN2359 ($\Delta ompT$) (Table 2; Figure 5A,B). The presence or absence of the protease made no difference to the phenotypes of the site-2 mutants, which implies that the enzyme does not affect FepA *in vivo*.
- (ii). Less degradation occurred in cell lysates of OKN2359 ($\Delta ompT$), so high concentrations of full-length FepA polypeptides were present in all the mutants (Figure 2C). Despite the fact that ~50% of total FepA was intact and un-degraded, numerous site-2 Ala constructs (R75A, R75A_R126A, E567A, E511_567A) were devoid of FeEnt uptake, and highly resistant to ColB killing. These data demonstrate that the transport defects of site-2 Ala substitutions derive from mechanistic impairment created by the mutations themselves.
- (iii). Fluorescence scans of SDS-PAGE gels of lysates from the mutant derivatives of FepA_698C-FM revealed proteolytic cleavage at sites within the NTLD (residues 37–38 and 48–49 (see below; Figures S8 and S9)). These regions of FepA normally reside inside the CT β B (Figures 1 and S9 and S10), where they are inaccessible to OmpT as it exists in the OM of living cells.
- (iv). Most FhuA Ala mutants were not proteolytically degraded, but R93A, E522A and R93A_R133A did not transport Fc. FhuA_E522A_E571A was partially proteolyzed, but a large amount of intact FhuA_E522A_E571A remained in the cells, so its complete loss of Fc transport ability derived from the mutation itself.

In both *ompT*+

The increased susceptibility to proteolysis of the Ala substitution mutants of FepA gave insight into the structural effects of charge ablation at site-2. The degradation of FepA site-2 charge mutants led to two predominant products: ~77 kDa and ~75 kDa. The locations of the cleavage sites (Figures 2 and S8 and S9), between residues R37–K38 (77 kDa product) and K48–I49 (75 kDa) were apparent from the known target specificity of OmpT [83–87] and the fact that the FepA orthologs of *E. coli* B and *Salmonella typhimurium* LT2 are not cleaved by OmpT [57]. Cleavage at the former site persisted in the $\Delta ompT$ host, whereas the latter was absent in the $\Delta ompT$ strain. Sites R37–K38 and K48–I49 became more

accessible in FepA_R75A, FepA_E567A and their combinations. These targets are in the NTLD (Figures S9 and S10), so the enhanced proteolysis suggested that ablation of charge interactions at site-2 at least partially released the NTLD from the CT β B, where it became more susceptible to proteases in the cell lysate.

It is of interest to consider these data in light of the phenotypes of mutants containing disulfide linkages either within the NTLD, or linking it to the CT β B [42]. Disulfide bonds that linked the luminal domain to the β -barrel (near sites-1, -2, -3 and -4; Figure 1) did not prevent FeEnt transport. These data infer that parts of the NTLD may remain fixed within the CT β B during ligand uptake, which concurs with the notion of its electrostatic stabilization of the NTLD in the β -barrel by the ion pair network that we investigated. Our findings further underscore that stabilizations at site-2 are necessary for ferric siderophore uptake by both FepA and FhuA. On the other hand, the interactions at sites-1, -3 and -4 are not a requisite, and may be eliminated without disrupting the iron transport activities of FepA. Stated another way, dynamics involving the NTLD and the CT β B at sites-1, -3 and -4 are permissible. Majumdar et al. [42] concluded that conformational dynamics must occur within the NTLD, because disulfide bonds between the β -strands of its central β -sheet, that preclude conformational motion, prevented FeEnt uptake. Thus, both site-directed Ala substitution mutagenesis and site-directed disulfide bonds suggested the same conclusions: (i) the NTLD remains within the CT β B during TBDR transport activity, and (ii) conformational motion in the NTLD triggers the internalization of bound iron complexes into the periplasm. In FepA and FhuA, and likely all TBDRs, the NTLD adheres to the CT β B at site-2, but it undergoes conformational dynamics in other regions as iron passes through the transmembrane channel. The identification of five evolutionarily conserved glycines in the N-domain, surrounding the charge cluster at site-2, [40], implies conformational flexibility, as a result of their minimized ϕ/ψ steric hindrance. These outcomes generally agree with molecular dynamic simulations of Fiu-mediated uptake of degraded FeEnt (FeEnt*), an iron complex that is proposed to travel a defined pathway between the NTLD and the CT β B in Fiu [43]. This route involves binding poses in the protein interior that ultimately position FeEnt* above a narrow opening between the N-domain and the β -barrel (Figure S10). In its resting state [30], this pore is too small to permit passage of FeEnt*, implying that the role of TonB activity is to transiently change the conformation of the NTLD in a way that enlarges the channel, either allowing or compelling FeEnt* into the periplasm.

4. Materials and Methods

4.1. Bacterial Strains and Plasmids

E. coli strains with precise, site-directed deletions of *entA*, *fiu*, *fepA*, *fhuA*, *cir*, and/or *tonB* [78] derived from MG1655 [88]. These constructs did not synthesize siderophores nor relevant OM TBDR; we used them as hosts for plasmids expressing TBDRs of interest. The strains included OKN359 ($\Delta entA$, $\Delta fepA$, Δfiu , Δcir), OKN1359 ($\Delta entA$, $\Delta tonB$, $\Delta fepA$, Δfiu , Δcir), OKN2359 ($\Delta entA$, $\Delta fepA$, Δfiu , Δcir , $\Delta ompT$), OKN7 ($\Delta entA$, $\Delta fhuA$) and OKN17 ($\Delta entA$, $\Delta tonB$, $\Delta fhuA$) [78].

We cloned and expressed OM TBDR in pITS23 [89] or pITS11 [89], that derive from the low-copy (1-3/cell) plasmid pHSG575 [90,91]. pITS23 and pITS11 carry intact, functional *fepA* and *fhuA* structural genes, respectively, under control of their natural Fur promoters [89]. We introduced site-directed substitution mutations into both *fepA* and *fhuA* structural genes for study in vivo.

4.2. Site-Directed Mutagenesis

We created site-directed substitutions in *fepA* or *fhuA*, carried on pITS23 and pITS11, respectively. With a pair of complementary primers that flank the target codon, we introduced mutations that encoded Cys or Ala residues in mature *FepA* or *FhuA*. After digesting the wild-type template DNA with DpnI, we transformed BN1071 [92] with the mutant plasmid, isolated transformant clones and sequenced their plasmids to confirm the substitutions in *fepA* or *fhuA*. We did not expect the Ala substitutions to strongly disrupt local secondary or tertiary structures, especially because among more than 200 other site-directed Ala or Cys substitutions in *FepA*, <2% noticeably altered its assembly, OM localization, or functionality [42,70,93–95].

4.3. Iron-Deficient Bacterial Culture Conditions

We initially grew OKN359, OKN1359 or OKN2359 harboring pHSG575 derivatives of interest at 37 °C in Luria–Bertani (LB) broth [96] to stationary phase. For host strain and plasmid selection, the media contained streptomycin (100 µg/mL) and chloramphenicol (20 µg/mL), respectively. To impose low-iron stress and induce the expression of Fur-regulated *FepA* or *FhuA*, we sub-cultured stationary-phase LB cultures at 0.5% into iron-deficient MOPS minimal medium [97] and shook the flasks at 200 rpm for ~20 h at 37 °C to a cell density of $1.5\text{--}2.5 \times 10^9$ cells/mL.

4.4. Siderophores

We prepared apo and iron complexes from purified siderophores [98]. For FeEnt we mixed a micromole of purified Ent in 0.5 mL of methanol with a micromole of FeCl₃ in 0.5 mL distilled water, allowed the mixture to incubate for a few minutes, added NaHPO₄, pH 7, to 50 mM, allowed the mixture to incubate at RT for an hour, and then purified the FeEnt complex over a column (1.5 × 50 cm) of Sephadex LH20 in 5 mM NaHPO₄, pH 7 [99]. For Fc, we diluted a micromole of crystals into 1 mL of 5 mM NaHPO₄, pH 7.

4.5. Siderophore Nutrition Tests

We qualitatively analyzed iron transport by microbiological nutrition tests [99]. Cells expressing wild-type *FepA*, *FhuA* or their mutant derivatives were grown overnight in LB broth to stationary phase and sub-inoculated at 1% into nutrient broth (NB) containing streptomycin (100 µg/mL) and chloramphenicol (20 µg/mL). After overnight growth in NB, we mixed 100 µL of NB culture with 3 mL of molten NB top agar containing 100 µM apoferrichrome A and appropriate antibiotics, and deposited the mixtures in a 6-well microplate. We applied a paper disc to the solidified agar and added 10 µL of 50 µM ferric siderophore to the disc. After 24 h at 37 °C, we measured the diameter of growth around the paper disc.

4.6. Colicin Susceptibility Assays

We determined the susceptibility of *FepA* and *FhuA* mutants to colicins B and M, respectively, by microbiological plate tests. In each case we grew the individual test strains in LB broth overnight and plated 10^8 cells in 2 mL of LB top agar on LB plates. For each bacteriocin we prepared a sequential dilution matrix in half of a 96-well microtiter plate, with serial 10-fold dilutions of the purified colicin in PBS down the first column of the plate, and then serial 2-fold dilutions in PBS across columns 2–6. We used a sterile, 48-pin Clonemaster (Immusine Inc., Walnut Creek, CA, USA) to transfer 5 µL of each well of the colicin dilution matrix to the test plates, and incubated them overnight at 37 °C. This procedure revealed the titer of the ColB and ColM solutions on each *FepA* or *FhuA* mutant

strain, as the reciprocal of the highest colicin dilution that gave clearance of the bacteria on the agar surface. We expressed these results as a percentage of wild-type titer.

4.7. Site-Directed Labeling with Fluorescein Maleimide (FM) [100]

We used FepA_A698C [94] and FhuA_S396C [71] for attachment of FM. In some experiments we combined these mutations with site-directed Ala substitutions (see below) for phenotypic analysis. For FM-labeling we cultured strains expressing the FepA or FhuA Cys mutant derivatives in 10 mL of MOPS iron-deficient minimal media for ~20 h to a density of $2\text{--}3 \times 10^9$ cells/mL. After collecting the bacteria by centrifugation at $7000 \times g$ for 10 min, we washed the pellet with, and resuspended it in 10 mL of 50 mM NaHPO₄, pH 6.7. We added FM to the cell suspension to 5 μ M, incubated the mixture for 15 min 37 °C, and quenched labeling by adding β -mercaptoethanol to 100 μ M. We pelleted the fluoresceinated bacteria by centrifugation at $7000 \times g$ for 10 min and then washed and resuspended them in 10 mL of PBS, pH 7.4. We analyzed the extent of fluorescence labeling by spectroscopic determinations of fluorescence intensity, and its specificity by SDS-PAGE/image analysis.

4.8. SDS-PAGE and Western Immunoblots

We analyzed protein expression and fluorescence labeling by SDS-PAGE [67,101] and western immunoblots [102]. For determinations of protein expression, we suspended 10^8 bacterial cells in 100 μ L of distilled water, added an equal volume of SDS-PAGE sample buffer containing 3% SDS and 0.3% β -mercaptoethanol, and heated the samples in a water bath at 100 °C for 5 min. We resolved the cell proteins on 12% acrylamide/0.3% bis acrylamide SDS-PAGE gels [67,101,103] and observed FM-labeled Cys mutant proteins by transillumination with UV light. For strains expressing FepA or its mutants, we transferred the proteins to nitrocellulose and imaged the blots with anti-FepA mAb 44 [102] and goat anti-mouse IgG-alkaline phosphatase.

4.9. Fluorescence Determinations of Ligand Binding

To measure the affinities of Ala substitution mutants in FepA, we engineered each mutation in the background of marker *fepA_A698C* (that does not affect FeEnt binding or transport [94]), and expressed them in *E. coli* OKN1359. To measure the affinities of Ala substitution mutants in FhuA, we engineered each of them in the background of marker *fhuA_S396C* (that does not affect Fc binding or transport [104]), and expressed them in OKN17 (Δ *tonB*, Δ *fhuA*). After FM-labeling of each of the strains (see above) we performed fluorescent determinations of ligand binding with an SLM AMINCO 8100 fluorescence spectrometer with an OLIS operating system (OLIS Inc., Bogart, GA, USA). We added 5×10^7 FM-labeled bacterial cells to 2 mL of PBS in a quartz cuvette, with stirring. Excitation and emission wavelengths were 488 nm and 520 nm, respectively. After measuring the initial fluorescence (F_0) of the sample, we titrated the labeled cells with sequentially increasing concentrations of ferric siderophore, and recorded their corresponding fluorescence (F). We collected data in triplicate, plotted the mean values of $1-F/F_0$ versus [ligand], and analyzed the data with the “1-site with background” binding model of Grafit 6.0.12 (Erithacus Ltd. West Sussex, UK). The titrations yielded apparent binding K_D values, as well as fitted curves of [bound ligand] as a function of [free ligand]:

$$[Bound] = \frac{Capacity[Free]}{K_d + [Free]} + Background$$

Throughout this report, affinities are “apparent” K_D values, in the sense that we did not directly measure binding of the iron complexes, but rather the fluorescence quenching that resulted from their binding.

4.10. Fluorescence Spectroscopic Uptake Measurements

We employed decoy sensors [71] to monitor TonB-dependent uptake of FeEnt or Fc by FepA and FhuA wild-type TBDRs, and their Ala substitution mutant derivatives. In the fluorescence spectroscopic uptake studies we used *E. coli* OKN1359 [71] as host for pITS23 encoding *fepA_A698C* [94] or OKN17 as host for pITS11 encoding *fhuA_S396C* [104]. After growing these strains in iron-deficient MOPS media, we labeled them with FM. The TonB-deficient host strains are incapable of ferric siderophore uptake, and acted as sensitive sensors of [FeEnt] or [Fc] in solution [71], that monitored the uptake of the ferric siderophores by test strains. We expressed the Ala substitution mutant FepA or FhuA proteins in OKN2359 ($\Delta entA$, $\Delta fepA$, $\Delta fhuA$, Δcir , $\Delta ompT$) or OKN7 ($\Delta entA$, $\Delta fhuA$), respectively, and grew them overnight in iron-deficient MOPS minimal media. For each assay we combined 10^7 sensor cells (e.g., OKN1359/pFepA-FM [71]) and 10^7 cells of the mutant test strain in a 2 mL quartz cuvette containing PBS + 0.2% glucose at 37 °C. After recording fluorescence intensity for 100 s, we added an iron complex (e.g., FeEnt) at a final concentration of ~10 nM. The siderophore bound to the sensor and quenched fluorescence (e.g., ~40% quenching for EcoFepA_A698C-FM). We monitored the time course of fluorescence emissions at 520 nm for 15–40 min, with stirring. Transport of the iron complex by the test strain caused an increase in fluorescence intensity as the cells depleted it from solution.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/ijms27042007/s1>.

Author Contributions: Conceptualizations, methodology, validation, formal analysis, investigation, writing—review and editing, S.M.N. and P.E.K.; resources, data curation, writing—original draft preparation, supervision, project administration, funding acquisition, P.E.K. All authors have read and agreed to the published version of the manuscript.

Funding: No external funding supported the research in this manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All relevant data are contained within Figures 1–5 of this manuscript and Figures S1–S10 of the Supplementary Materials.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

TBDR	TonB-dependent receptor
NTLD	N-terminal luminal domain
CT β B	C-terminal β -barrel
OM	outer membrane
FeEnt	ferric enterobactin
Fc	ferrichrome
ColB	colicin B
ColM	colicin M
FM	fluorescein maleimide
PMF	proton motive force

References

1. Neilands, J.B. A Crystalline Organo-iron Pigment from a Rust Fungus (*Ustilago sphaerogena*). *J. Am. Chem. Soc.* **1952**, *74*, 4846–4847. [\[CrossRef\]](#)
2. Neilands, J.B. Siderophores: Structure and function of microbial iron transport compounds. *J. Biol. Chem.* **1995**, *270*, 26723–26726. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Hider, R.C.; Kong, X. Chemistry and biology of siderophores. *Nat. Prod. Rep.* **2010**, *27*, 637–657. [\[CrossRef\]](#)
4. Harris, W.R.; Carrano, C.J.; Raymond, K.N. Spectrophotometric determination of the proton-dependent stability constant of ferric enterobactin. *J. Am. Chem. Soc.* **1979**, *101*, 2213–2214. [\[CrossRef\]](#)
5. Neilands, J.B. Microbial iron compounds. *Annu. Rev. Biochem.* **1981**, *50*, 715–731. [\[CrossRef\]](#)
6. Pollack, J.R.; Neilands, J.B. Enterobactin, an iron transport compound from *Salmonella typhimurium*. *Biochem. Biophys. Res. Commun.* **1970**, *38*, 989–992. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Neilands, J.B. Hydroxamic acids in nature. *Science* **1967**, *156*, 1443–1447. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Anderson, G.J.; Frazer, D.M. Current understanding of iron homeostasis. *Am. J. Clin. Nutr.* **2017**, *106*, 1559s–1566s. [\[CrossRef\]](#)
9. Chen, Y.; Fan, Z.; Yang, Y.; Gu, C. Iron metabolism and its contribution to cancer. *Int. J. Oncol.* **2019**, *54*, 1143–1154. [\[CrossRef\]](#)
10. Chifman, J.; Laubenbacher, R.; Torti, S.V. A systems biology approach to iron metabolism. *Adv. Exp. Med. Biol.* **2014**, *844*, 201–225. [\[CrossRef\]](#)
11. Noinaj, N.; Guillier, M.; Barnard, T.J.; Buchanan, S.K. TonB-dependent transporters: Regulation, structure, and function. *Annu. Rev. Microbiol.* **2010**, *64*, 43–60. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Di Masi, D.R.; White, J.C.; Schnaitman, C.A.; Bradbeer, C. Transport of vitamin B12 in *Escherichia coli*: Common receptor sites for vitamin B12 and the E colicins on the outer membrane of the cell envelope. *J. Bacteriol.* **1973**, *115*, 506–513. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Newton, S.M.; Trinh, V.; Pi, H.; Klebba, P.E. Direct measurements of the outer membrane stage of ferric enterobactin transport: Postuptake binding. *J. Biol. Chem.* **2010**, *285*, 17488–17497. [\[CrossRef\]](#)
14. Braun, V. Substrate Uptake by TonB-Dependent Outer Membrane Transporters. *Mol. Microbiol.* **2024**, *122*, 929–947. [\[CrossRef\]](#)
15. Buchanan, S.K.; Smith, B.S.; Venkatramani, L.; Xia, D.; Esser, L.; Palnitkar, M.; Chakraborty, R.; van der Helm, D.; Deisenhofer, J. Crystal structure of the outer membrane active transporter FepA from *Escherichia coli*. *Nat. Struct. Biol.* **1999**, *6*, 56–63. [\[PubMed\]](#)
16. Ferguson, A.D.; Chakraborty, R.; Smith, B.S.; Esser, L.; van der Helm, D.; Deisenhofer, J. Structural basis of gating by the outer membrane transporter FecA. *Science* **2002**, *295*, 1715–1719. [\[CrossRef\]](#)
17. Locher, K.P.; Rees, B.; Koebnik, R.; Mitschler, A.; Moulinier, L.; Rosenbusch, J.P.; Moras, D. Transmembrane signaling across the ligand-gated FhuA receptor: Crystal structures of free and ferrichrome-bound states reveal allosteric changes. *Cell* **1998**, *95*, 771–778. [\[CrossRef\]](#)
18. Bradbeer, C. The proton motive force drives the outer membrane transport of cobalamin in *Escherichia coli*. *J. Bacteriol.* **1993**, *175*, 3146–3150. [\[CrossRef\]](#)
19. Kadner, R.J. Vitamin B12 transport in *Escherichia coli*: Energy coupling between membranes. *Mol. Microbiol.* **1990**, *4*, 2027–2033. [\[CrossRef\]](#)
20. Wiener, M.C. TonB-dependent outer membrane transport: Going for Baroque? *Curr. Opin. Struct. Biol.* **2005**, *15*, 394–400. [\[CrossRef\]](#)
21. Bolam, D.N.; van den Berg, B. TonB-dependent transport by the gut microbiota: Novel aspects of an old problem. *Curr. Opin. Struct. Biol.* **2018**, *51*, 35–43. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Eisenbeis, S.; Lohmiller, S.; Valdebenito, M.; Leicht, S.; Braun, V. NagA-dependent uptake of N-acetyl-glucosamine and N-acetyl-chitin oligosaccharides across the outer membrane of *Caulobacter crescentus*. *J. Bacteriol.* **2008**, *190*, 5230–5238. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Pollet, R.M.; Martin, L.M.; Koropatkin, N.M. TonB-dependent transporters in the Bacteroidetes: Unique domain structures and potential functions. *Mol. Microbiol.* **2021**, *115*, 490–501. [\[CrossRef\]](#)
24. Wang, C.C.; Newton, A. Iron transport in *Escherichia coli*: Relationship between chromium sensitivity and high iron requirement in mutants of *Escherichia coli*. *J. Bacteriol.* **1969**, *98*, 1135–1141. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Schauer, K.; Rodionov, D.A.; de Reuse, H. New substrates for TonB-dependent transport: Do we only see the ‘tip of the iceberg’? *Trends Biochem. Sci.* **2008**, *6*, 6. [\[CrossRef\]](#)
26. Wu, J.Y.; Srinivas, P.; Pogue, J.M. Cefiderocol: A Novel Agent for the Management of Multidrug-Resistant Gram-Negative Organisms. *Infect. Dis. Ther.* **2020**, *9*, 17–40. [\[CrossRef\]](#)
27. Saier, M.H., Jr. Families of proteins forming transmembrane channels. *J. Membr. Biol.* **2000**, *175*, 165–180. [\[CrossRef\]](#)
28. Nikaido, H. Porins and specific diffusion channels in bacterial outer membranes. *J. Biol. Chem.* **1994**, *269*, 3905–3908. [\[CrossRef\]](#)
29. Buchanan, S.K.; Lukacik, P.; Grizot, S.; Ghirlando, R.; Ali, M.M.; Barnard, T.J.; Jakes, K.S.; Kienker, P.K.; Esser, L. Structure of colicin I receptor bound to the R-domain of colicin Ia: Implications for protein import. *Embo J.* **2007**, *26*, 2594–2604. [\[CrossRef\]](#)

30. Grinter, R.; Lithgow, T. The structure of the bacterial iron-catecholate transporter Fiu suggests that it imports substrates via a two-step mechanism. *J. Biol. Chem.* **2019**, *294*, 19523–19534. [\[CrossRef\]](#)
31. Shultis, D.D.; Purdy, M.D.; Banchs, C.N.; Wiener, M.C. Outer membrane active transport: Structure of the BtuB:TonB complex. *Science* **2006**, *312*, 1396–1399. [\[CrossRef\]](#)
32. Cowan, S.W.; Schirmer, T.; Rummel, G.; Steiert, M.; Ghosh, R.; Paupit, R.A.; Jansonius, J.N.; Rosenbusch, J.P. Crystal structures explain functional properties of two *E. coli* porins. *Nature* **1992**, *358*, 727–733. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Nikaido, H.; Rosenberg, E.Y. Effect on solute size on diffusion rates through the transmembrane pores of the outer membrane of *Escherichia coli*. *J. Gen. Physiol.* **1981**, *77*, 121–135. [\[CrossRef\]](#)
34. Braun, V.; Ratliff, A.C.; Celia, H.; Buchanan, S.K. Energization of Outer Membrane Transport by the ExbB ExbD Molecular Motor. *J. Bacteriol.* **2023**, *205*, e0003523. [\[CrossRef\]](#)
35. Celia, H.; Botos, I.; Ni, X.; Fox, T.; De Val, N.; Lloubes, R.; Jiang, J.; Buchanan, S.K. Cryo-EM structure of the bacterial Ton motor subcomplex ExbB-ExbD provides information on structure and stoichiometry. *Commun. Biol.* **2019**, *2*, 358. [\[CrossRef\]](#)
36. Ferguson, A.D.; Hofmann, E.; Coulton, J.W.; Diederichs, K.; Welte, W. Siderophore-mediated iron transport: Crystal structure of FhuA with bound lipopolysaccharide. *Science* **1998**, *282*, 2215–2220. [\[CrossRef\]](#)
37. Chakraborty, R.; Lemke, E.A.; Cao, Z.; Klebba, P.E.; van der Helm, D. Identification and mutational studies of conserved amino acids in the outer membrane receptor protein, FepA, which affect transport but not binding of ferric-enterobactin in *Escherichia coli*. *Biometals Int. J. Role Met. Ions Biol. Biochem. Med.* **2003**, *16*, 507–518. [\[CrossRef\]](#)
38. Endriss, F.; Braun, M.; Killmann, H.; Braun, V. Mutant analysis of the *Escherichia coli* FhuA protein reveals sites of FhuA activity. *J. Bacteriol.* **2003**, *185*, 4683–4692. [\[CrossRef\]](#)
39. Klebba, P.E. Three paradoxes of ferric enterobactin uptake. *Front. Biosci.* **2003**, *8*, 1422–1436. [\[CrossRef\]](#) [\[PubMed\]](#)
40. Klebba, P.E.; Newton, S.M.; Six, D.A.; Kumar, A.; Yang, T.; Nairn, B.L.; Munger, C.; Chakravorty, S. Iron acquisition systems of Gram (−) bacterial pathogens define TonB-dependent pathways to novel antibiotics. *Chem. Rev.* **2021**, *121*, 5193–5239. [\[CrossRef\]](#) [\[PubMed\]](#)
41. Eisenhauer, H.A.; Shames, S.; Pawelek, P.D.; Coulton, J.W. Siderophore transport through *Escherichia coli* outer membrane receptor FhuA with disulfide-tethered cork and barrel domains. *J. Biol. Chem.* **2005**, *280*, 30574–30580. [\[CrossRef\]](#)
42. Majumdar, A.; Trinh, V.; Moore, K.J.; Smallwood, C.R.; Kumar, A.; Yang, T.; Scott, D.C.; Long, N.J.; Newton, S.M.; Klebba, P.E. Conformational rearrangements in the N-domain of *Escherichia coli* FepA during ferric enterobactin transport. *J. Biol. Chem.* **2020**, *295*, 4974–4984. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Yang, T.; Zou, Y.; Ng, H.L.; Kumar, A.; Newton, S.M.; Klebba, P.E. Specificity and Mechanism of TonB-dependent Ferric Catecholate Uptake by Fiu. *Front. Microbiol.* **2024**, in press. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Jordan, L.D.; Zhou, Y.; Smallwood, C.R.; Lill, Y.; Ritchie, K.; Yip, W.T.; Newton, S.M.; Klebba, P.E. Energy-dependent motion of TonB in the Gram-negative bacterial inner membrane. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 11553–11558. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Rieu, M.; Krutyholowa, R.; Taylor, N.M.I.; Berry, R.M. A new class of biological ion-driven rotary molecular motors with 5:2 symmetry. *Front. Microbiol.* **2022**, *13*, 948383. [\[CrossRef\]](#)
46. Somboon, K.; Melling, O.; Lejeune, M.; Pinheiro, G.M.S.; Paquelin, A.; Bardiaux, B.; Nilges, M.; Delepelaire, P.; Khalid, S.; Izadi-Pruneyre, N. Dynamic interplay between a TonB-dependent heme transporter and a TonB protein in a membrane environment. *mBio* **2024**, *15*, e0178124. [\[CrossRef\]](#)
47. Webby, M.N.; Williams-Jones, D.P.; Press, C.; Kleanthous, C. Force-Generation by the Trans-Envelope Tol-Pal System. *Front. Microbiol.* **2022**, *13*, 852176. [\[CrossRef\]](#)
48. Williams-Jones, D.P.; Webby, M.N.; Press, C.E.; Gradon, J.M.; Armstrong, S.R.; Szczepaniak, J.; Kleanthous, C. Tunable force transduction through the *Escherichia coli* cell envelope. *Proc. Natl. Acad. Sci. USA* **2023**, *120*, e2306707120. [\[CrossRef\]](#)
49. Zinke, M.; Lejeune, M.; Mechaly, A.; Bardiaux, B.; Boneca, I.G.; Delepelaire, P.; Izadi-Pruneyre, N. Ton motor conformational switch and peptidoglycan role in bacterial nutrient uptake. *Nat. Commun.* **2024**, *15*, 331. [\[CrossRef\]](#)
50. Klebba, P.E. ROSET Model of TonB Action in Gram-Negative Bacterial Iron Acquisition. *J. Bacteriol.* **2016**, *198*, 1013–1021. [\[CrossRef\]](#)
51. Grinter, R.; Lithgow, T. Determination of the molecular basis for coprogen import by Gram-negative bacteria. *IUCr* **2019**, *6 Pt 3*, 401–411. [\[CrossRef\]](#)
52. Lukacik, P.; Barnard, T.J.; Keller, P.W.; Chaturvedi, K.S.; Seddiki, N.; Fairman, J.W.; Noinaj, N.; Kirby, T.L.; Henderson, J.P.; Steven, A.C.; et al. Structural engineering of a phage lysin that targets gram-negative pathogens. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 9857–9862. [\[CrossRef\]](#)
53. Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; McGettigan, P.A.; McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; et al. Clustal W and Clustal X version 2.0. *Bioinformatics* **2007**, *23*, 2947–2948. [\[CrossRef\]](#)
54. Little, J.; Chikina, M.; Clark, N.L. Evolutionary rate covariation is a reliable predictor of co-functional interactions but not necessarily physical interactions. *eLife* **2024**, *12*, RP93333. [\[CrossRef\]](#) [\[PubMed\]](#)

55. Chatellier, J.; Mazza, A.; Brousseau, R.; Vernet, T. Codon-based combinatorial alanine scanning site-directed mutagenesis: Design, implementation, and polymerase chain reaction screening. *Anal. Biochem.* **1995**, *229*, 282–290. [[CrossRef](#)]
56. Fiss, E.H.; Hollifield, W.C., Jr.; Neilands, J.B. Absence of ferric enterobactin receptor modification activity in mutants of *Escherichia coli* K-12 lacking protein a. *Biochem. Biophys. Res. Commun.* **1979**, *91*, 29–34. [[CrossRef](#)] [[PubMed](#)]
57. Fiss, E.H.; Stanley-Samuelson, P.; Neilands, J.B. Properties and proteolysis of ferric enterobactin outer membrane receptor in *Escherichia coli* K12. *Biochemistry* **1982**, *21*, 4517–4522. [[CrossRef](#)] [[PubMed](#)]
58. Hollifield, W.C., Jr.; Fiss, E.H.; Neilands, J.B. Modification of a ferric enterobactin receptor protein from the outer membrane of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **1978**, *83*, 739–746. [[CrossRef](#)]
59. Rupperecht, K.R.; Gordon, G.; Lundrigan, M.; Gayda, R.C.; Markovitz, A.; Earhart, C. omp T: *Escherichia coli* K-12 structural gene for protein a (3b). *J. Bacteriol.* **1983**, *153*, 1104–1106. [[CrossRef](#)]
60. Cho, Y.H.; Fadle Aziz, M.R.; Malpass, A.; Sutradhar, T.; Bashal, J.; Cojocari, V.; McPhee, J.B. OmpT Proteases of Enterobacterales Show Conserved Regulation by the PhoPQ Two-Component System but Exhibit Divergent Protection from Antimicrobial Host Peptides and Complement. *Infect. Immun.* **2023**, *91*, e0051822. [[CrossRef](#)]
61. Hui, C.Y.; Guo, Y.; He, Q.S.; Peng, L.; Wu, S.C.; Cao, H.; Huang, S.H. *Escherichia coli* outer membrane protease OmpT confers resistance to urinary cationic peptides. *Microbiol. Immunol.* **2010**, *54*, 452–459. [[CrossRef](#)]
62. Thomassin, J.L.; Brannon, J.R.; Gibbs, B.F.; Gruenheid, S.; Le Moual, H. OmpT outer membrane proteases of enterohemorrhagic and enteropathogenic *Escherichia coli* contribute differently to the degradation of human LL-37. *Infect. Immun.* **2012**, *80*, 483–492. [[CrossRef](#)]
63. Thomassin, J.L.; Brannon, J.R.; Kaiser, J.; Gruenheid, S.; Le Moual, H. Enterohemorrhagic and enteropathogenic *Escherichia coli* evolved different strategies to resist antimicrobial peptides. *Gut Microbes* **2012**, *3*, 556–561. [[CrossRef](#)]
64. Akiyama, Y.; Ito, K. SecY protein, a membrane-embedded secretion factor of *E. coli*, is cleaved by the ompT protease in vitro. *Biochem. Biophys. Res. Commun.* **1990**, *167*, 711–715. [[CrossRef](#)]
65. Baneyx, F.; Georgiou, G. In vivo degradation of secreted fusion proteins by the *Escherichia coli* outer membrane protease OmpT. *J. Bacteriol.* **1990**, *172*, 491–494. [[CrossRef](#)]
66. Grodberg, J.; Dunn, J.J. ompT encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. *J. Bacteriol.* **1988**, *170*, 1245–1253. [[CrossRef](#)] [[PubMed](#)]
67. Lugtenberg, B.; Meijers, J.; Peters, R.; van der Hoek, P.; van Alphen, L. Electrophoretic resolution of the “major outer membrane protein” of *Escherichia coli* K12 into four bands. *FEBS Lett.* **1975**, *58*, 254–258. [[CrossRef](#)]
68. Luckey, M.; Pollack, J.R.; Wayne, R.; Ames, B.N.; Neilands, J.B. Iron uptake in *Salmonella typhimurium*: Utilization of exogenous siderochromes as iron carriers. *J. Bacteriol.* **1972**, *111*, 731–738. [[CrossRef](#)] [[PubMed](#)]
69. Wayne, R.; Frick, K.; Neilands, J.B. Siderophore protection against colicins M, B, V, and Ia in *Escherichia coli*. *J. Bacteriol.* **1976**, *126*, 7–12. [[CrossRef](#)] [[PubMed](#)]
70. Cao, Z.; Qi, Z.; Sprencel, C.; Newton, S.M.; Klebba, P.E. Aromatic components of two ferric enterobactin binding sites in *Escherichia coli* fepA. *Mol. Microbiol.* **2000**, *37*, 1306–1317. [[CrossRef](#)]
71. Chakravorty, S.; Shipelskiy, Y.; Kumar, A.; Majumdar, A.; Yang, T.; Nairn, B.L.; Newton, S.M.; Klebba, P.E. Universal fluorescent sensors of high-affinity iron transport, applied to ESKAPE pathogens. *J. Biol. Chem.* **2019**, *294*, 4682–4692. [[CrossRef](#)] [[PubMed](#)]
72. Charbit, A.; Wang, J.; Michel, V.; Hofnung, M. A cluster of charged and aromatic residues in the C-terminal portion of maltoporin participates in sugar binding and uptake. *Mol. Gen. Genet.* **1998**, *260*, 185–192. [[CrossRef](#)]
73. Klebba, P.E.; Hofnung, M.; Charbit, A. A model of maltodextrin transport through the sugar-specific porin, LamB, based on deletion analysis. *Embo J.* **1994**, *13*, 4670–4675. [[CrossRef](#)]
74. Mourez, M.; Hofnung, M.; Dassa, E. Subunit interactions in ABC transporters: A conserved sequence in hydrophobic membrane proteins of periplasmic permeases defines an important site of interaction with the ATPase subunits. *Embo J.* **1997**, *16*, 3066–3077. [[CrossRef](#)]
75. Raffy, S.; Sassoon, N.; Hofnung, M.; Betton, J.M. Tertiary structure-dependence of misfolding substitutions in loops of the maltose-binding protein. *Protein Sci.* **1998**, *7*, 2136–2142. [[CrossRef](#)] [[PubMed](#)]
76. Szmelcman, S.; Sassoon, N.; Hofnung, M. Residues in the alpha helix 7 of the bacterial maltose binding protein which are important in interactions with the Mal FGK2 complex. *Protein Sci.* **1997**, *6*, 628–636. [[CrossRef](#)]
77. Hickman, S.J.; Cooper, R.E.M.; Bellucci, L.; Paci, E.; Brockwell, D.J. Gating of TonB-dependent transporters by substrate-specific forced remodelling. *Nat. Commun.* **2017**, *8*, 14804. [[CrossRef](#)]
78. Ma, L.; Kaserer, W.; Annamalai, R.; Scott, D.C.; Jin, B.; Jiang, X.; Xiao, Q.; Maymani, H.; Massis, L.M.; Ferreira, L.C.; et al. Evidence of ball-and-chain transport of ferric enterobactin through FepA. *J. Biol. Chem.* **2007**, *282*, 397–406. [[CrossRef](#)]
79. Haiko, J.; Suomalainen, M.; Ojala, T.; Lähdenmäki, K.; Korhonen, T.K. Invited review: Breaking barriers—attack on innate immune defences by ompT surface proteases of enterobacterial pathogens. *Innate Immun.* **2009**, *15*, 67–80. [[CrossRef](#)]
80. Hritonenko, V.; Stathopoulos, C. OmpT proteins: An expanding family of outer membrane proteases in Gram-negative *Enterobacteriaceae*. *Mol. Membr. Biol.* **2007**, *24*, 395–406. [[CrossRef](#)] [[PubMed](#)]

81. Kukkonen, M.; Korhonen, T.K. The omptin family of enterobacterial surface proteases/adhesins: From housekeeping in *Escherichia coli* to systemic spread of *Yersinia pestis*. *Int. J. Med. Microbiol.* **2004**, *294*, 7–14. [\[CrossRef\]](#)
82. Vandeputte-Rutten, L.; Kramer, R.A.; Kroon, J.; Dekker, N.; Egmond, M.R.; Gros, P. Crystal structure of the outer membrane protease OmpT from *Escherichia coli* suggests a novel catalytic site. *Embo J.* **2001**, *20*, 5033–5039. [\[CrossRef\]](#)
83. Dekker, N.; Cox, R.C.; Kramer, R.A.; Egmond, M.R. Substrate specificity of the integral membrane protease OmpT determined by spatially addressed peptide libraries. *Biochemistry* **2001**, *40*, 1694–1701. [\[CrossRef\]](#)
84. McCarter, J.D.; Stephens, D.; Shoemaker, K.; Rosenberg, S.; Kirsch, J.F.; Georgiou, G. Substrate specificity of the *Escherichia coli* outer membrane protease OmpT. *J. Bacteriol.* **2004**, *186*, 5919–5925. [\[CrossRef\]](#)
85. Okuno, K.; Yabuta, M.; Kawanishi, K.; Ohsuye, K.; Ooi, T.; Kinoshita, S. Substrate specificity at the P1' site of *Escherichia coli* OmpT under denaturing conditions. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 127–134. [\[CrossRef\]](#)
86. Sugimura, K.; Nishihara, T. Purification, characterization, and primary structure of *Escherichia coli* protease VII with specificity for paired basic residues: Identity of protease VII and OmpT. *J. Bacteriol.* **1988**, *170*, 5625–5632. [\[CrossRef\]](#)
87. White, C.B.; Chen, Q.; Kenyon, G.L.; Babbitt, P.C. A novel activity of OmpT. Proteolysis under extreme denaturing conditions. *J. Biol. Chem.* **1995**, *270*, 12990–12994. [\[CrossRef\]](#) [\[PubMed\]](#)
88. Blattner, F.R.; Plunkett, G., III; Bloch, C.A.; Perna, N.T.; Burland, V.; Riley, M.; Collado-Vides, J.; Glasner, J.D.; Rode, C.K.; Mayhew, G.F. The complete genome sequence of *Escherichia coli* K-12. *Science* **1997**, *277*, 1453–1462. [\[CrossRef\]](#)
89. Scott, D.C.; Cao, Z.; Qi, Z.; Bauler, M.; Igo, J.D.; Newton, S.M.; Klebba, P.E. Exchangeability of N termini in the ligand-gated porins of *Escherichia coli*. *J. Biol. Chem.* **2001**, *276*, 13025–13033. [\[CrossRef\]](#) [\[PubMed\]](#)
90. Takeshita, S.; Sato, M.; Toba, M.; Masahashi, W.; Hashimoto-Gotoh, T. High-copy-number and low-copy-number plasmid vectors for lacZ α -complementation and chloramphenicol-or kanamycin-resistance selection. *Gene* **1987**, *61*, 63–74. [\[CrossRef\]](#) [\[PubMed\]](#)
91. Hashimoto-Gotoh, T.; Kume, A.; Masahashi, W.; Takeshita, S.; Fukuda, A. Improved vector, pHSG664, for direct streptomycin-resistance selection: cDNA cloning with G:C-tailing procedure and subcloning of double-digest DNA fragments. *Gene* **1986**, *41*, 125–128.
92. Klebba, P.E. *Regulation of the Biosynthesis of the Iron-Related Membrane Proteins in Escherichia coli*; University of California: Berkeley, CA, USA, 1981.
93. Annamalai, R.; Jin, B.; Cao, Z.; Newton, S.M.; Klebba, P.E. Recognition of ferric catecholates by FepA. *J. Bacteriol.* **2004**, *186*, 3578–3589. [\[CrossRef\]](#) [\[PubMed\]](#)
94. Smallwood, C.R.; Jordan, L.; Trinh, V.; Schuerch, D.W.; Gala, A.; Hanson, M.; Shipelskiy, Y.; Majumdar, A.; Newton, S.M.; Klebba, P.E. Concerted loop motion triggers induced fit of FepA to ferric enterobactin. *J. Gen. Physiol.* **2014**, *144*, 71–80. [\[CrossRef\]](#) [\[PubMed\]](#)
95. Smallwood, C.R.; Marco, A.G.; Xiao, Q.; Trinh, V.; Newton, S.M.; Klebba, P.E. Fluoresceination of FepA during colicin B killing: Effects of temperature, toxin and TonB. *Mol. Microbiol.* **2009**, *72*, 1171–1180. [\[CrossRef\]](#)
96. Bertani, G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bacteriol.* **1951**, *62*, 293–300. [\[CrossRef\]](#)
97. Neidhardt, F.C.; Bloch, P.L.; Smith, D.F. Culture medium for enterobacteria. *J. Bacteriol.* **1974**, *119*, 736–747. [\[CrossRef\]](#)
98. Balhasteros, H.; Shipelskiy, Y.; Long, N.J.; Majumdar, A.; Katz, B.B.; Santos, N.M.; Leaden, L.; Newton, S.M.; Marques, M.V.; Klebba, P.E. TonB-Dependent Heme/Hemoglobin Utilization by *Caulobacter crescentus* HutA. *J. Bacteriol.* **2017**, *199*, e00723. [\[CrossRef\]](#) [\[PubMed\]](#)
99. Wayne, R.; Neilands, J.B. Evidence for common binding sites for ferrichrome compounds and bacteriophage phi 80 in the cell envelope of *Escherichia coli*. *J. Bacteriol.* **1975**, *121*, 497–503. [\[CrossRef\]](#)
100. Newton, S.M.; Klebba, P.E. Fluorescent Binding Protein Sensors for Detection and Quantification of Biochemicals, Metabolites, and Natural Products. *Bio-Protoc.* **2022**, *12*, e4543. [\[CrossRef\]](#)
101. Hancock, R.E.; Braun, V. The colicin I receptor of *Escherichia coli* K-12 has a role in enterochelin-mediated iron transport. *FEBS Lett.* **1976**, *65*, 208–210. [\[CrossRef\]](#) [\[PubMed\]](#)
102. Murphy, C.K.; Kalve, V.I.; Klebba, P.E. Surface topology of the *Escherichia coli* K-12 ferric enterobactin receptor. *J. Bacteriol.* **1990**, *172*, 2736–2746. [\[CrossRef\]](#) [\[PubMed\]](#)
103. Ames, G.F. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. Membrane, soluble, and periplasmic fractions. *J. Biol. Chem.* **1974**, *249*, 634–644. [\[CrossRef\]](#) [\[PubMed\]](#)
104. Kumar, A.; Yang, T.; Chakravorty, S.; Majumdar, A.; Nairn, B.L.; Six, D.A.; Marcondes Dos Santos, N.; Price, S.L.; Lawrenz, M.B.; Actis, L.A.; et al. Fluorescent sensors of siderophores produced by bacterial pathogens. *J. Biol. Chem.* **2022**, *298*, 101651. [\[CrossRef\]](#) [\[PubMed\]](#)

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.