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Ins and Outs: Recent Advancements in Membrane Protein-Mediated Prokaryotic Ferrous Iron Transport

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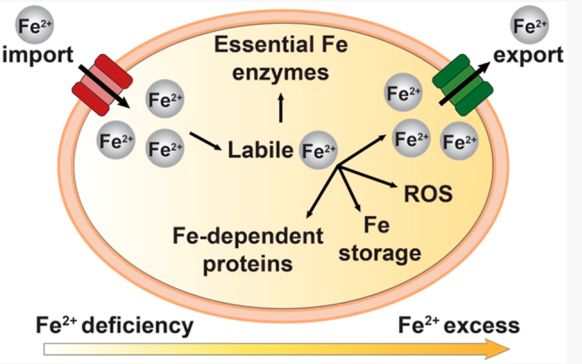
ABSTRACT: Iron is an essential nutrient for virtually every living organism, especially pathogenic prokaryotes. Despite its importance, however, both the acquisition and the export of this element require dedicated pathways that are dependent on oxidation state. Due to its solubility and kinetic lability, reduced ferrous iron (Fe^{2+}) is useful to bacteria for import, chaperoning, and efflux. Once imported, ferrous iron may be loaded into apo and nascent enzymes and even sequestered into storage proteins under certain conditions. However, excess labile ferrous iron can impart toxicity as it may spuriously catalyze Fenton chemistry, thereby generating reactive oxygen species and leading to cellular damage. In response, it is becoming increasingly evident that bacteria have evolved Fe^{2+} efflux pumps to deal with conditions of ferrous iron excess and to prevent intracellular oxidative stress. In this work, we highlight recent structural and mechanistic advancements in our understanding of prokaryotic ferrous iron import and export systems, with a focus on the connection of these essential transport systems to pathogenesis. Given the connection of these pathways to the virulence of many increasingly antibiotic resistant bacterial strains, a greater understanding of the mechanistic details of ferrous iron cycling in pathogens could illuminate new pathways for future therapeutic developments.



Iron is a versatile and necessary micronutrient for nearly all organisms, as it is essential for biological processes such as nitrogen fixation, respiration, oxygen transport, and even DNA biosynthesis.[11] Notwithstanding its importance, this essential element is difficult to obtain from today's oxygen-rich environment, and this conundrum is especially tricky for bacteria that are unable to control their surroundings or their elemental feedstocks. For pathogenic bacteria within a host, populations of both ferric (Fe^{3+}) and ferrous (Fe^{2+}) iron may exist under different physiological conditions and in different forms (labile, inert, chelated, loosely bound, etc.). In oxygen-replete (oxic) environments, the formation of iron hydroxides/oxides that are notoriously insoluble occurs spontaneously and represents a thermodynamic sink. To access Fe^{3+}, bacteria have adapted to scavenge iron from their surroundings using low-molecular weight siderophore-based approaches for downstream biological processes.[1,12] Infectious bacteria may also release proteins that will bind to either extracellular free ferric iron ions and/or molecules or will utilize transferrin/lactoferrin-binding proteins that will sequester ferric iron from their respective proteins.[13,14] Additionally, pathogenic bacteria may scavenge iron protoporphyrin IX (heme *b*) from erythrocytes and muscle tissue, if available.[11,15,16] Both siderophore- and heme-based iron acquisition strategies are being actively studied by multiple research groups, have been nicely reviewed (see, e.g., refs 17 and 18), and have been the target of recent exploits to attenuate bacterial virulence.[19−21] However, there is another form of iron cycling that is less well studied but equally important.[22,23]

Bacteria may also experience oxygen-depleted (anoxic) and/or acidic conditions that support reducing environments within the host in which Fe^{2+} is the dominant iron oxidation state.[24−26] Examples of these locations include acidic portions of the gut,[27] such as the stomach, reducing environments such as the mammalian duodenum,[28] and anoxic locations such as the biofilms of the subgingival tissue or the lungs of late-stage cystic fibrosis (CF) patients.[29,30] In these locations, ferrous iron is often prevalent, and the utilization of ferrous iron acquisition strategies for virulence becomes paramount.[14,24,25] Even though ferrous iron transport is critical under these conditions, the mechanisms by which bacteria obtain ferrous

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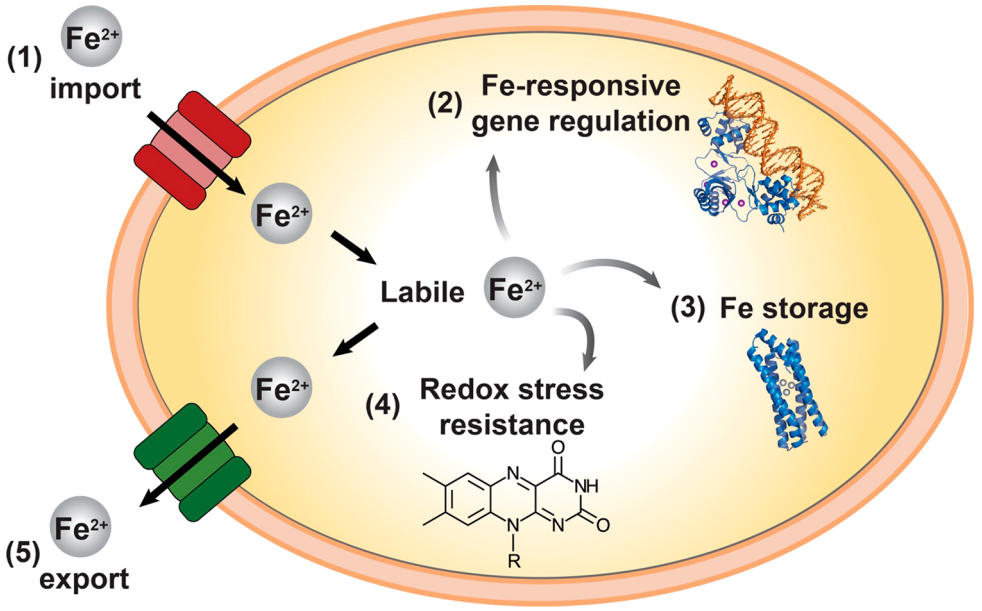

Figure 1. Cartoon schematic of strategies employed by bacteria to maintain iron homeostasis.[1] (1) Import of Fe^{2+} increases the size of labile intracellular Fe^{2+} pools. Intracellular labile Fe^{2+} can be (2) sensed by Fe-responsive gene regulatory systems, including the ferric uptake regulator (FUR; PDB entry 4RB2; purple spheres represent bound Mn^{2+} ions),[6] the fumarate and nitrate reductase regulator (FNR), the peroxide operon regulator (PerR), and the iron response regulator (Irr) for repression or derepression of import and export machinery; (3) stored in ferritins (PDB entry 1EUM; gray spheres represent Fe-binding sites),[10] bacterioferritins, and DNA-binding proteins from starved cells (Dps) for use when the cells face iron-replete conditions; and/or (4) used in conjunction with flavins to resist redox stress introduced from reaction with reactive oxygen species (ROS). Finally, (5) iron intoxication or iron excess may be relieved by efflux via Fe^{2+} export systems.

iron are not well elucidated, necessitating a closing of this knowledge gap.

Regardless of the precise acquisition pathway, bacteria use several common approaches to control iron homeostasis: (1) iron import systems that are tuned to acquire iron from the environment, (2) iron-responsive gene regulation, (3) internal storage systems for use when iron is scarce, (4) redox stress resistance systems, and (5) iron efflux systems (Figure 1).[1] This tight level of control is required, as excess and misregulated levels of iron can result in Fenton-like chemistry.[31,32] Specifically, cytoplasmic, labile iron may serve as a catalyst to reduce H_2O_2, producing reactive oxygen species (ROS) such as hydroxyl radicals that can damage proteins, membranes, and DNA.[31,33] As homeostasis wanes, further destruction of [Fe-S] clusters and/or their release into the cytosol can upend electron transport and even trigger bacterial ferroptosis.[33−37] These results highlight the importance of iron homeostasis for the survival of unicellular pathogens.

If famine becomes feast, bacteria may also use several approaches to compensate for high intracellular iron levels, including the attenuation of iron import, the storage of excess iron, the alteration of global metal transport, and even the efflux of iron.[32,38] Many of these strategies have been extensively reviewed (see, e.g., refs 1 and 12), with the exception of ferrous iron exporters. The concept of ferrous iron "intoxication" is beginning to gain traction in the field, and ferrous iron efflux pumps are generally accepted as active transporters that are utilized by bacteria to export Fe^{2+} to alleviate excess labile intracellular iron and to lower levels of oxidative stress.[32] Although ferrous iron exporters are thought to be found across all kingdoms of life, including most pathogenic bacteria,[9] many ferrous iron efflux systems are only beginning to be understood, and their contribution to virulence remains unclear.

This Perspective highlights recent advancements in our biochemical, mechanistic, and structural characterizations of ferrous iron import and efflux. Much of our understanding derives from *in vitro* recombinant work complemented by studies at the organismal level. Unfortunately, as the majority of these transport systems are membrane proteins and/or membrane protein complexes, structural advancements in this field have been slow but significant (Table 1).

However, with the major leap forward in the accessibility of alternative strategies for, in particular, the structural determination of particulate proteins (e.g., cryo-EM), we anticipate advancements in this field to accelerate at a rapid pace to complement what is known at the *in vivo* and *in vitro* levels. We believe a greater understanding at the atomic level of the underpinnings that control the cycling of ferrous iron within pathogenic bacteria could afford the targeting of these systems for the attenuation of virulence, similar to strategies that have been developed for ferric siderophore-based and heme-based iron acquisition systems.

FERROUS IRON IMPORTERS

The first step in the bacterial ferrous iron cycle is import across a lipid bilayer. The relevant reported ferrous iron transporters that have been described include the MntH, ZupT, EfeUOB, IroT, YfeABCDE, FutABC, and Feo systems (Figure 2). Despite the ostensibly large number of ferrous iron importers that have been characterized, few appear to be dedicated to the uptake of only ferrous iron, and even fewer (chiefly Feo) are widespread across the prokaryotic domain. In this section, we summarize what is currently known about bacterial ferrous iron import.

Promiscuous Iron Importers. The H^+-dependent manganese transport system (MntH) membrane protein is encoded by the *mntH* gene and may transport Fe^{2+} in addition

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Table 1. Structures of Known and Proposed Prokaryotic Fe^{2+} Importers, Exporters, and Their Associated Fragmentary Domains

protein	organism	function	PDB entry
MntH	*Deinococcus radiodurans*	import	5KTE,[a] 6D9W,[a] 6C3I,[b] 6BU5,[c] 6D91[d]
EfeO	*Yersinia pestis*	import	5TW9
EfeB	*Escherichia coli*	import	2Y4D,[f] 3O72[g]
NFeoB[e]	*E. coli*	import	3HYR,[f] 5FH9,[f] 3I8S,[f] 3I92[h]
NFeoB	*Gallionella capsiferriformans*	import	3W5I,[f] 3W5J[i]
NFeoB	*Klebsiella pneumoniae*	import	2WIA,[f] 2WIB,[i] 2WIC[j]
NFeoB	*Legionella pneumophila*	import	3IBY
NFeoB	*Methanocaldococcus jannaschii*	import	2WJH,[f] 2WJG,[i] 2WJI,[j] 2WJJ[k]
NFeoB	*Pyrococcus furiosus*	import	3K53
NFeoB	*Streptococcus thermophilus*	import	3B1Z,[f] 3B1Y,[i] 3B1W,[i] 3LX8,[i] 3B1X,[j] 3SS8,[l] 7BWV,[l] 7BVU,[l] 3LX5,[m] 3B1V,[m] 3TAH[n]
NFeoB	*Thermatoga maritima*	import	3A1V,[f] 3A1S,[i] 3A1T,[i] 3A1U,[j] 3A1W[o]
FieF	*E. coli*	export	3H90,[p] 2QFI[p]
FieF	*Shewanella oneidensis*	export	3J1Z,[a] 5VRF,[p] 7KZX,[q] 7KZZ[r]
$MamB_{CTD}$[s]	*Desulfamplus magnetovallimortis*	export	6QFJ
$MamM_{CTD}$	*D. magnetovallimortis*	export	6QEK
$MamM_{CTD}$	*Magnetospirillum gryphiswaldense*	export	3W5X,[f] 3W5Y,[f] 6GP6,[t] 6GMT,[u] 6GMV[v]

[a]Inward-facing, open, apo. [b]Inward-facing, occluded, apo. [c]Outward-facing, open, Mn^{2+}-bound. [d]Outward-facing, open, apo. [e]NFeoB is the soluble G-protein-like domain at the N-terminus of FeoB. [f]Apo form. [g]Heme-bound form. [h]GCP-bound form. [i]GDP-bound form. [j]GMP-PNP-bound form. [k]GDP-bound selenomethionine form. [l]GDP:AlF_4^-- and K^+-bound form. [m]mantGMP-PNP-bound form. [n]mantGDP-bound form. [o]ΔGDI form. [p]Zn^{2+}-bound form. [q]Apo form complexed with fragment antigen-binding (Fab) protein. [r]Zn^{2+}-bound form complexed with Fab. [s]CTD represents the C-terminal, cytosolic domain of Mam proteins. [t]Cu^{2+}-bound form. [u]Cd^{2+}-bound form. [v]Ni^{2+}-bound form.

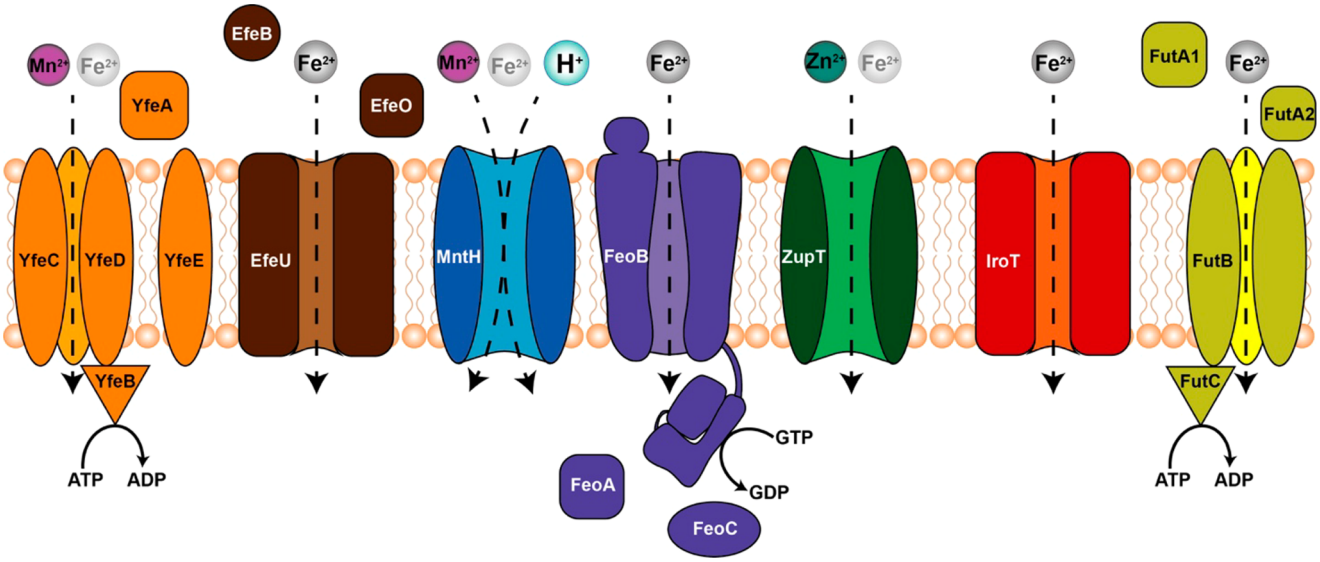

Figure 2. Cartoon overview of the major bacterial ferrous iron importers. From left to right are the YfeABCDE (orange), EfeUOB (brown), MntH (blue), FeoABC (purple), ZupT (green), IroT (red), and FutABC (yellow) systems, respectively. Ferrous iron ions are represented by gray spheres. Manganese is represented by a purple sphere. Zinc is represented by a green sphere. A proton is represented by a blue sphere. In several cases (e.g., MntH and ZupT), Fe^{2+} is not the primary metal substrate but rather an auxiliary substrate.

to its chief function as a Mn^{2+} importer. Despite its name, the sequence of MntH is ≈36% identical to that of eukaryotic natural resistance-associated macrophage protein 1 (NRAMP1),[39] a transmembrane, proton-dependent divalent metal and proton-dependent co-transporter with broad metal specificity, suggesting that other divalent metals such as Fe^{2+} may be MntH substrates. The *Deinococcus radiodurans* R1 MntH structure has been determined (PDB entry 6C3I), and the overall architecture is composed of 10 α-helices that resemble a pore (Figure 3).[40] The Mn^{2+}-bound MntH structure reveals that Mn^{2+} is coordinated by the backbone carbonyl of Ala53 and the side chains of Asp56, Asn59, and Met230 as well as two water molecules.[40] In addition to its canonical substrate (Mn^{2+}), studies utilizing inducible *Escherichia coli mntH* have revealed the intracellular uptake of radioactive Fe^{2+} in a proton-dependent manner, establishing that the MntH transmembrane protein can also transport Fe^{2+}.[39] MntH-mediated metal transport activity is higher under acidic conditions, and protons accumulate intracellularly, indicating that dedicated symport is operative.[41] However, despite the ability of MntH to transport Fe^{2+}, experiments performed with radioactive Mn^{2+} demonstrated a preference for Mn^{2+} as a substrate over Fe^{2+}. In fact, MntH is capable of binding and transporting a wide swath of divalent metals in a proton-dependent manner with the following affinities: Mn^{2+} > Cd^{2+} > Co^{2+} > Fe^{2+} > Zn^{2+} > Ni^{2+} > Cu^{2+}. Such a broad

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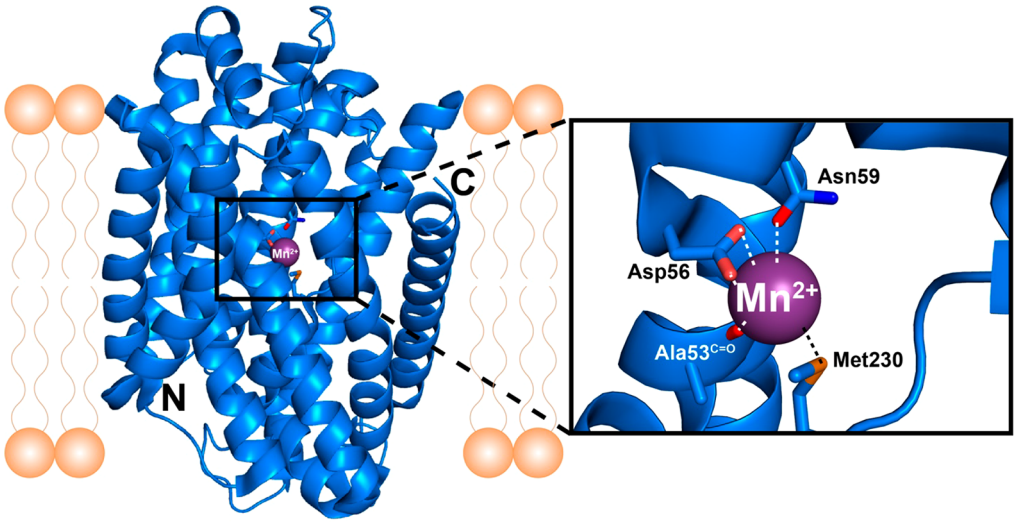

Figure 3. Structure of *D. radiodurans* MntH bound to Mn^{2+} (PDB entry 6BU5). The MntH structure is composed of 10 α-helices that adopt a pore-like structure. The metal-binding site (right) reveals that MntH coordinates Mn^{2+} via Asp56, Asp59, Met230, and the backbone carbonyl of Ala53. Two additional molecules of water coordinate Mn^{2+} but are not displayed for the sake of clarity. N and C represent the amino and carboxy termini, respectively.

specificity demonstrates the promiscuous nature of MntH. Given MntH's sequence identity and similar broad specificity to that of the eukaryotic NRAMP family of proteins, it has been suggested that MntH may represent a NRAMP bacterial ancestor, leading many to refer to MntH as an NRAMP-like transporter.[39,42]

MntH has been shown to be important for some pathogenic strains, although a consensus remains opaque. For example, Makui et al. found that a frameshift deletion of the *mntH* gene in *E. coli* did not affect cell growth in either minimal or nutrient-rich medium.[39] This finding led to the conclusion that *E. coli* MntH is not required for cell growth or virulence, meaning that *E. coli* relies heavily on metal transporters other than MntH to meet its needs for certain divalent metals.[39] Similarly, *in vivo* studies of *Yersinia pestis*-infected mice found that a Δ*mntH* strain had no significant loss of virulence in a bubonic mouse model, and only a double knockout of *yfe* and *mntH* led to a 133-fold loss of virulence; however, reduced virulence was not observed in the pneumonic plague model in mice.[43] Virulence testing of *Mycobacterium tuberculosis* MntH using bone marrow-derived macrophages demonstrated no significant loss of virulence.[43,44] Another study performed with *Brucella abortus*, the leading cause of undulant fever in humans, showed that a deletion of the *mntH* gene led to attenuated growth in both rich and minimal media but was later rescued by introducing high concentrations of Mn^{2+}.[43] Given the established role of Mn^{2+} in protecting bacteria from oxidative stress,[38] these findings may link MntH to a role in oxidative stress resistance. Consistent with this notion, deletion of the *mntH* gene within *Yersinia pseudotuberculosis* and *Salmonella typhimurium* led to higher sensitivity to H_2O_2 under manganese-limited conditions, signifying a MntH dependence of oxidative stress resistance in bacteria.[24,41,45] While MntH is the main Mn^{2+} transporter in *B. abortus* and potentially other species of bacteria,[46] the extent to which auxiliary Mn^{2+} transporters, and even a secondary function in Fe^{2+} transport, contribute to virulence is unclear, thus warranting further characterization.

The zinc uptake transporter ZupT is a part of the ZRT- and IRT-like protein (ZIP) family of proteins involved chiefly in Zn^{2+} transport, but these proteins may also transport Fe^{2+} in addition to other divalent metal ions.[47,48] The crystal structure of *Bordetella bronchiseptica* ZIP with bound Zn^{2+} has been determined and reveals a membrane-imbedded eight-α-helical pore with two Zn^{2+} ions forming an intriguing binuclear metal center (PDB entry 5TSA).[49] While there is currently no structure of ZupT, there is speculation based on sequence conservation with that of *B. bronchiseptica* that ZupT may be composed of a similar architecture. *In vivo* radioactive Zn^{2+} transport studies have demonstrated definitively that ZupT mediates Zn^{2+} uptake in *E. coli*;[48] however, whether ZupT has a binuclear metal center like other members of the ZIP family has yet to be determined. Like MntH, ZupT also exhibits substrate promiscuity, having been shown to transport Fe^{2+}, Co^{2+}, and Mn^{2+}, but ZupT appears to retain its highest affinity for Zn^{2+}.[47,48] While the mechanism of how ZupT transports divalent metals is still not clearly understood, it is suggested to function as a permease, similar to other ZIP family proteins. In addition, ZupT maintains a constitutive and low expression level even in the presence of the aforementioned divalent metals.[47] In terms of its connection to virulence, deletion of the *zupT* gene alone does not disable infection in pathogens.[50,51] For example, *in vivo* studies of *Salmonella enterica* demonstrate that virulence is decreased only when both *zupT* and an additional zinc ATP-binding casette (ABC) transporter gene, *znuABC* (zinc uptake ABC), are deleted but not *zupT* alone. These observations suggest that ZupT is not the primary Zn^{2+} transporter in bacteria and thus is not strictly required for colonization.[50] Additionally, phenotypic changes do not appear to be related to Fe^{2+} homeostasis, suggesting that ZupT plays a minimal role at best in maintaining intracellular stores of ferrous iron.[47]

The *Yersinia* ferrous iron transport (Yfe) ABCDE and *Salmonella* iron transport (Sit) ABCD systems have homology and are involved in the import of Mn^{2+} and Fe^{2+}.[52,53] The Yfe system is an inner membrane ABC transporter that utilizes ATP for the active transport of both Mn^{2+} and Fe^{2+}. Studies have shown that Yfe can transport both metals but has a higher affinity for Mn^{2+}, suggesting that Fe^{2+} transport may be an auxiliary function in *S. enterica*.[54] YfeA is a periplasmic protein

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predicted to bind both Mn^{2+} and Fe^{2+}, YfeC and YfeD are inner membrane permeases, and YfeB is an ATPase.[52] In addition to its typical ABC transporter architecture that consists of a four-protein complex including two cytosolic nucleotide-binding domains and two transmembrane α-helical domains,[55] the Yfe system bears an additional gene, *yfeE*, which is not part of the same operon as *yfeABCD* but encodes a separate inner membrane protein YfeE that has an unknown function.[24,52,56] Knockout studies report that a *yfeE* deletion in *Y. pestis* did not result in a decreased level of cell growth, suggesting that YfeE may only be accessory to the Yfe system and not essential for homeostasis.[52] Interestingly, *in vivo* knockout studies reveal that a deletion of the *yfeABCD* operon resulted in only a modest decrease in *Y. pestis* cell growth under iron-deficient conditions, indicating that Yfe does not act as a primary Fe^{2+} transporter.[52] In contrast, metal transport studies of the pathogenic *E. coli* SitABCD system demonstrated a stronger preference for Fe^{2+} than Mn^{2+}, and Fe^{2+} was transported with a higher affinity than Fe^{3+}.[54,57] Taken together, these findings highlight the need for additional studies of the specific determinants of substrate recognition within these transporters.

Dedicated Iron Importers. The ferric uptake (Fut) ABC system is primarily found in cyanobacteria and is an inner membrane ABC transporter system that appears to be a dedicated iron transporter;[56,58,59] however, there is no consensus about iron oxidation state specificity. The Fut system contains two separate periplasmic proteins named FutA1 and FutA2, with FutB being an inner membrane pore and FutC an ATPase.[59] Evidence suggests each part of the ABC transporter may have differing iron specificities, with FutB preferentially binding Fe^{3+}, while periplasmic proteins FutA1 and FutA2 preferentially bind Fe^{2+}.[58−60] However, the crystal structure of a *Trichodesmium erythraeum* FutA1 homologue reveals that FutA1 binds Fe^{3+} coordinated by a strongly Lewis acidic, octahedral ligand sphere (three Tyr hydroxyls/hydroxides and three oxygens from water molecules), which was confirmed by electron paramagnetic resonance spectroscopy.[61] It is important to note that these experiments were performed under oxic conditions; thus, it is possible that FutA1 is capable of binding Fe^{2+} via a different ligand set under anoxic conditions, but these experiments remain unrealized.[61]

While there is still no consensus about the Fut mechanism, Kranzler et al. have suggested that the Fut system works in conjunction with the alternate respiratory terminal oxidase (ARTO) membrane protein in a reductive iron uptake pathway, similar to a ferric reductase approach (*vide infra*).[58] It is hypothesized that FutA1 and/or FutA2 binds Fe^{3+} and delivers ferric iron to FutB for transport, or the Fut system interacts with ARTO and reduces Fe^{3+} to Fe^{2+}, allowing the ferrous iron to be transported in another manner. While a consensus on the substrate oxidation state remains unresolved, knockout of any components of the Fut system led to modest decreases in the level of iron accumulation within *Synechocystis* 6803.[58] While cyanobacteria are generally not regarded as pathogenic, this information could be useful and extrapolated to infectious homologues.

A more common pathogenic iron transport system is the elemental ferrous iron uptake (Efe) UOB system. This system resembles the Fe^{2+} transporter Ftr1P found in yeast, with EfeU being an inner transmembrane pore, and EfeO and EfeB both being periplasmic. EfeO has a C-terminal peptidase domain (PDB entry 5TW9) and a predicted N-terminal cupredoxin-like domain, while EfeB is a homodimer in which each protomer is composed of 12 α-helices and eight β-strands (PDB entry 2Y4D). Crystal structures of *E. coli* EfeB reveal the protein to be complexed with a *b*-type heme, showing that it is part of the heme peroxidase superfamily (PDB entry 3O72).[62] Because of the high degree of sequence homology to Ftr1P, it has been suggested that EfeU acts as a permease to transport both Fe^{2+} and Fe^{3+}, with a higher affinity for Fe^{2+}.[63,64] Interestingly, the *efe* operon is induced under low-pH conditions, possibly due to the fact that these acidic environments have higher concentrations of Fe^{2+} than of Fe^{3+}. Despite its connection to Fe^{2+} import, deletions to the *efe* operon do not seem to restrict cell growth (at least in *E. coli*), indicating that other systems may be the primary drivers of Fe^{2+} uptake or that these systems are capable of compensating in lieu of the Efe system.[62]

A more specialized set of dedicated Fe^{2+} transporters are the iron transporter (IroT) membrane proteins, which are homologues of the previously defined "more regions allowing vacuolar co-localization N" (MavN) proteins. IroT is found chiefly in the *Legionella* genus of bacteria, including *Legionella pneumophila*, the human pathogen that causes Legionnaires' disease.[65] This bacterium infects macrophage cells and relies on the formation of an intracellular compartment named the *Legionella*-containing vacuole (LCV). To meet the iron requirements of the *L. pneumophila*, IroT is recruited to the LCV membrane.[66,67] IroT is predicted to contain eight transmembrane α-helices and has also been shown to transport a wide array of divalent metals in addition to Fe^{2+}, including Mn^{2+}, Co^{2+}, and Zn^{2+}, with equivalent proficiency at least *in vitro*; however, IroT is suggested to function only for Fe^{2+} transport *in vivo*.[68] While the exact mechanism of IroT-mediated metal transport has not been elucidated, several mutagenesis experiments display the importance of key amino acids for metal translocation. For example, an ExxE motif that is conserved among IroT proteins has been found to affect iron transport using an E439A variant.[68] However, this motif is notably found within the lumen side of the LCV, indicating that it may play a role in either initial iron binding or mediating iron translocation through the pore.[67] An additional variant of the predicted transmembrane His (H412A) showed a considerable decrease in the level of growth within the LCV, implying that divalent metals may be coordinated by His412 during metal transport.[68] Interestingly, *in vivo* knockout studies of LCV-independent *L. pneumophila* cells show impaired growth, suggesting that IroT may also be incorporated into the bacterial membrane for iron uptake.[65] However, knockout of the LCV IroT affected growth more drastically, indicating a stronger reliance on this strategy in the vacuole.[68] Regardless, these results strongly suggest that targeting this Fe^{2+} transporter could be a viable strategy in the fight to curb Legionnaires' disease.

The ferrous iron transport (Feo) system is the most widely distributed and dedicated Fe^{2+} uptake system across the prokaryotic domain. Unlike many of the aforementioned iron transporters, the Feo system has been shown to have the highest affinity for Fe^{2+},[25,56,69−71] and Feo is connected most widely to the virulence of pathogenic prokaryotes.[56,65,71,72] Interestingly, studies of FeoB2, a second protein annotated as FeoB that is found in mostly the *Porphyromonas* class of bacteria, indicate that this protein can transport Mn^{2+} and not Fe^{2+}, whereas FeoB1 in *Porphyromonas gingivalis* is strictly an

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Fe^{2+} transporter.[73] These results suggest FeoB2 may behave like MntH; however, structural and metal specificity studies of FeoB2 have yet to be carried out *in vitro* in great detail. The Feo system, initially identified in *E. coli*, consists of three separate proteins: FeoA, FeoB, and FeoC. FeoA and FeoC are both small (~8 kDa each), cytosolic proteins, while FeoB is a large (~80 kDa) transmembrane, nucleotide-hydrolyzing protein (most commonly GTP) with eukaryotic G-protein orthologs.[25]

The functions of both FeoA and FeoC remain enigmatic. FeoA has been structurally characterized and bears a conserved SH3-like domain, which is characterized by a small β-barrel.[74] The conservation of this protein−protein interaction fold has led to the hypothesis that FeoA acts as either a GTPase-activating protein (GAP; increases the rate of GTP hydrolysis) or a guanine exchange factor (GEF; helps to exchange hydrolyzed GDP for GTP). However, for this function to be operative, direct protein−protein interactions must be made, and no such interactions have been characterized at the atomic level, although they have been hypothesized and inferred.[74] FeoC has also been structurally characterized and contains a conserved helix−turn−helix (HTH) motif,[75] suggesting that FeoC may function as a transcriptional regulator. Adding to its complexity, FeoCs from both *E. coli* and *K. pneumoniae* have been shown to bind a redox-active [4Fe-4S] cluster under strict anoxic conditions.[76] This cluster is sensitive to O_2 and may be used as an oxygen sensor to regulate ferrous iron transport, in a manner somewhat similar to that of the fumarate and nitrate reduction regulatory protein (FNR).[76] However, there are FeoC proteins that do not have the ability to bind [Fe-S] clusters, and there are no reports indicating that FeoC binds to DNA; therefore, the function of FeoC remains to be elucidated.[25]

FeoB is the most important component of the Feo system, and this large, polytopic transmembrane protein is hypothesized to bind and to transport Fe^{2+} in a GTP-dependent manner. The recently released AlphaFold database proposes that FeoB (Uniprot entry P33650; AlphaFold ID AF-P33650-F1) is composed of three domains: a soluble N-terminal G-protein-like domain (termed NFeoB) (six β-strands that form a central β-sheet surrounded by five or six α-helices and a GDI domain featuring five α-helices), a transmembrane domain consisting of 10 or 11 α-helices, and a carboxylate-rich periplasmic loop (Figure 4).[5] However, there is no full-length structure of FeoB; instead, most research has historically focused on soluble NFeoB, which has been structurally characterized in the apo and guanosine-bound forms.[69,77,78] In addition, GTPase activity analyses have been performed on both NFeoB and full-length FeoB proteins, with the full-length protein displaying higher activity (in some cases), implicating the existence of important contacts between the soluble domain and the transmembrane region.[70,79,80] Even so, full-length FeoB still exhibits slow GTP hydrolysis rates (maximal reported k_{cat}^{GTP} of ~0.1 s^{-1}), calling into question whether Fe^{2+} is translocated via an active or passive process.[70,80,81] While the precise mechanism of Fe^{2+} import remains to be elucidated, Seyedmohammad et al. proposed that FeoB features a Cys-lined, GTP-gated channel as opposed to a transporter;[81] alternatively, our lab has postulated that a Met-rich pathway may be involved in metal translocation,[25] which is a common theme among many Fe^{2+} transporters. These conflicting proposals underscore the need for additional studies to define the mechanism of Fe^{2+} transport by FeoB.

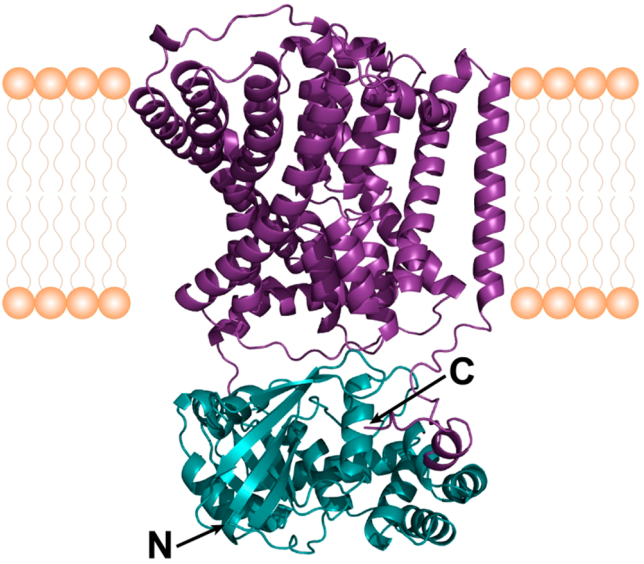

Figure 4. Predicted AlphaFold model of *E. coli* FeoB. FeoB (Uniprot entry P33650; AlphaFold ID AF-P33650-F1) is predicted to contain three distinct regions: a soluble N-terminal G-protein-like domain (NFeoB, teal), a transmembrane region consisting of 10 or 11 α-helices, and a soluble periplasmic loop (both purple).[5] N and C represent the amino and carboxy termini, respectively.

Adding to FeoB's complexity, recent discoveries have indicated that despite structural similarity between FeoB and eukaryotic G-proteins, some FeoBs may actually be NTPases.[82,83] Recent results from Shin et al. have demonstrated that a subset of NFeoB proteins have the ability to hydrolyze ATP in addition to GTP.[82,83] It has been proposed that the adenosine/guanosine discriminatory factor is a single amino acid difference within the FeoB G5 motif of the G-domain.[82] This result is both intriguing and surprising, as NFeoB is not structurally similar to known ATPases. Whether this motif is a defining factor of nucleotide distinction remains to be seen, as it is not yet known how NFeoB binds adenosine nucleotides.

While a consensus on mechanism has yet to be reached, a number of *in vivo* studies have demonstrated the connection between the presence of Feo and pathogenesis. With a particular emphasis of its role as a major virulence factor, the infectivity of problematic pathogens such as *E. coli* O78:H10, *Helicobacter pylori, Y. pestis, Campylobacter jejuni*, and even *P. gingivalis* (to name a few) all have shown significant decreases upon the deletion (either in part or in whole) of the Feo system. While outside of the scope of this perspective, a more comprehensive report on Feo virulence has been laid out previously by Lau et al.[56] Thus, as the most widely distributed and dedicated Fe^{2+} uptake system across the prokaryotic domain, a greater understanding of the function of Feo at the atomic level would make major impacts in targeting this essential system in an effort to fight bacterial pathogenesis.

Ferric Reductases and Ferrous Iron Transport. Intriguingly, the widely distributed Feo system is expressed when bacteria live in both anoxic and oxygen-replete environments, despite the reactivity of Fe^{2+} in the presence of oxygen.[84] While living under oxic conditions, many bacteria notably employ Fe^{3+} transporters to uptake ferric siderophore complexes. While these chelators bind and solubilize Fe^{3+} effectively, ferric siderophore complexes are notoriously stable

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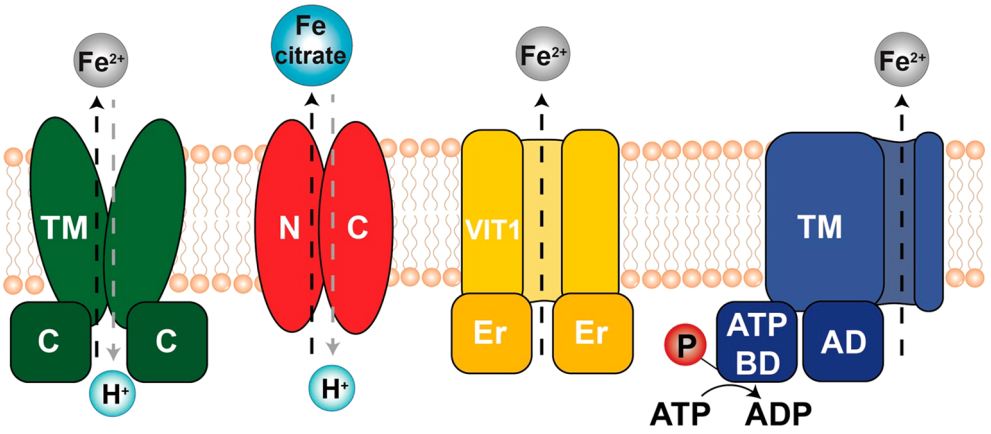

Figure 5. Cartoon overview of the major ferrous iron efflux systems in bacteria. Cation diffusion facilitator proteins (CDF, green) generally form a homodimer featuring an N-terminal transmembrane domain (TM) followed by a soluble C-terminal domain (C) and use the proton motive force (PMF) to transport divalent metal ions. Major facilitator superfamily (MFS, red) proteins are implicated in the transport of ferric/ferrous iron citrate and citrate alone (teal).[3] MFS proteins often have distinct N- and C-terminal domains. Iron-transporting MFS proteins are homologous to proton antiporters, and it is proposed that an exchange with the iron citrate and protons occurs in this system.[8] Membrane-bound ferritin-like proteins (yellow) feature a soluble, dimeric, N-terminal ferritin-like Er domain (Er) and a C-terminal vacuolar iron transporter domain (VIT1). P_{1B}-ATPase proteins (blue) are composed of a transmembrane domain (TM), an ATP-binding domain (ATP-BD), and an actuator domain (AD). Ferrous iron ions are represented by gray spheres; protons are represented by blue spheres, and phosphate is represented by a red sphere. Figure adapted from ref 9.

and recalcitrant to iron release. To circumvent this issue, many bacteria use soluble (flavin-based) and/or membrane-bound (heme-based) ferric iron reductases, which reduce Fe^{3+} to Fe^{2+}, creating a more labile feedstock for ferrous iron uptake pathways. Given its presence in most bacteria, it has thus been hypothesized that ferric iron reductases and the Feo system may work in conjunction with one another to fulfill bacterial iron needs. Indeed, a study has implicated the involvement of membrane-bound ferric reductases in Feo-mediated uptake pathways.[85] Given that membrane-bound ferric reductases are typically localized to the cytoplasmic membrane, it is tempting to speculate that these small, α-helical hemoproteins could be co-localized with FeoB to orchestrate ferrous iron import under oxic conditions; however, more work is needed to probe this hypothesis.[86] While ferrous iron uptake is principal to the survival of bacteria, a careful control of the intracellular iron levels must be achieved. To prevent the risk of intracellular oxidative damage, ferrous iron efflux systems are expressed and utilized when intracellular labile iron levels exceed a certain threshold.

■ FERROUS IRON EFFLUX SYSTEMS

Although iron is a necessary nutrient for nearly all bacteria, excess iron can be both toxic and dangerous, especially O_2-reactive ferrous iron. In oxygen-rich environments, this toxicity is commonly attributed to oxidative damage resulting from Fenton-like chemistry that creates reactive radicals from Fe^{2+} redox chemistry;[31] however, the precise cause of damage under anoxic conditions has yet to be determined. To prevent toxicity, iron is typically sequestered by proteins, including enzymes and chaperones, and excess iron can trigger the expression of iron storage vessels such as heme-containing bacterioferritins, traditional ferritins, and even the DNA-binding proteins from starved cells (Dps) family of mini-ferritins.[9] However, should the level of intracellular iron exceed this buffering capacity, its egress is warranted, and versatile ferrous iron efflux systems have been recently identified in bacteria and are used to attenuate deleterious iron concentrations. These exporters are divided into four main families: (1) cation diffusion facilitator (CDF) proteins, (2) major facilitator superfamily (MFS) proteins, (3) membrane-bound ferritin-like proteins, and (4) P-type ATPases. In this section, we summarize what is currently known about bacterial ferrous iron export.

Cation Diffusion Facilitator (CDF) Proteins. Cation diffusion facilitator (CDF) proteins export a range of divalent metal ions, including Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, and Mn^{2+}.[87−89] Bacterial CDF proteins have been structurally characterized and are generally homodimers that feature a transmembrane domain composed of six α-helices connected to a C-terminal cytoplasmic domain (Figures 5 and 6).[2,87,90] To date, only the structures of metal-bound (Zn^{2+}), full-length CDF proteins from *E. coli* and *Shewanella oneidensis* are known (Table 1).[2,91] Phylogenetic and functional studies have identified factors for substrate selectivity, leading to a division of CDF proteins into three groups: those that transport only Zn^{2+}, those that transport Fe^{2+} and Zn^{2+}, and those that transport only Mn^{2+}.[92] However, nearly 15 years have elapsed since the most recent effort to characterize metal selectivity based on phylogenetics,[92] and an updated analysis is warranted. In general, transport by CDF proteins is stimulated by pH changes and chemiosmosis,[93] and Fe^{2+}-translocating CDF proteins use the proton motive force (PMF) for efflux.[89,94]

The field is conflicted regarding metal binding and selectivity.[9] For example, *E. coli* ferrous iron efflux protein (FieF, previously reported as YiiP) was first identified as a Zn^{2+} transporter,[89,94] as FieF expression was induced by the presence of Zn^{2+} in a concentration-dependent manner. However, this behavior did not explain the inherent contradiction that Δ*fieF* strains did not exhibit a decrease in Zn^{2+} tolerance, suggesting that FieF may serve to transport a different cation.[95] Later, FieF was found to transport iron on the basis of studies in which the level of iron accumulation was decreased upon *in trans* expression of FieF in Δ*fieF E. coli*,[90] and biophysical studies demonstrated that *E. coli* FieF

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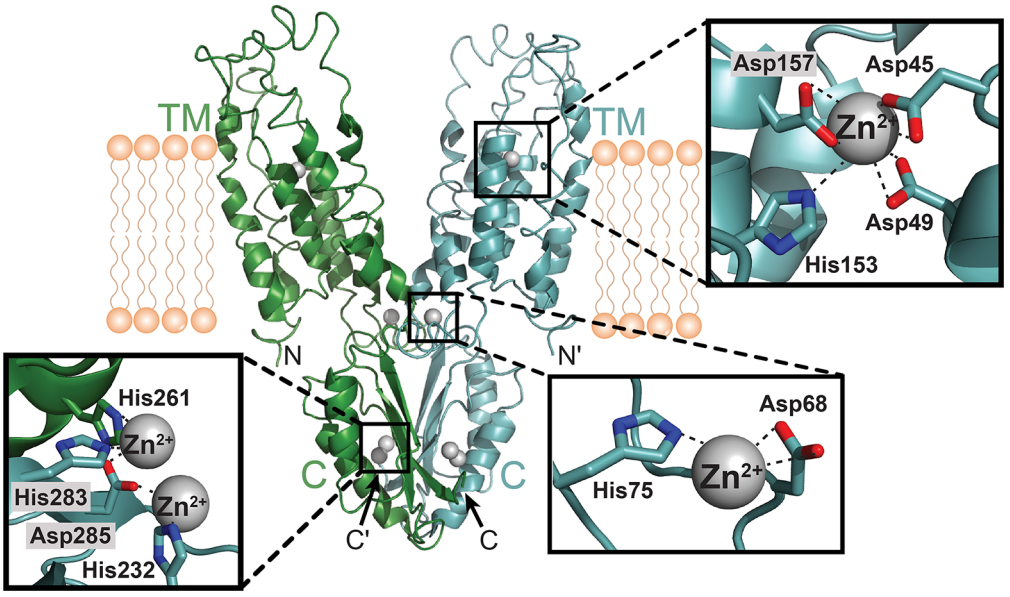

Figure 6. X-ray crystal structure of the iron-transporting *E. coli* FieF (YiiP) protein in the presence of Zn^{2+} (PDB entry 2QFI). FieF is often considered a model for other CDF proteins.[2] The homodimer adopts a "Y"-like shape, where each protomer (colored green and teal separately) includes a transmembrane (TM) domain and a soluble, C-terminal domain (C). The TM domain features a six-helix bundle, and the C-terminal domain features two α-helices and a three-stranded β-sheet. The structure of FieF in the presence of Zn^{2+} reveals multiple metal-binding sites: one in the TM domain (coordinated by Asp45, Asp49, His153, and Asp157), one in the linker between the TM and C domains (coordinated by Asp68 and His75), and an interesting dizinc site in the C-terminal domain (coordinated by His232, His261, His283, and Asp285). It is possible that Fe^{2+} may bind in similar locations. N and C represent the amino and carboxy termini, respectively.

selectively bound both Zn^{2+} and Cd^{2+}.[96] This discord in metal-binding specificity of FieF highlights the possibility that FieF recognizes and/or transports a diverse set of metals. Despite the challenges in determining its function, *E. coli* FieF is still considered a model of both CDF protein structure and function, having been studied structurally by X-ray crystallography (Figure 6) and even recently by cryo-EM to gain insight into the mechanism of metal efflux. Specifically, cryo-EM studies have shown the rotating and "rocking" motions of the transmembrane helices that are necessary for Zn^{2+} transport,[91,97,98] which may be involved in Fe^{2+} efflux by extrapolation.

Other iron-transporting CDF proteins have been identified in both pathogenic and nonpathogenic bacteria but remain poorly understood. For example, *S. oneidensis* FeoE, a homologue of *E. coli* FieF, transports Fe^{2+} to maintain cell vitality during iron respiration,[99] but biochemical characterization is lacking. In *Magnetospirillum gryphiswaldense*, MamB and MamM comprise a heterodimeric CDF protein complex that is relevant for transporting Fe^{2+} into magnetosome membrane vesicles, which are thought to drive biomineralization of magnetite crystals.[100−103] The opportunistic pathogen *Pseudomonas aeruginosa* encodes AitP, a CDF that directly participates in the homeostasis of both Fe^{2+} and Co^{2+},[104] but little else is known beyond this extent. In fact, despite the identification of CDF proteins across several pathogenic bacterial strains, and their likely contribution to virulence, a consensus model for metal specificity of CDF proteins has yet to be developed.[9] Thus, further investigation of the CDF metal transport mechanism is warranted.

Major Facilitator Superfamily (MFS). The major facilitator superfamily (MFS) of proteins is the largest group of secondary active transport proteins that are linked to pathogenesis and are involved in translocation (both import and efflux) of nutrients, small molecules, and metal ions (Figure 5).[3,105] Subdivided into three categories on the basis of the direction and type of energy sources used to support substrate transport,[3] MFS-mediated transport may be carried out by a facilitator (uniporter) that supports diffusion across the membrane based on a concentration gradient, a symporter (co-transporter in the same direction), or an antiporter (exchanger in opposite directions).[3] Generally, MFS proteins are composed of 12 transmembrane α-helices arranged in distinct N- and C-terminal domains with 2-fold pseudosymmetry that are connected by an unstructured linker and cycle through distinct conformations to complete transport.[3] Structural analysis of an iron-transporting MFS protein remains elusive, but other MFS transporters have been well-studied by crystallography.[3,106−109] These studies have demonstrated that α-helices 1, 4, 7, and 10 are oriented toward the center of the two domains to form a path for transport, while the interface between the N- and C-terminal domains is formed by α-helices 2, 5, 8, and 11; α-helices 3, 6, 9, and 12 compose the outer surface of the protein to provide structural support.[3] Substrates of MFS transporters are a diverse but essential set of molecular building blocks, and evidence has linked MFS transport to the uptake of amino acids, ions, lipids, nucleosides, peptides, and even iron complexes.[3]

The iron citrate efflux transporter (IceT, formerly MdtD) is a member of the *S. typhimurium* MFS family that facilitates the efflux of citrate or iron citrate and has sequence homology to proteins belonging to the drug:proton antiporter-2.[8] *S. typhimurium* causes foodborne illness, as the pathogen requires intestinal inflammation in the host for survival.[110] IceT is encoded by *mdtD*, part of the *mdtABCD-baeSR* gene cluster that also expresses multidrug transporter (Mdt) domains A, B, and C as well as BaeSR, a two-component system that

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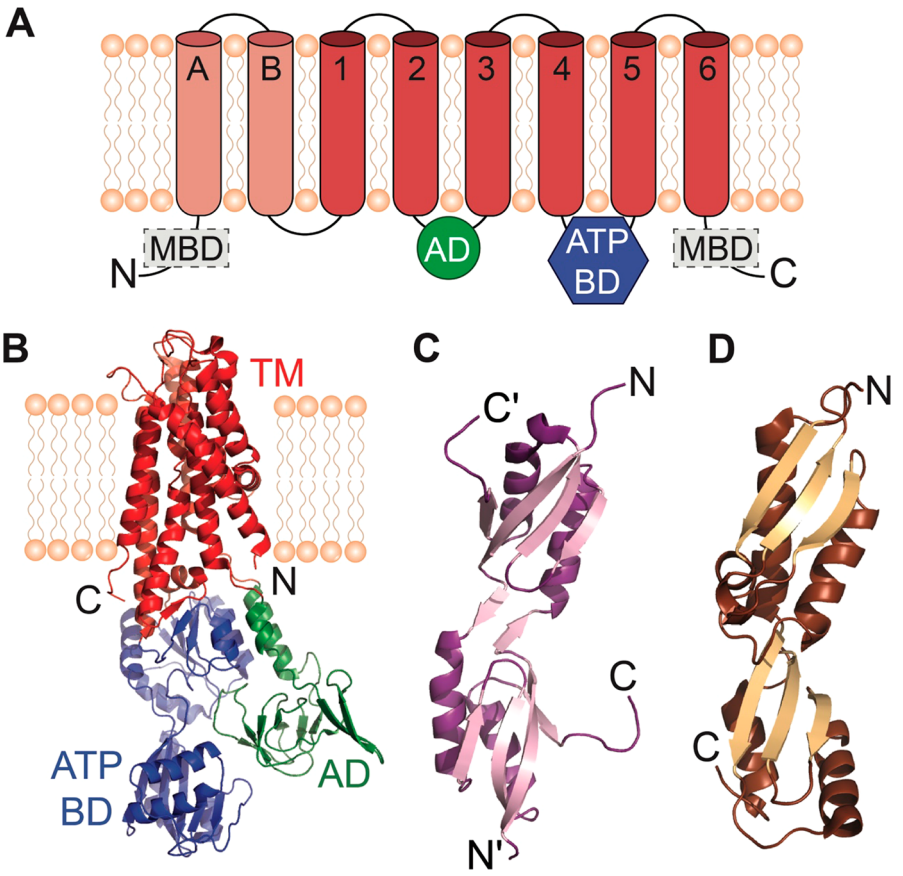

Figure 7. Structural elements of P_{1B}-ATPases. (A) Cartoon topology of a P_{1B}-type ATPase. Soluble N- and/or C-terminal metal-binding extensions (MBDs, gray) are proposed to regulate P_{1B}-ATPase activity. The transmembrane region may feature N-terminal accessory helices (cylinders labeled A and B, salmon), but six helices are generally conserved across P_{1B}-ATPases (cylinders 1−6, red). The soluble actuator domain (AD, green) generally lies between helices 2 and 3, and the ATP-binding domain (ATP-BD, blue) is generally located between helices 4 and 5. Figure adapted from ref 4. (B) X-ray crystal structure of the archetypal metal-transporting P_{1B}-type ATPase CopA (PDB entry 3RFU) highlighting the fold of the six α-helices comprising the transmembrane region (TM, red), the ATP-BD (blue), and the AD (green).[7] (C and D) P_{1B}-ATPases can have one or several metal-binding domains (MBDs) at either terminus. (C) The CopA C-terminal MBD (PDB entry 3FRY) features a ferredoxin-like domain-swapped dimer with α-helices colored purple and β-strands colored pink. (D) The CzcP N-terminal MBD (PDB entry 4U9R) adopts a duplication of the ferredoxin-like fold and a second metal-binding site located at the domain−domain interface. N and C represent the amino and carboxy termini, respectively.

influences antibiotic tolerance and export in *S. typhimurium*.[9,111] Both of these operons comprising this gene cluster are promoted by the increase in the intracellular ROS level.[8] In addition, IceT expression has been linked to tolerance to streptonigrin, an iron-sensitive antibiotic.[8,112] Expression of IceT has also been shown to reduce cellular iron levels, which has been proposed to be the result of obstruction of Feo import in addition to export of iron citrate.[8] While this mode of export is intriguing, cytoplasmic interactions between iron and citrate have not been previously reported, it is unclear whether and to what extent IceT and Feo may interact or have cross-talk, and the mechanism by which Fe^{2+} is selected over Fe^{3+} for chelation via citrate has yet to be established.[32] Considering that IceT is not homologous to previously characterized MFS transporters or other metal citrate transporters, a better structural and mechanistic understanding of IceT would shed light onto this unique subset of MFS transporters present in problematic pathogens.[32]

Membrane-Bound Ferritin-like Proteins. Membrane-bound ferritin A (MbfA) belongs to the erythrin-vacuolar iron transport (Er-VIT1) family and is involved in ferrous iron efflux.[113−115] Members of the ferritin-like superfamily, the Er-VIT1-like proteins generally consist of two major domains: a soluble N-terminal ferritin-like domain (also called Er) that houses a diiron-binding site and a membrane-imbedded VIT1 domain that is proposed to function in the transport of iron to vacuoles based on sequence homology to *Arabidopsis* VIT1.[113,115] In bacteria that utilize MbfA, expression is regulated by the transcriptional iron response regulator (Irr) protein.[114,116] In addition, biochemical studies of *Agrobacterium tumefaciens* suggest that the level of MbfA expression is increased in response to low pH (5.5) and H_2O_2 as well as iron.[113,115] Similarly, *Bradyrhizobium japonicum* MbfA expression is promoted by high-iron conditions and has been shown to remediate iron excess, as cells lacking *mbfA* had elevated iron levels.[117] Furthermore, MbfA appears to be relevant in some organisms for general intracellular iron homeostasis, rather than simply correcting for major iron imbalances.[117] Taken together, these findings indicate a role for MbfA in iron export to reduce potential cellular damage.[9,117]

P_{1B}-Type ATPases. P_{1B}-type ATPases make up a subdivision of the P-type ATPase family of membrane proteins that are found across all kingdoms, including pathogenic

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prokaryotes, and are utilized to transport a number of metal substrates, including Fe^{2+}.[4,118,119] The architecture of a P_{1B}-type ATPase is generally conserved and features three domains: (1) several α-helices (typically at least six) that comprise a transmembrane region for metal transport, (2) a soluble ATP-binding domain (ATP-BD) that includes nucleotide-binding (N-) and phosphorylation (P-) subdomains, and (3) a soluble actuator domain (AD) that is hypothesized to communicate changes in the ATP-BD, aid in dephosphorylation, and alter protein dynamics (Figures 5 and 7).[4] Two P_{1B}-type ATPases have been structurally characterized using X-ray crystallography: the Cu^{+}-transporting *L. pneumophilia* CopA and the Zn^{2+}-transporting *Shigella sonnei* ZntA.[7,120] Additionally, many (but not all) P_{1B}-type ATPases have a variable number of soluble metal-binding domains (MBDs) that can be found at the N- and/or C-termini, may function as allosteric sites, and may even participate in metal hand-off to the transmembrane domain (Figure 7).[4,121,122] Structural comparisons to other P-type ATPases as well as bioinformatics-based studies of hundreds of sequences demonstrated conservation of the structure and multiple residues that contact ATP/ADP or facilitate interdomain interactions across the P_{1B}-type subfamily, indicating a common and general mechanism despite the wide distribution of substrate diversity.[4,123,124]

Our understanding of the metal specificity (or even promiscuity in some cases) of P_{1B}-type ATPases is constantly evolving and has led to the identification of at least seven subclasses (P_{1B1}−P_{1B7}) that transport numerous metal substrates,[4] although Fe^{2+} as a substrate remains controversial. The P_{1B1}−P_{1B3} subclasses have been thoroughly studied historically and are known to be involved most commonly in Cu^{+} and Zn^{2+} homeostasis.[4] The P_{1B4}-type ATPases exhibit the greatest substrate diversity of all of the P_{1B}-type ATPases.[7,119,121,125−129] Though P_{1B4}-ATPases are originally considered to transport exclusively Co^{2+}, a growing body of evidence demonstrates they are also capable of transporting Cd^{2+}, Fe^{2+}, Ni^{2+}, and Zn^{2+}.[119,130−136] Recent reports have suggested that several of the P_{1B4}-ATPases may be ferrous iron exporters that function in concert with regulators to mitigate iron intoxication (i.e., excess intracellular iron). Work has shown that the peroxide operon regulator (PerR) and the ferric uptake regulator control transcription of the P_{1B4}-ATPases *Bacillus subtilis* peroxide-induced ferrous efflux transporter (PfeT) and the *Listeria monocytogenes* Fur-regulated virulence determinant FrvA.[131,132,137] PerR appears to regulate export by *Streptococcus pyogenes* Per-regulated metal transporter A (PmtA).[135] In addition, expression of several P_{1B}-type ATPases also appears to be induced by ROS. Specifically, *B. subtilis* PfeT, *M. tuberculosis* CtpD, and group A *Streptococcus* PmtA expression levels have all been linked to the presence and concentration of H_2O_2.[9] However, despite this potential connection to ferrous iron export, Michaelis constants (i.e., $K_{1/2}^{Fe}$) indicate that Fe^{2+} is a weak substrate (ranging from 92 to 640 μM) for these transporters compared to other metal ions ($K_{1/2}^{Co}$ ranging from 3.4 to 58 μM), suggesting that Fe^{2+} may be exported by this subfamily only when homeostatic conditions are severely disrupted.[131−133,138] Subclasses P_{1B5}−P_{1B7} are less well-characterized but are implicated in the transport of Fe^{2+} and/or Ni^{2+}.[4] Specifically, the P_{1B5}-ATPase subfamily's substrate specificity is still unknown, but approximately 25% of P_{1B5} proteins have a C-terminal diiron-binding hemerythrin-like domain,[4] implicating iron as a likely substrate.[139,140] Additionally, some members of the P_{1B6} subfamily are found downstream of Feo genes, prompting the hypothesis that this subfamily may export excess Fe^{2+} that is imported via FeoB.[4] Undoubtedly more *in vivo* and *in vitro* work is necessary to determine the metal specificity of these recently identified subfamilies.

CONCLUSIONS AND FURTHER PERSPECTIVES

Currently, there are several reported prokaryotic ferrous iron importers; however, only three of them (Efe, IroT, and Feo) are specific for iron, and all three remain understudied. These systems, and Feo in particular, have been implicated in bacterial pathogenesis, and further research into these importers could reveal novel approaches and potential therapeutic targets for attenuating virulence. Given its broad distribution, additional studies of Feo in particular may yield the greatest benefit, although advancements on any of these systems would be a boon for the development of therapeutic strategies. Excitingly, novel inhibitors against *Staphylococcus aureus* FeoB were recently described and may offer insight for future antibiotic development strategies to target this or other Fe^{2+} transporters.[141,142] However, like Efe and IroT, there are currently no experimentally determined structures of the full-length FeoB protein, representing a major bottleneck. While some advancement has been made in determining metal−protein interactions for IroT, it is not known how Efe and FeoB bind and transport Fe^{2+}. In addition, the roles of auxiliary proteins (i.e., EfeB, EfeO, FeoA, and FeoC) in Fe^{2+} uptake are still largely unknown. Thus, more work, including additional structural information, is needed to elucidate mechanisms of prokaryotic ferrous iron import, especially to target these uptake systems in pathogens.

The identification of dedicated ferrous iron exporters is a newer concept, but homologues are spread across all kingdoms of life, are necessary for cellular iron homeostasis, and are found in several infectious and problematic prokaryotes. If better characterized, these efflux pumps could present a target for antibiotic development against pathogenic bacteria; however, our lack of knowledge regarding structures, mechanisms, and metal selectivities represents a major (but not insurmountable) barrier. While bioinformatics analyses have proven to be especially insightful in expanding our understanding of P_{1B}-type ATPases, other classes of Fe^{2+} exporters have not been similarly explored. Additionally, like those of ferrous iron importers, few experimentally determined structures are known for the ferrous iron efflux pumps. Critically, the structure of any of these membrane proteins in the presence of Fe^{2+} would greatly expand our knowledge of structure and mechanism and could inform selectivity. Despite these major hurdles, we anticipate that the increased availability and widespread instrumentation of cryo-EM techniques could fill this knowledge gap sooner rather than later.

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■ ABBREVIATIONS

ABC, ATP-binding cassette; AD, actuator domain; ARTO, alternate respiratory terminal oxidase; ATP, adenosine 5′-triphosphate; ATP-BD, ATP-binding domain; CDF, cation diffusion facilitator; CF, cystic fibrosis; cryo-EM, cryogenic electron microscopy; Dps, DNA-binding protein from starved cells; Efe, elemental ferrous iron; Er-VIT1, erythrin-vacuolar iron transport; Fab, fragment antigen-binding; Feo, ferrous iron transport; Fie, ferrous iron efflux; FNR, fumarate and nitrate reduction regulatory protein; Frv, Fur-regulated virulence; Fut, ferric uptake system; GAP, GTPase-activating protein; GCP, phosphomethylphosphonic acid guanylate; GDI, guanosine 5′-diphosphate dissociation domain; GDP, guanosine 5′-diphosphate; GEF, guanine exchange factor; GMP-PNP, guanosine 5′-[β,γ-imido]triphosphate; GTP, guanosine 5′-triphosphate; HTH, helix−turn−helix; IceT, iron citrate efflux transporter; IroT, iron transporter; Irr, iron response regulator; LCV, *Legionella*-containing vacuole; mantGDP, 2′/3′-(*N*-methyl-anthraniloyl)-guanosine 5′-diphosphate; mantGMP-PNP, 2′/3′-(*N*-methyl-anthraniloyl)-guanosine 5′-[(β,γ)-imido]triphosphate; MavN, more regions allowing vacuolar co-localization N; MBD, metal-binding domain; MbfA, membrane-bound ferritin A; Mdt, multidrug transporter; MFS, membrane facilitator superfamily; MntH, H^+-dependent manganese transport; NFeoB, N-terminal, G-protein-like domain of the ferrous iron transport protein; NRAMP, natural resistance-associated macrophage protein; PDB, Protein Data Bank; PerR, peroxide operon regulator; PfeT, peroxide-induced ferrous efflux transporter; PMF, proton motive force; PmtA, Per-regulated metal transporter A; ROS, reactive oxygen species; Sit, *Salmonella* iron transport; Yfe, *Yersinia* ferrous iron transport; Zip, ZRT- and IRT-like protein; Znu, zinc uptake; ZupT, zinc uptake transporter.

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