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Toward a Mechanistic Understanding of Feo-Mediated Ferrous Iron Uptake

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Abstract

Virtually all organisms require iron and have evolved to obtain this element in free or chelated forms. Under anaerobic or low pH conditions commonly encountered by numerous pathogens, iron predominantly exists in the ferrous (Fe^{2+}) form. The ferrous iron transport (Feo) system is the only widespread mechanism dedicated solely to bacterial ferrous iron import, and this system has been linked to pathogenic virulence, bacterial colonization, and microbial survival. The canonical *feo* operon encodes for three proteins that comprise the Feo system: FeoA, a small cytoplasmic β -barrel protein; FeoB, a large, polytopic membrane protein with a soluble G-protein domain capable of hydrolyzing GTP; and FeoC, a small, cytoplasmic protein containing a winged-helix motif. While previous studies have revealed insight into soluble and fragmentary domains of the Feo system, the chief membrane-bound component FeoB remains poorly studied. However, recent advances have demonstrated that large quantities of intact FeoB can be overexpressed, purified, and biophysically characterized, revealing glimpses into FeoB function. Two models of full-length FeoB have been published, providing starting points for hypothesis-driven investigations into the mechanism of FeoB-mediated ferrous iron transport. Finally, *in vivo* studies have begun to shed light on how this system functions as a unique multicomponent complex. In light of these new data, this review will summarize what is known about the Feo system, including recent advancements in FeoB structure and function.

Graphical Abstract

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Conflicts of Interest

There are no conflicts of interest to declare.

The ferrous iron transport (Feo) system is the predominant mode of bacterial Fe^{2+} import. Advancements in the structure and function of FeoB provide glimpses into the mechanism of Fe^{2+} uptake.

Iron is an incredibly versatile element that nature has adopted for indispensable biological processes such as aerobic cellular respiration, nitrogen fixation, and even DNA biosynthesis (1). Acquisition of iron is thus vital for the survival of most organisms, and bacteria have adapted to obtain several forms of this element. Under aerobic conditions iron predominantly exists in the ferric (Fe^{3+}) form and is chiefly found in complex oxides, which are rather kinetically inert and insoluble (10^{-18} M at pH=7) (2), whereas under anaerobic conditions iron predominantly exists in the ferrous (Fe^{2+}) form, which is significantly more soluble (reaching as high as $\sim 10^{-3}$ M, depending on pH) (2) and more kinetically labile (1). The maintenance of cellular iron homeostasis is essential, as high concentrations of intracellular iron are necessary for normal physiological function; however, if iron concentrations are left unregulated, reduced iron in particular can be cytotoxic by promoting the formation of reactive oxygen species (ROS), which can damage DNA, proteins, and lipids (3, 4). To keep iron soluble and to prevent its toxicity within the organism, virtually all cytosolic iron is bound by proteins as part of a prosthetic group, such as in iron protoporphyrin IX (heme), complexed to proteins in the form of iron sulfur clusters, or directly bound by a protein, such as in transferrin, lactoferrin, and ferritin (5). However, these scenarios all require intracellular iron stores that must be established from the organism's environment.

During infection, pathogenic bacteria must acquire iron from their surrounding environment, which frequently comprises a niche within the host organism. At this location begins a tug-of-war over iron, and an array of regulatory mechanisms are employed by both the host and the invading bacterium to achieve and to maintain iron homeostasis (6). As part of an innate immune response to infection, mammalian hosts employ regulatory mechanisms that attempt to limit the concentration of bioavailable iron to invading organisms (a strategy known as nutritional immunity) by sequestration of iron into macrophages, hepatocytes, enterocytes and proteins (such as transferrin) with a concomitant reduction of dietary iron absorption (3, 5, 6). In response, bacteria employ high-affinity sequestration and transport systems to fulfill their iron needs, especially in the iron-deprived niches often created by the host (6).

One common method for the bacterium to scavenge for iron involves the synthesis of siderophores, high-affinity ferric iron chelators that specifically coordinate Fe^{3+} with catecholate, hydroxamate, or hydroxyl-carboxylate ligands in an octahedral geometry. After ferric iron binding, the iron-siderophore complex is transported into the cell and the metal ion is retrieved from the siderophore, either by reduction of ferric iron to ferrous iron, or by hydrolysis of the siderophore backbone (5, 7, 8). The utilization of siderophores enables bacteria to bind ferric iron tightly ($K_D \sim 10^{-20}$ M), in order to compete with host iron-binding proteins (5, 8). Additionally, bacteria have adapted to utilize heme, distributed widely within the human host, as an iron source (9, 10). To acquire heme, bacteria synthesize hemophores—proteins that scavenge for heme iron—and heme receptors—proteins that directly bind host hemoproteins (9, 10). Once heme is transported across the

bacterial outer membrane via energy provided by the membrane complex TonB/ExbB/ExbD, heme is then actively transported across the inner membrane by ATP-binding cassette transporters (9, 10). Once heme enters the cytoplasm, heme oxygenases most commonly release iron by degradation of the macrocycle (9, 10).

Although ferric iron and heme serve as the chief sources of iron under aerobic conditions, the invading pathogen may frequently encounter highly acidic, reducing, and anaerobic conditions where ferrous iron predominates. Within this environment, siderophore- and heme-mediated iron uptake systems are frequently inoperative, yet pathogens must (and do) acquire iron under these conditions. To fulfill their need for iron under anaerobic and/or reducing conditions, bacteria most frequently use the ferrous iron transport (Feo) system (Fig. 1) (11). Though other ferrous iron transport systems have been identified and characterized to some degree, Feo is the dominant prokaryotic system dedicated solely to ferrous iron transport (11). This review will examine the Feo system as a whole, and how this unique complex contributes to virulence. Additionally, this work will focus on recent developments regarding the full-length, intact membrane protein, FeoB, including putative mechanisms of FeoB-mediated Fe^{2+} movement across a prokaryotic membrane.

The *feo* Operon

The canonical *feo* operon, which was originally identified in *Escherichia coli* K-12 (12, 13) (Fig. 1A), encodes for three proteins that comprise the Feo system (Fig. 1B): FeoA, a small cytoplasmic β -barrel protein (14); FeoB, a large, polytopic membrane protein containing a soluble G-protein domain capable of hydrolyzing GTP (15) (termed NFeoB) covalently linked to approximately 7–12 transmembrane (TM) helices (16) through a GDP dissociation inhibitor (GDI) domain (17); and FeoC, a small, cytoplasmic protein containing a winged-helix motif (18, 19). Although the *feoABC* organization of the operon was the first to be discovered, it is not the most prevalent organization and only occurs in ~13% of the bacterial genomes that have been sequenced (11). The most frequent organization of the *feo* operon is that of *feoAB* (~54%) (Fig. 1A), while other common organizations include *feoB* alone (~11%), and an *feoAB* fusion (~3%) (Fig. 1A) (11). In organisms for which it has been determined (12, 20, 21), these genes are also under control of the ferric uptake transcriptional regulator (FUR) (22), which represses *feo* expression in the presence of iron, and the fumarate and nitrate reductase transcriptional regulator (FNR) (23), which serves as an oxygen sensor to activate *feo* expression under anaerobic conditions (11). These observations indicate that ferrous iron transport is a tightly controlled process, which is unsurprising, given both the necessity and the reactivity of this nutrient.

The sequenced genomic arrangements of the *feo* operon indicate FeoB is an indispensable component of the ferrous iron transport process, whereas FeoA and FeoC may only be accessories to this process (Fig. 1B). A comprehensive understanding of the mechanism of ferrous iron transport, including how FeoA, FeoB, and FeoC function in concert to move Fe^{2+} across the bacterial membrane, and how GTP hydrolysis is coupled to this transport process, remains unknown. However, insights into this process have been garnered through studies of individual soluble proteins as well as some fragmentary, soluble domains.

FeoA

FeoA is the most common protein present alongside of FeoB within the *feo* operon, yet very little is known about the function of this small (~8 kDa) protein. NMR structural studies of *E. coli* FeoA (2LX9) have shown the presence of a Src homology 3 (SH3)-like fold, a small β -barrel (Fig. 2) that commonly mediates protein-protein interactions and comprises two curved, antiparallel β -sheets (14, 24). Additionally, *Ec*FeoA contains two α -helices that flank the β -barrel fold and resemble a lid (14). While some FeoA proteins show high sequence identity, such as *Ec*FeoA and *Klebsiella pneumoniae* FeoA (*Kp*FeoA) (14), large-scale sequence alignments generally show poor sequence similarity among FeoA proteins despite the strongly conserved β -barrel structure (14, 25). For example, this fold is also observed in numerous, unpublished NMR structures of FeoA: 3E19 (*E. coli*; solved by Hughes *et al.*), 2K5L (*Clostridium thermocellum*; solved by Zeri *et al.*), 2K5I (*C. thermocellum*; solved by Liu *et al.*), 2K4Y (*C. acetobutylicum*; solved by Singarapu *et al.*), 2K5F (*Chlorobaculum tepidum*; solved by Eletsky *et al.*), and 2GCX (*K. pneumoniae*; solved by Hung *et al.*), suggesting that the structure of the FeoA protein is an essential link to FeoA function (11). Intriguingly, the soluble GTP-binding domain (GTPBD) of FeoB (NFeoB) contains an xPxxP motif, which is a common epitope recognized by SH3-like folds (24). To date, no structure of FeoA in the presence of iron has been solved, so it is currently unknown whether FeoA is capable of binding ferrous or ferric iron (14). Perhaps alluding to function, FeoA has an isoelectric point (pI) of ~9 (12), which may facilitate potential interactions with NFeoB by targeting FeoA to the inner leaflet of the membrane (11), allowing FeoA to scan the membrane for NFeoB in order to affect FeoB function. However, this scenario remains hypothetical.

Despite the presence of the SH3 domain and the xPxxP motif on NFeoB, there is no consensus as to whether FeoA interacts with FeoB. Studies have shown that FeoA is necessary for ferrous iron uptake in *Vibrio cholerae* (26) as well as in *Salmonella enterica* (27), prompting investigations into potential FeoA-FeoB interactions. In *V. cholerae*, a bacterial two-hybrid analysis (BACTH) was used to determine whether *Vc*FeoA-*Vc*FeoB interactions were occurring; however, no *in vivo* interaction was indicated (26). In a later study of *V. cholerae* that utilized *in vivo* formaldehyde crosslinking, interactions of *Vc*FeoA (and *Vc*FeoC) with *Vc*FeoB were observed (28). The discrepancies between experiments in the same organism is interesting and could suggest that the BACTH assays were performed under conditions in which FeoA was not able to interact with FeoB. In support of the *V. cholerae* crosslinking studies, BACTH analysis within *S. enterica* demonstrated that *Se*FeoA interacts with *Se*FeoB *in vivo*, and that this interaction is essential for FeoB-mediated ferrous iron uptake (27). Though the authors identified an FeoA variant, L26Q, that appeared to abolish both the *Se*FeoA-*Se*FeoB interaction and ferrous iron uptake (27), the atomic-level details of the *Se*FeoA-*Se*FeoB interactions remain unclear. The importance of this Leu residue is currently unknown, but a hydrophobic residue appears to be present in this location in the majority of bacterial FeoAs (Fig. 2) (29). Interestingly, hydrophobic interactions between SH3 domains and binding partners are thought to be essential for the protein-protein interactions to occur (24). If hydrophobic interactions between FeoA and FeoB are necessary for binding, it is plausible that replacement of the hydrophobic side

chain of Leu with the more hydrophilic side chain of Gln is sufficient to disrupt the potential interactions between these two polypeptides.

Further indication that FeoA and FeoB are capable of interacting comes from analyses of *Porphyromonas gingivalis* (30) and *Bacteroides fragilis* (20), both of which contain *feo* operons where FeoA and FeoB are encoded as a single peptide and FeoA is covalently tethered to the N-terminal GTPBD of FeoB (Fig. 1A). These naturally occurring fusions suggest that FeoA could serve as a regulator for GTPase activity and/or ferrous iron transport. Though intriguing, no study has examined the nature of the interactions of FeoA with heterologously expressed, full-length FeoB, and no study has revealed the exact role of FeoA in ferrous iron transport.

FeoC

X-ray crystallographic and NMR structural studies on FeoC (Fig. 3) have identified the presence of a winged-helix motif within this small (~8 kDa) protein. This structural motif is similar to that of the LysR transcriptional regulator (LTTR) family (19), and this fold has also been identified in an unpublished NMR structure of *EcFeoC* (1XN7, Shen *et al.*) and an unpublished structure of *KpFeoC* (2K02, Hung *et al.*). LTTR family proteins consist of two domains: the first domain is a trihelical helix-turn-helix (HTH) motif responsible for binding to DNA; the second domain consists of the wing, responsible for co-inducer binding, which typically regulates the affinity of the HTH domain for DNA (19). It is thought that FeoC could be a putative transcriptional regulator for the Feo system because the HTH motif is located at the N-terminus of FeoC (11, 19). Intriguingly, partial sequence alignments demonstrate the presence of four strongly conserved Cys residues that may be capable of binding a [Fe-S] cluster, and evidence for cluster binding has been demonstrated with purified *KpFeoC* (31). It is possible that the [Fe-S] cluster could serve as a co-inducer. In this scenario, incorporation of the [Fe-S] cluster into the unstructured wing region could induce conformational changes within the protein that cause the wing region to become structured, allowing for FeoC to have a greater affinity for DNA. Thus, FeoC could function as an important regulatory factor for ferrous iron transport, which could relate to the bioavailability of ferrous iron within the cell. However, no interactions between FeoC and DNA have been observed (11, 31), and some bacteria, such as *V. cholerae*, do not contain these strongly conserved Cys residues, precluding cluster binding (26, 28). Furthermore, given that FeoC is only present in ~13% of bacterial *feo* operons (11, 32, 33), it seems unlikely that this protein is necessary for the direct binding and transport of ferrous iron into the cytosol, although it cannot be ruled out that FeoC (or an FeoC-like protein) may be present elsewhere in bacterial genomes.

Despite its poor conservation, FeoC has been shown to be important for ferrous iron uptake in tripartite (*feoABC*) systems (Fig. 1A) (26), and some evidence indicates that FeoC can interact with FeoB. In the aforementioned *V. cholerae* BACTH study, *VcFeoC* was also probed for *in vivo* interactions with either *VcFeoA* or *VcFeoB* (26). While no interaction was observed between *VcFeoA* and *VcFeoC*, an interaction was observed between *VcFeoC* and full-length *VcFeoB* (26). In an attempt to identify where on the FeoB polypeptide the interaction with FeoC occurred, three different truncated FeoBs were probed for interactions

with FeoC: the GTPase domain alone, the GDI domain alone, and the full N-terminal cytoplasmic domain (VcNFeoB) (26). An interaction was only observed between VcFeoC and VcNFeoB, leading the authors to suggest that either both the GTPase and GDI domains are essential for FeoC-FeoB interactions, or that the truncated domains had not adopted the correct conformation to facilitate FeoC-FeoB interactions (26). This study also demonstrated that FeoC variants E29G and M35A abolished FeoC-FeoB interactions. While the importance of these residues is unknown, it is interesting to note that the analogous Glu²⁹ is conserved in other *Vibrio* species, but not in *E. coli*, *S. enterica*, *K. pneumoniae*, or *Y. pestis*, whereas the analogous Met³⁵ is conserved in all of these species (Fig. 3) (26). In support of this study, the aforementioned *V. cholerae* *in vivo* formaldehyde crosslinking study also revealed an interaction between FeoC and FeoB (28). Additionally, a significantly truncated form of FeoC from *K. pneumoniae*, which did not contain the winged-helix region of the protein, has been crystallized in the presence of KpNFeoB (32). This truncated FeoC was observed to bind between the G domain and the GDI domain of the respective KpNFeoB, suggesting that FeoC might be capable of regulating GTPase activity (32). Several NMR structures of KpFeoC were superposed onto this crystal structure, and the modeled winged-helix suggested that this domain of FeoC could interact with the GTP-binding site of NFeoB, which could also be dependent on the state of the [Fe-S] cluster. Though intriguing, it remains unclear whether the presence of the intact winged-helix region in KpFeoC would either facilitate or hinder FeoC-NFeoB interactions.

NFeoB

The lion's share of published literature on the Feo system has focused on the soluble NFeoB component, which comprises the combined N-terminal GTP-binding and GDI domains of FeoB (Fig. 4). Shortly after the discovery of the Feo system, FeoB was classified as an ATPase based on sequence alignments of NFeoB with those of other ATP-utilizing enzymes (12). At that time, it was hypothesized that ATP hydrolysis drove Feo-mediated ferrous iron transport in an active manner (12). To support this initial observation, a study of *H. pylori* FeoB revealed three potent inhibitors of ferrous iron transport, two of which were common inhibitors of ATP synthesis and ATP hydrolysis (34). Shortly thereafter, an important study revealed that NFeoB was misidentified as an ATPase (15). Contrary to previous sequence alignments, Marlovits *et al.* (15) showed that *Ec*FeoB shared higher sequence homology to GTP-binding proteins (35). The authors then confirmed NFeoB's specificity for guanine nucleotides by mixing NFeoB with fluorescent, non-hydrolyzable nucleotide analogs of GTP and ATP, and they only observed a fluorescent signal in the presence of NFeoB and the GTP analog, not the ATP analog (15). Finally, the authors discovered that the G protein domain was essential for ferrous iron uptake as only a full-length, intact FeoB was able to rescue iron uptake in an *feoB*-deficient strain (15).

A suite of X-ray crystal structures was subsequently determined for NFeoB (36–44), confirming the structural similarity between NFeoB and the GTP-binding domains of eukaryotic G-proteins (Fig. 4). In *Thermotoga maritima*, the G domain comprises 7 β -strands that form a core β -sheet surrounded by 6 α -helices, while the GDI domain comprises 4 α -helices (39) (Fig. 4). Dali searches of the G-domain revealed structural similarity to several bacterial and eukaryotic GTPases, while Dali searches of the GDI domain did not

reveal any structural matches, suggesting that the GDI domain adopts a novel fold (39, 45). This fold is often referred to as a “valve” and is believed to transfer information about the state of the G-domain to the TM domain. However, this hypothesis has yet to be substantiated, and the function of the GDI domain remains to be determined. NFeoB also contains two critical switch regions, termed Switch I and Switch II. Switch I has been extensively characterized and undergoes conformational changes upon nucleotide binding and release (Fig. 4), but both likely play an important role in the activity and the function of FeoB (37, 39, 42).

In the first crystal structure of *Methanococcus jannaschii* NFeoB, the Switch I loop was found to be disordered in the GMPPNP-bound state of the protein and pointed away from the nucleotide binding pocket, but it was found to be ordered in the GDP-bound form (37). Conversely, the opposite effect was seen in the crystal structure of GDP-bound NFeoB (3LX8) and the mant-GMPPNP-bound NFeoB (3LX5) from *Streptococcus thermophilus* NFeoB (42) (Fig. 4). In the GDP-bound form, the Switch I loop is oriented away from the nucleotide-binding site and makes no contact with the nucleotide; however, in the mant-GMPPNP-bound form, the Switch I loop undergoes significant conformational changes and moves to cover the nucleotide-binding site, forming a “lid” that covers the nucleotide phosphate groups while exposing the nucleotide base (see *e.g.*, Fig. 4A vs. Fig. 4B). These observations suggest that GTPase activation by an arginine finger, similar to that of Ras-like G proteins may not be possible (42). For the Switch I loop to undergo the conformational change, a bend is induced at Asn19 and four hydrogen bonds are formed with the nucleotide phosphates (42). This Asn residue and bend is typical of G proteins from the TEES superfamily, but not for Ras-like G proteins (42). The bend induced by Asn19 places another conserved Asn residue, Asn11, near the nucleotide binding site; Asn11 was also found to be essential for potassium-activation of *S*NFeoB GTPase activity (42). In the *Tm*NFeoB structure, the Switch I region was positioned away from the nucleotide binding site, independent of the nucleotide state of the G-protein domain (39). To date, the function of the switch region conformational changes in NFeoB and the effect it has on GTP hydrolysis regulation are unknown. It is plausible that the conformational changes in NFeoB transduce a signal to the rest of the protein, particularly the TM domain, to transport ferrous iron (42), but this hypothesis remains untested.

It is also unclear whether additional stimulatory factors may be present in the cell to control GTP hydrolysis. To date, no effector proteins have been determined for FeoB; however, FeoA and/or FeoC could potentially fulfill these roles by interacting with FeoB. Either of these proteins could function as either a G-activating protein (GAP) or a guanine nucleotide exchange factor (GEF). GAPs act by accelerating the rate of hydrolysis of GTP to GDP in an irreversible manner by inserting a positive locus of charge (often an Arg or Lys side chain) into the GTPBD and polarizing the phosphodiester bond (46). If either FeoA or FeoC upregulates GTP hydrolysis, then there should be significantly enhanced rates of GTP hydrolysis in their presence, similar to the potassium-activated GTP hydrolysis for *S*NFeoB ($k_{\text{cat}}^{\text{GTP}} \sim 0.04 \text{ s}^{-1}$) (42) instead of the relatively slow GTP hydrolysis rate for *Ec*NFeoB ($k_{\text{cat}}^{\text{GTP}} \sim 0.002 \text{ s}^{-1}$) (15), which is similar to typical G proteins. Interestingly, *Ec*NFeoB also exhibits potassium-dependent GTP hydrolysis as monitored by ^{31}P NMR (~5-fold rate increase in KCl compared to NaCl); however, the presence of affinity-tagged *Ec*FeoA had no

effect on GTP hydrolysis by *Ec*NFeoB at equimolar stoichiometry (14). Alternatively, GEFs are responsible for accelerating the dissociation of GDP from G proteins by increasing the exchange of GDP for GTP in a reversible manner (46). If FeoA or FeoC is instead responsible for decreasing the affinity of the G protein domain for GDP, then it would be expected that dissociation constants for GDP would be significantly larger than what is typical for G proteins (*e.g.*, $K_D \sim 0.01 \mu\text{M}$ for H-Ras p21(47)). Since the dissociation constants observed for NFeoB are already several orders of magnitude larger than those for typical G proteins (*e.g.*, $K_D \sim 40 \mu\text{M}$ for *Sn*NFeoB(42), $K_D \sim 5.3 \mu\text{M}$ for *Ec*NFeoB(15) at 10°C), it is plausible that FeoA and/or FeoC do not function as a GEF, but serve a different role.

To date, published data on NFeoB remain puzzling, as most GTP hydrolysis rates of this domain suggest that ferrous iron transport cannot proceed in an active manner (15, 39), while others propose that active transport may be possible (42, 48). It has been speculated that ferrous iron transport may be tied to a G protein-coupled channel due to the slow rates of GTP hydrolysis (36). If this were the case, in the absence of an effector protein to regulate GTP hydrolysis, this channel would remain in an “open,” or GTP-bound state, for long periods of time, potentially allowing for an overflow of ferrous iron into the cell (36, 42). However ferrous iron excess could be counterbalanced by metal export, and recent studies have indicated that Fe^{2+} efflux pumps can be operative during metal overload, even contributing to virulence (49–53). In light of these data, and despite the breadth of literature on NFeoB (including over 28 structures of this domain alone), a paucity of mechanistic information exists vis-à-vis ferrous iron transport, emphasizing the need to investigate the properties of this cytoplasmic domain in the context of intact FeoB.

FeoB

While fundamentally insightful, the studies of NFeoB alone have failed to elucidate the ferrous iron transport mechanism. This bottleneck undoubtedly stems from the few expression systems that can provide sufficient quantities of intact FeoB for biophysical and kinetic studies. To date, only three heterologous systems exist: one study has demonstrated purification and spin labeling of intact FeoB (54), two studies have demonstrated both the purification and kinetic analysis of intact FeoB (48, 55), and none have demonstrated how GTP hydrolysis is linked to the uptake of ferrous iron.

The functional oligomeric state of FeoB has been debated, as several oligomeric states of NFeoB have been observed in crystal structures. To help elucidate this process, Hagelueken *et al.* demonstrated that they could heterologously express sufficiently pure quantities of DDM-solubilized *Ec*FeoB for biophysical analysis (54). The authors then proceeded to demonstrate spin labeling of *Ec*FeoB for electron paramagnetic resonance (EPR) studies with para-acetylphenylalanine (pAcF), an unnatural amino acid (54). Incorporation of pAcF into full-length *Ec*FeoB is necessary as *Ec*FeoB contains 12 native Cys residues, rendering it somewhat intractable to more traditional Cys-based spin labeling (54). During the 5-step purification of *Ec*NFeoB and the 3-step purification of *Ec*FeoB, the authors suggested that GTP and/or GDP from *E. coli* overexpression are separated from the protein (54). This observation has implications for performing and for optimizing GTP hydrolysis assays, as

spuriously bound nucleotide may dramatically affect observed GTP hydrolysis rates. Unfortunately, no functional analyses were performed on this system. However, using gel filtration and X-band EPR spectroscopy, the authors estimated the size of two DDM-solubilized oligomers of *Ec*FeoB to be ~480 kDa and ~300 kDa, respectively (54). While the authors were unable to define the exact oligomeric state of *Ec*FeoB in solution, they suggested that the protein forms either a tetramer or a trimer, the latter of which would agree with the *Ec*NFeoB crystal structure (3HYT) (54).

Functional analysis was performed on *Pseudomonas aeruginosa* FeoB (*Pa*FeoB) that was heterologously expressed in *E. coli* (55). Seyedmohammad *et. al* successfully optimized and purified intact WT and D123N *Pa*FeoB from inside-out membrane vesicles, and these proteins were also reconstituted into proteoliposomes (55). The authors examined GTP hydrolysis of full-length *Pa*FeoB and observed a $k_{\text{cat}}^{\text{GTP}} \sim 0.004 \text{ s}^{-1}$ and $k_{\text{cat}}^{\text{GTP}} \sim 0.001 \text{ s}^{-1}$ for the WT and D123N *Pa*FeoB, respectively (55). These GTP hydrolysis rates are similar to those observed for the soluble NFeoB component and the corresponding D123N variants from other organisms. To ensure that slow GTP hydrolysis rates were not due to an improperly folded FeoB, circular dichroism (CD) spectroscopy was performed and results indicated that the intact proteins did in fact contain a chiefly α -helical secondary structure, as predicted (55). After reconstitution into proteoliposomes, stimulated GTP hydrolysis was observed for WT *Pa*FeoB, with a $k_{\text{cat}}^{\text{GTP}} \sim 0.005 \text{ s}^{-1}$, indicating only a minimal change in the rate of GTP hydrolysis (55), and suggesting that intact *Pa*FeoB recapitulates the slow GTP hydrolysis rates observed for NFeoB alone.

Similar to *Ec*FeoB and *Pa*FeoB, we have optimized the expression and purification of intact *Kp*FeoB. As previously noted, DDM and C₁₂E₈ were able to solubilize suitable quantities of intact FeoB for subsequent biophysical and kinetic analyses (48). CD spectroscopy revealed that intact *Kp*FeoB in the presence of both detergents retained its chiefly α -helical secondary structure, which matched the expected α -helical percentage when deconvoluted (48). In stark contrast with previous results, *Kp*FeoB displays significantly faster rates of GTP hydrolysis in both DDM and C₁₂E₈, $k_{\text{cat}}^{\text{GTP}} \sim 0.09 \text{ s}^{-1}$ and $k_{\text{cat}}^{\text{GTP}} \sim 0.03 \text{ s}^{-1}$, respectively (48). To assess *Kp*FeoB for potassium stimulation, similar to what has been observed with *SN*FeoB, activity assays were also performed in the presence of potassium, with no significant alteration in rate (48). These results are the first to indicate that *Kp*FeoB may in fact be capable of driving ferrous iron transport in an active manner, in contrast with the GTP-coupled channel hypothesis. How ferrous iron transport is linked to GTP hydrolysis remains a mystery, but these expression and purification systems have provided the necessary first steps toward answering this key question.

While recent publications demonstrate that various expression, solubilization, and purification protocols can yield pure, intact FeoB from multiple organisms, structural information will be necessary to decipher the mechanism of ferrous iron transport. While the determination of the three-dimensional structure of a large membrane protein such as FeoB is a substantial undertaking, models can aid in the design of meaningful experiments to enhance our understanding of the Feo system, and to test mechanistic hypotheses. Two models of FeoB have been proposed: one of *Ec*FeoB (Fig. 5, 6A) (56) and one of *Pa*FeoB (Fig. 6B) (57). The model of full-length FeoB from *P. aeruginosa*, which appeared in 2016,

predicted that FeoB may trimerize and form a pore containing three Cys residues (one from each monomer), which are logical Lewis bases for ferrous iron binding (57) (Fig. 6B). This model also proposes that a key Cys residue (Cys⁶⁷⁵), modeled such that it is exposed to the periplasm, may be essential to the transport process (Fig. 6B). Support for this model came from the analysis of a C675S *PaFeoB* variant, which demonstrated significantly reduced GTPase activity (57). Based on the predicted location of this residue and the effect of the C675S variation on GTPase activity, the authors suggest that Cys⁶⁷⁵ could play an important role in ferrous iron recognition in the periplasm, thereby transducing a signal to NFeoB that could cause the protein to switch from an “off” state to an “on” state (57). In contrast, a 2017 model of *E. coli* FeoB, which does not presuppose oligomerization, predicts the homologous Cys⁶⁷⁵ (Cys⁶⁷⁷ in *E. coli*) to be well within the core of the protein, consistent with hydropathy analyses, suggesting that this residue is not located on the protein surface, but rather extends within the TM domain of FeoB (56). Additionally, this model of *EcFeoB* reveals a series of highly conserved Met residues within the metallotransporter that could be key to binding and to transporting ferrous iron (Fig. 6A) (56). Notwithstanding these contradictions, these models provide a platform from which to test whether any (or all) of these conserved residues interact directly with ferrous iron and/or are involved in iron transport, which will undoubtedly provide insight into the mechanism of metal transport.

Contribution of the Feo System to Virulence

Despite our lack of mechanistic understanding, the importance of Feo in numerous pathogenic (and non-pathogenic) bacterial species is underscored by deletion and/or mutations of the genes responsible for *feo* expression, especially under anaerobic conditions. Here we highlight important key findings regarding *feo*-linked virulence. However, for a more comprehensive analysis, the authors point the reader to Ref. (11).

Bacterial species such as *Campylobacter jejuni* (58) and *Helicobacter pylori* (34), which have been subjected to *feo* mutations or deletions exhibit loss of ferrous iron uptake activity, deficiencies in bacterial colonization, and impaired intracellular survival. *C. jejuni*, the causative agent of gastroenteritis (58), and *H. pylori*, the causative agent of chronic active type B gastritis and gastric and duodenal ulceration (34), colonize in the gastrointestinal tract of humans and animals, where the acidic and oxygen-reduced environment of the gut favors the ferrous form of iron (58). *C. jejuni* is also dependent on FeoB for intracellular survival, as *feoB* mutants were attenuated for intracellular survival in human embryonic intestinal cells and porcine small intestinal epithelial cells (58). Chick and newborn piglet models were used to determine the role of FeoB in *C. jejuni* colonization, and these models demonstrated that *feoB* mutants were additionally attenuated in their ability to colonize in the chick cecum and the piglet intestine, respectively (58). Similarly, *H. pylori feoB* mutants were also impaired towards ferrous iron accumulation (34). A mouse model of infection was performed to determine the importance of FeoB for the colonization of *H. pylori* within the mouse gut. In this study, *H. pylori feoB* mutants were unable to establish infection within the mouse stomach, indicating FeoB is necessary for *H. pylori* infection (34).

Some highly lethal bacterial species possess multiple ferrous, ferric, and/or heme transport systems, such as *Francisella tularensis* (59, 60) and *Yersinia pestis* (61), and at least double

mutations of the transport genes are necessary to affect ferrous iron uptake, bacterial colonization, and intracellular survival. For example, *F. tularensis*, the causative agent of tularemia, which has the potential to be manufactured as bioweapon, infects and replicates in hepatocytes within the lungs and the liver of the host (59). *F. tularensis* has multiple iron uptake systems: the *Francisella* siderophore locus (Fsl), which comprises six genes responsible for the synthesis of a rhizoferrin-like siderophore and its receptor, as well as Feo (59, 60). In the live vaccine strain, deletion of the *fslC* gene (*fslC*), one of the Fsl system genes responsible for production of this siderophore, did not prevent this bacterium from growing in murine hepatocytes, whereas *feoB* strains displayed abrogated growth (59). However, all strains were able to grow in human macrophages, demonstrating a necessity for FeoB to grow in some, but not in all, host cells (59). Unlike *C. jejuni* and *H. pylori*, *feoB* *F. tularensis* strains were still able to cause lethal disease in mice, but importantly they were impaired in their ability to establish infection (59). A follow-up study using the Schu S4 strain of *F. tularensis* further demonstrated a need for a double mutant in *feo* and *fsl* to attenuate *in vivo* growth and virulence (60). *Y. pestis*, the causative agent of bubonic, septicemic, and pneumonic plague, grows extracellularly in its host, is capable of growing in non-phagocytic cells, and encodes 12 different ferrous, ferric, and heme transport systems (61). A deletion of both sets of genes for the ferrous iron ABC transporter (known as *yfe*) and *feo* were necessary to attenuate growth of *Y. pestis* in a murine macrophage cell line; a mutation of only one gene alone did not exhibit a significant difference in growth (61).

It has been shown that *P. aeruginosa*, an opportunistic pathogen and common infectious agent of patients suffering from cystic fibrosis, can physically adapt the shape of its cell and its iron uptake systems depending on the stage of cystic fibrosis within the host, where it contributes to the formation of biofilms in the lungs (62). Within these biofilms, the concentration of oxygen is reduced to an extent where ferrous iron is significantly more prevalent (~39 μ M (63)) than ferric iron, and the bacterium has been shown to employ the Feo system to acquire iron under these conditions (62). *P. aeruginosa* living within these biofilms also exhibits a switch in the iron uptake systems characterized by a loss or a reduction of ferric iron uptake genes and a concomitant upregulation of ferrous iron uptake genes (64).

Additionally, deletions in, or mutations of, other components of the Feo system can affect bacterial virulence. Strikingly, an *feoA* deletion in *E. coli* K12 that maintains the presence of *feoB* resulted in ~60% reduced iron uptake when compared to the wild type (12). Further underscoring the importance of FeoA, ferrous iron uptake in either *V. cholerae* or *Salmonella enterica* was found to be dependent on the presence of a fully functional *feoA* (26). In the *S. enterica* study, the authors found that *feo* strains lacking a functional *feoB* exhibited the same decrease in ferrous iron uptake as those lacking a functional *feoA* when compared to the wild type (~3-fold lower); when both genes were deleted, ferrous iron uptake could only be rescued by the complementation of both *feoA* and *feoB* (27). The role of *feoA* in pathogenesis has also recently been characterized in *Acinetobacter baumannii*, an opportunistic pathogen that infects the lungs and is becoming increasingly multidrug-resistant (65), warranting its recent classification as a Priority 1 pathogen on the World Health Organization's global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics (66). In *A. baumannii*, *feoA* was found to be

necessary for bacterial growth, as well as the formation of biofilms and cellular adhesion. Strains lacking the *feoA* gene were attenuated in bacterial growth, colonization, and infection. Interestingly, these strains were also more susceptible to oxidative stress, reducing the overall virulence of the pathogen (65).

Similarly, the deletion of the gene encoding the natural FeoA-FeoB fused polypeptide (FeoAB) appears to affect bacterial virulence. *P. gingivalis*, the causative agent of chronic periodontitis, is distinct from most bacteria and encodes for two FeoB homologs: FeoB1 and FeoB2 (30). FeoB1 is predicted to be 844 amino acids in length and to have an eight TM α -helical segment with FeoA naturally tethered to its N-terminus (30). FeoB2 is predicted to be 725 amino acids in length and is present in an operon with and FeoA-DtxR/MntR fusion protein, indicating it is likely responsible for manganese uptake and divergent from a canonical FeoB (30). *P. gingivalis* FeoB1 gene mutants were attenuated in their ability to acquire iron (30). Furthermore, FeoB1 mutants were avirulent when tested by a mouse lesion model of infection, suggesting that FeoB1-mediated ferrous iron transport is essential for *P. gingivalis* virulence and growth (30). Similar to organisms with stand-alone FeoA and FeoB proteins, *B. fragilis* FeoAB is predicted to contain the conserved SH3 domain, the G-protein domain, and the chiefly α -helical transmembrane region (20). In a cursory study of *B. fragilis*, FeoAB appears to be essential for bacterial growth, as *feoAB* deletion strains were incapable of growing in an intraperitoneal mouse model (20). Taken all together, these collection of *in vivo* data indicate that the Feo system is intimately involved in bacterial infection and bacterial virulence, suggesting that this system could be the target for future therapeutic intervention, including novel antibiotic development.

Future Outlook

To date, several X-ray crystal structures have been solved for NFeoB (38, 42, 67), some NMR and X-ray crystal structures have been solved for FeoA (14, 25), 2 NMR structures have been solved for FeoC (33) and PDB ID 1XN7, and one X-ray crystal structure has been solved for a truncated FeoC-NFeoB complex (32). However, no structure of an intact FeoB has been determined, which represents a pressing gap in this field. Obtaining structural information on an intact FeoB will be invaluable for deciphering the mechanism of ferrous iron transport and for discerning the potential regulation of this process.

As there is no consensus regarding which part(s) of FeoA and/or FeoC interact(s) with the FeoB polypeptide, the site(s) of these interactions must be determined to elucidate how these proteins may contribute to ferrous iron transport. There is an abundant amount of evidence supporting the importance of the membrane spanning portion of FeoB in the import of bacterial ferrous iron (34, 58, 59, 61, 68–74), yet little is known regarding key residues that are important for this process (15, 42, 57, 67, 75). Most published works have focused on the mechanism of GTP hydrolysis, while residues that are critical for iron binding and iron transport remain chiefly unknown. The available homology models of FeoB provide a useful starting point for identifying putative residues involved in ferrous iron binding and transport.

Finally, while numerous studies have shed light on key components of the Feo system, a comprehensive understanding of the mechanism of ferrous iron transport, including how this

process is coupled and/or linked to GTP hydrolysis is still lacking. Whether GTP hydrolysis serves as the energy source for Feo-mediated ferrous iron transport, or if FeoA and/or FeoC can serve as a GAP or GEF, have not been determined. Though the existing FeoB models provide two putative routes for ferrous iron transport, neither has been substantiated experimentally. To this end, additional biophysical and kinetic studies on the full-length FeoB are necessary to further unravel the complexities of the Feo system. Closing several of these gaps will be crucial to understand how pathogenic bacteria procure ferrous iron to meet their metabolic demands, and this information should prove invaluable in order to exploit the Feo system to attenuate or to abrogate bacterial pathogenesis.

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Abbreviations

ATP	adenosine triphosphate
BACTH	bacterial two hybrid analysis
C₁₂E₈	octaethylene glycol monododecyl ether
CD	circular dichroism
DDM	<i>n</i> -dodecyl β-D-maltopyranoside
EPR	electron paramagnetic resonance
Feo	ferrous iron transport system
FNR	fumarate and nitrate reductase
Fsl	<i>Francisella</i> siderophore locus
FUR	ferric uptake regulator
GAP	G-activating protein
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GTP	guanosine triphosphate
GTPBD	GTP binding domain
heme	iron protoporphyrin IX
HTH	helix turn helix
LTTR	LysR transcriptional regulator

NFeoB	soluble N-terminal G-protein domain of FeoB
pAcF	para-acetylphenylalanine
ROS	reactive oxygen species
SH3	Src homology 3
TM	transmembrane

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Figure 1.

(A) Most prevalent bacterial *feo* operons. (i) The *feo* operon originally identified in *E. coli* K-12 encodes for the soluble, cytosolic proteins FeoA and FeoC and the integral membrane protein FeoB. FeoA and FeoB are strongly conserved, whereas FeoC is found in only ~13% of operons; this lack of conservation is indicated by a dashed line about the FeoC gene. In several bacteria, the *feo* genes are downstream of binding sites for fumarate and nitrate reductase (FNR) and ferric uptake transcriptional regulators (FUR). (ii) The most common *feo* operon encodes for only FeoA and FeoB. (iii) Some *feo* operons encode for FeoA covalently tethered to the N-terminal GTPDB of FeoB, as in *B. fragilis* and *P. gingivalis*. (iv) Some *feo* operons only contain the *feoB* gene, underscoring the essential nature of FeoB to that of the *feo* operon. (B) Cartoon illustration of Feo-mediated ferrous iron transport. Membrane bound FeoB (purple) allows for the movement of ferrous iron (Fe²⁺, gray sphere) across the bacterial membrane into the cytosol. GTP hydrolysis by NFeoB (teal) may serve as an energy source for this transport process; alternatively, the state of GTP bound to this domain could regulate the opening and closing of the FeoB protein. It is currently unclear how the soluble, cytoplasmic proteins FeoA (red) and FeoC (green) participate in ferrous iron transport.

Figure 2.

Lowest energy solution NMR structure of *E. coli* FeoA (PDB ID 2LX9, conformer 1). All structurally-characterized FeoA proteins adopt a β -barrel fold that is part of the SH3 domain. The N- and C-termini are labeled (N-, C-), and the right panel represents a 60° rotation of the left panel. The conserved hydrophobic residue implicated in FeoA-FeoB interactions (Leu²⁶) is colored in cyan.

Figure 3.

Lowest energy solution NMR structure of *E. coli* FeoC (PDB ID 2K02, conformer 1). FeoC contains a winged helix motif, which is a combination of a helix-turn-helix (HTH) motif and an elongated, unstructured wing region. The side chains of four highly-conserved Cys residues (colored gray and yellow) are believed to bind a [4Fe-4S] cluster, which could structure the wing region and control FeoC function. The N- and C-termini are labeled (N-, C-) and the conserved Met³⁶ residue implicated in FeoC-FeoB interactions is colored in cyan.

Figure 4.

X-ray crystal structures of NFeoB from *Streptococcus thermophilus* demonstrating structural changes within the Switch I region (yellow) due to nucleotide binding. (A) In the GDP-bound state of *S. thermophilus* NFeoB (PDB ID 3LX8) the Switch I loop points away from the nucleotide binding pocket and away from GDP (colored by heteroatom). (B) In the mant-GMPPNP-bound state of *S. thermophilus* NFeoB (PDB ID 3LX5) the Switch I region covers the phosphate base of the nucleotide, forming a cap over GMPPNP (colored by heteroatom). In both panels, the GDP dissociation inhibitor (GDI) domain is indicated by a label, and the N- and C-termini are labeled (N-, C-).

Figure 5. Three-dimensional model of *E. coli* FeoB derived from metagenomic data (56). The cytoplasmic domain of *Ec*NFeoB is colored in teal and labeled, the periplasmic loop and the transmembrane (TM) domain are both colored in purple but are differentially labeled, and the N- and C-termini are labeled (N-, C-).

Figure 6. Cartoon representation of putative FeoB-mediated ferrous iron transport mechanisms. **(A)** Based on the *EcFeoB* model (56), we propose a Met-lined channel mechanism in which conserved Met residues *within* the FeoB polypeptide bind and transport ferrous iron across the TM region. **(B)** A Cys-lined pore mechanism is proposed based on the oligomerized, trimeric model of *PaFeoB* (57).