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# Ferrous Iron Efflux Systems in Bacteria

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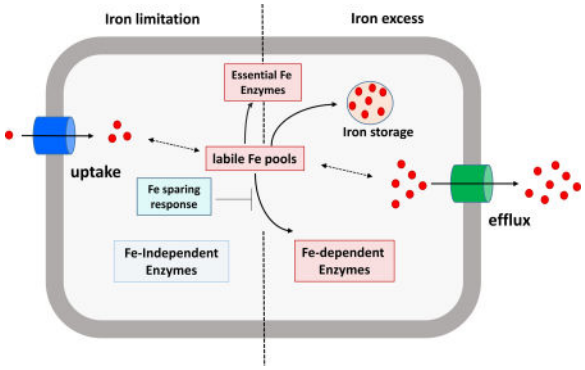
## Abstract

Bacteria require iron for growth, with only a few reported exceptions. In many environments, iron is a limiting nutrient for growth and high affinity uptake systems play a central role in iron homeostasis. However, iron can also be detrimental to cells when it is present in excess, particularly under aerobic conditions where its participation in Fenton chemistry generates highly reactive hydroxyl radicals. Recent results have revealed a critical role for iron efflux transporters in protecting bacteria from iron intoxication. Systems that efflux iron are widely distributed amongst bacteria and fall into several categories: $P_{1B}$-type ATPases, cation diffusion facilitator (CDF) proteins, major facilitator superfamily (MFS) proteins, and membrane bound ferritin-like proteins. Here, we review the emerging role of iron export in both iron homeostasis and as part of the adaptive response to oxidative stress.

## Graphical abstract




## Introduction

Iron is critical for cell growth and survival. However, when present in excess, it is also detrimental to cells. Under aerobic conditions, iron toxicity is closely related to oxidative stress through Fenton chemistry[1]. Hydrogen peroxide ($H_2O_2$) reacts with ferrous iron ($Fe^{2+}$) to generate highly reactive hydroxyl radicals that damage macromolecules such as DNA, proteins and fatty acids, resulting in disruption of cell metabolism and ultimately cell death[2]. Therefore, the toxicity of reactive oxygen species (ROS) is generally thought to be exacerbated by conditions that elevate the intracellular iron pool. Conversely, high levels of intracellular iron may also be toxic independent of ROS, presumably due to the ability of

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iron to compete with other transition metals, such as manganese, for binding to metal-dependent enzymes or regulators, resulting in mismetallation and inactivation of these proteins[3, 4]. ROS such as $H_2O_2$ and superoxide radical can disrupt iron-sulfur clusters and mononuclear iron centers of iron-enzymes, thereby leading to iron release[5, 6]. Therefore, iron intoxication may also be exacerbated by an elevation in ROS. Clearly, the toxicity of iron and ROS are closely intertwined, with each potentially increasing the toxicity of the other.

Bacteria adapt to environmental stresses by activation of specific transcriptional programs. In the case of iron homeostasis, bacteria monitor intracellular iron levels using metal-sensing (metalloregulatory) proteins[7, 8]. The ferric uptake regulator (Fur) protein is the most widespread bacterial iron sensor[9], but it can be replaced by functionally analogous proteins such as IdeR (in actinomycetes)[10, 11] and Irr (in alpha-proteobacteria)[12–14]. Fur helps to maintain iron homeostasis by regulating genes implicated in iron uptake, storage, and efflux[15]. Typically, Fur is considered to function as an $Fe^{2+}$-activated transcriptional repressor for most of its targets, but there are increasing examples where Fur functions as a transcriptional activator or where it binds DNA in the absence of bound iron[16–18].

Iron-sensing regulators such as Fur play a central role in the control of iron homeostasis[19]. The *Escherichia coli* Fur regulon illustrates the diverse roles that Fur may play. *E. coli* Fur ($Fur_{EC}$) binds to DNA when associated with $Fe^{2+}$ and serves to repress the expression of target operons[20]. This repression is relieved under iron-limited conditions, and this results in the derepression of iron uptake systems, including the synthesis of the high-affinity iron-chelating compound siderophore known as enterobactin and its cognate import system[21]. Fur also helps bacteria to remodel their proteomes to prioritize the utilization of iron, in a process known as "iron-sparing" (Fig. 1)[22–24]. In *E. coli*, the loss of $Fur_{EC}$ DNA-binding activity (under low iron conditions) results in expression of the RyhB small RNA (sRNA) that represses translation of non-essential iron-enzymes[22–24]. Fur also participates in the regulation of gene expression under conditions of iron excess. For example, $Fur_{EC}$ positively regulates expression of the iron storage protein ferritin by occluding the binding of the H-NS transcriptional repressor[25]. In general, adaptation to iron excess often involves expression of iron storage functions (including heme-containing bacterioferritins, ferritins, and Dps-family mini-ferritins) but may additionally require iron efflux systems (Fig. 1). In light of the central role of Fur in coordinating iron homeostasis, it is not surprising that some iron efflux systems are induced by Fur in response to iron excess[26, 27].

Bacteria also adapt to oxidative stress by the induction of specific defensive genes. For example, $H_2O_2$ induces a specific peroxide-stress response that is regulated by the OxyR repressor in *E. coli*[28] and by the PerR repressor in *Bacillus subtilis*[29]. In both model organisms, a rise in intracellular $H_2O_2$ triggers the induction of defensive enzymes such as catalase and alkyl hydroperoxide reductase, which can directly detoxify $H_2O_2$. In addition, cells scavenge excess iron from the cytosol by sequestration into mini-ferritin proteins, including Dps in *E. coli*[30] and the Dps ortholog MrgA in *B. subtilis*[31]. The co-regulation of $H_2O_2$ degradation enzymes and iron-sequestering proteins further highlights the central role of iron in peroxide intoxication. In addition to scavenging iron, peroxide stress also frequently modulates metal uptake and efflux systems[32]. In *E. coli*, $H_2O_2$ induces an OxyR-

activated $Mn^{2+}$ uptake system (MntH)[33, 34], and in *B. subtilis* $H_2O_2$ induces a PerR-regulated iron efflux system, PfeT[35, 36]. PfeT is a member of the $P_{1B4}$-type ATPases, and recent results indicate that several close homologs also function as $Fe^{2+}$ efflux pumps[27, 37–39]. $Fe^{2+}$ efflux pumps have now been documented in a wide variety of bacteria, and include $P_{1B}$-type ATPases, cation diffusion facilitator (CDF) proteins, major facilitator superfamily (MFS) proteins, and membrane bound ferritin-like proteins (Table 1 & Fig. 2). Here, we summarize the emerging role of these ferrous iron efflux pumps in helping ameliorate the deleterious effects of excess iron and peroxide.

## P-type ATPases

The P-type ATPases are a large group of transmembrane proteins that transport ions and lipids across cellular membranes, energetically driven by ATP hydrolysis[40]. Five subgroups of P-type ATPases have been defined based on sequence homology and substrate specificity[41]. These are the $P_1$-type ($K^+$ and transition metal transporters), $P_2$-type ($Ca^{2+}$, $Na^+/K^+$, and $H^+/K^+$ pumps), $P_3$-type ($H^+$ pumps), $P_4$-type (phospholipid transporters), and $P_5$-type ATPases (unknown substrate). The $P_2$-type ATPases have been well studied and are more prevalent in eukaryotes than in prokaryotes. The majority of $P_3$-type ATPases are $H^+$ pumps found in plants and fungi. Some of the $P_4$-type ATPases have been revealed to be phospholipid transporters[42, 43]. No specific substrate has yet been identified for the $P_5$-type ATPases that are only found in eukaryotes.

The $P_1$-type ATPases exist predominately in prokaryotes but are omnipresent across all domains of life[44]: $P_{1A}$-ATPases are involved in $K^+$ transport whereas $P_{1B}$-ATPases are important for maintaining transition metal homeostasis. $P_{1B}$-ATPases are known to transport $Cu^+$ [45, 46], $Ag^+$ [47], $Zn^{2+}$ [48], $Cd^{2+}$ [49], $Cu^{2+}$ [50], $Co^{2+}$ [51] and $Fe^{2+}$ [27, 36, 37]. The structure of a typical $P_{1B}$-ATPase includes a transmembrane domain with 6–8 helices, a soluble actuator domain, and an ATP-binding domain[52] (Fig. 2). The $P_{1B}$-ATPases can be further divided into seven subclasses based on sequence similarity and metal substrate specificity[52]. The $P_{1B4}$-type ATPases were originally assigned a role in $Co^{2+}$ export, based on the properties of some of the first characterized members[53]. However, $P_{1B4}$-type ATPases have recently been found to function instead, or in addition, as $Fe^{2+}$ efflux transporters including *Bacillus subtilis* PfeT[36], *Listeria monocytogenes* FrvA[27], *Mycobacterium tuberculosis* CtpD[37], and group A *Streptococcus* PmtA[38, 39].

### PfeT in *Bacillus subtilis*

*B. subtilis* is a Gram-positive soil microorganism and encodes two transcriptional regulators critical for iron homeostasis, $Fur_{Bs}$ and PerR. $Fur_{Bs}$ is a global transcriptional regulator of iron homeostasis analogous to $Fur_{EC}$[54] and PerR mediates the adaptive response to peroxide stress by regulating genes involved in iron storage and peroxide detoxification[29]. The regulons for both $Fur_{Bs}$ and PerR have been well defined[55, 56]. $Fur_{Bs}$ senses intracellular iron sufficiency and represses genes that are involved in siderophore synthesis and uptake[54, 57]. $Fur_{Bs}$ also regulates an iron sparing response mediated by the small non-coding RNA FsrA (Fig. 1) and its coregulators FbpA, FbpB and FbpC[58–60]. This system, analogous to RyhB in *E. coli*, blocks the translation of non-essential iron-containing enzymes such as

aconitase and succinate dehydrogenase[58–60]. PerR regulates peroxide detoxification enzymes (catalase, alkyl hydroperoxide reductase), iron sequestration (MrgA) and the $P_{1B4}$-type ATPase (PfeT). Although the Fur and PerR regulons are largely non-overlapping, *pfeT* is the exception and is regulated by both proteins[26]. The result is that *pfeT* is induced by either peroxide stress or by iron excess (unpublished data, Pinochet-Barros A & Helmann JD).

PfeT is one of three $P_{1B}$ ATPases encoded by *B. subtilis*. CopA is a $P_{1B1}$-ATPase that functions as a $Cu^+$ efflux transporter and, appropriate to its function, is regulated by the CsoR $Cu^+$ sensor[61]. CadA is a $P_{1B2}$-ATPase that confers resistance to $Cd^{2+}$, $Zn^{2+}$, and $Co^{2+}$ and is regulated by the divalent cation sensor CzrA[62]. PfeT (formerly named as ZosA) is a $P_{1B4}$-type ATPase and was discovered as a transporter induced by $H_2O_2$ that plays a role in protecting cells against oxidative stress[35]. Initial results indicated that deletion of *pfeT* enhanced $Zn^{2+}$ tolerance, as monitored in cells lacking the CadA efflux system[35]. This led to the proposal that PfeT might function as a $Zn^{2+}$ importer under oxidative stress conditions, consistent with the idea that $Zn^{2+}$ has a role in protecting cells against oxidative damage[35]. As a result, PfeT was originally named for this proposed role as ZosA ($Zn^{2+}$ uptake under oxidative stress)[35].

Contrary to this model, most $P_{1B}$-type ATPases function in metal export rather than import, which motivated a reinvestigation of the role of PfeT. Further study revealed that a *pfeT* null mutant is sensitive to $Fe^{2+}$ and $Fe^{3+}$, particularly under acidic media conditions, but not to $Zn^{2+}$ or $Co^{2+}$. Moreover, a *pfeT* null mutant accumulates elevated levels of intracellular $Fe^{2+}$, as judged by sensitivity to the $Fe^{2+}$-activated antibiotic streptonigrin and by direct chemical measurement[36]. Biochemical studies confirmed that the ATPase activity of PfeT is induced the most by $Fe^{2+}$, with modest induction by $Co^{2+}$ but not with other metals, including $Zn^{2+}$. In addition to $H_2O_2$, *pfeT* is strongly and specifically induced by iron, but not by other metals. Together, these findings indicate that PfeT function as a peroxide- and iron induced ferrous efflux transporter[36]. The ability of PfeT to protect against $H_2O_2$ is secondary to that of the detoxification enzymes catalase and alkyl hydroperoxide reductase. However, PfeT plays a dominant role in protecting cells from iron overload with the MrgA miniferritin playing a secondary role[36]. The revelation that PfeT functions in $Fe^{2+}$ efflux, in turn, prompted a re-evaluation of the roles of several other $P_{1B4}$-type ATPases in bacterial iron homeostasis.

## FrvA in *Listeria monocytogenes*

*L. monocytogenes* is the causative agent of the foodborne disease listeriosis, which is associated with central nervous system infections and bacteraemia. FrvA (Lmo0641) is a $P_{1B4}$-ATPase originally described as a Fur-regulated virulence factor[63]. FrvA was proposed to function as a heme exporter that was suggested to be induced by iron deficiency and to be under negative regulation of both Fur and PerR[63, 64]. However, a different transcriptome study showed a downregulation of *frvA* in a *fur* null mutant[65], indicating a positive regulatory role of Fur in *frvA* expression.

To resolve these contradictory reports of iron regulation, and to test if FrvA might function in $Fe^{2+}$ efflux, the mutant phenotype was reinvestigated and the FrvA protein was purified

for biochemical studies[27]. As predicted based on studies of *pfeT*, a *frvA* null mutant was sensitive to iron intoxication, but not to other metals or heme. Like *B. subtilis pfeT, frvA* is positively regulated by Fur in response to high $Fe^{2+}$ levels and is repressed by PerR[27, 64]. Biochemical studies indicate the FrvA ATPase activity is stimulated most strongly by $Fe^{2+}$ with weaker stimulation in the presence of $Co^{2+}$ or $Zn^{2+}$. Based on the $Fe^{2+}$ concentration dependence of ATPase activity, FrvA seems to have a higher affinity for $Fe^{2+}$ than *B. subtilis* PfeT. Consistent with this, not only does FrvA complement the iron-sensitive phenotype of a *B. subtilis pfeT* null mutant, its expression depletes the cytosol of iron (even under iron-rich conditions) thereby leading to derepression of the Fur regulon[27]. These results support the hypothesis that FrvA functions as a $Fe^{2+}$ efflux transporter that protects cells from $Fe^{2+}$ intoxication[27].

FrvA is required for virulence in murine and insect (*Galleria mellonella*) infection models[63]. The *frvA* null mutant strain shows strong attenuation in virulence, but is still able to invade and propagate inside antigen-presenting cells[66], suggesting an important link between iron homeostasis and virulence, but it is not clear at which stage(s) of the *L. monocytogenes* life cycle FrvA is important. The phagocytic vacuole is generally considered to be an iron-limited environment. One possibility is that the expression of high affinity iron uptake systems by iron limitation during infection or in the phagocytic vacuole can contribute to iron overload upon escape of cells into the relatively iron-rich cytosol. Alternatively, the imposition of oxidative damage from host immune cells may trigger iron release from listerial iron enzymes and this may lead to iron overload. The points in the infection cycle where FrvA plays a critical role are not yet clearly defined and provide an interesting avenue for future research.

### CtpD in *Mycobacterium tuberculosis*

*M. tuberculosis* is an obligate pathogen and the causative agent of human tuberculosis. Nearly one-third of the world's population is infected with *M. tuberculosis*, which can persist in a latent state for decades and then later emerge (in ~10% of cases) as an active lung infection. *M. tuberculosis* encodes a total of 11 P-type ATPases, which have been suggested to be possible targets for therapeutic intervention[67]. Of these, two encode $P_{1B4}$-ATPases: CtpD (Rv1469) and CtpJ (Rv3743)[37]. CtpD, but not CtpJ, was found to be important for survival in macrophages and the mouse lung[37]. Biochemical studies had previously highlighted the activity of these two $P_{1B4}$-ATPases with $Co^{2+}$, but it was not clear why *M. tuberculosis* would encode two such proteins, nor was it understood why $Co^{2+}$ efflux would be important for survival in the host.

In light of the finding that PfeT functions as an $Fe^{2+}$ efflux transporter, the roles of CtpD and CtpJ were reinvestigated. Biochemical studies indicated that the ATPase activity of CtpD is most strongly activated by $Fe^{2+}$. Although $Co^{2+}$ also activates ATPase activity, the maximal activity ($V_{max}$) is 10-fold lower than with ferrous iron[37]. CtpD also binds $Fe^{2+}$ with 3-fold higher affinity than $Co^{2+}$. In contrast, the CtpJ ATPase activity is activated by both $Fe^{2+}$ and $Co^{2+}$, and has a slightly higher affinity for $Co^{2+}$ than $Fe^{2+}$. To better understand their roles in vivo, metal accumulation and sensitivity was monitored for strains lacking either *ctpD* or *ctpJ*. The *ctpD* mutant strain did not accumulate $Co^{2+}$ and was impaired in growth in iron-

amended medium, consistent with a primary role in resistance to iron intoxication[37]. Mutation of *ctpJ* led to a significant increase in $Co^{2+}$ accumulation and expression was induced by $Co^{2+}$, consistent with a primary role in $Co^{2+}$ resistance[37]. However, the *ctpJ* mutant was also growth impaired in the presence of excess iron. Thus, these two paralogous transporters seem to have overlapping metal selectivity, but largely distinct physiological roles. Further studies are needed to understand the molecular mechanism of substrate specificity, but based on X-ray absorption spectroscopy (XAS) analysis, it is likely that distinct metal coordination geometry plays an important role[37].

During infection, *M. tuberculosis* propagates in the host macrophages, which are considered iron-poor environments. Just as noted for *L. monocytogenes*, it is not yet clear where in the infection process cells experience iron intoxication. Further studies are needed to better understand the conditions that lead to induction of *ctpD*. In prior work *ctpD* was not induced by metals such as $Co^{2+}$, $Zn^{2+}$, and $Ni^{2+}$, but its cognate substrate $Fe^{2+}$ was not tested[68]. It might be induced by $Fe^{2+}$ and, by analogy with its orthologs, this might involve an iron-sensing transcription factor. IdeR, a member of DtxR family, is the major iron-dependent transcriptional regulator in *M. tuberculosis*[10, 11]. IdeR represses transcription of genes involved in iron uptake and siderophore biosynthesis and activates expression of genes encoding iron-storage proteins such as bacterioferritin and a ferritin-like protein[10, 11]. Since *M. tuberculosis* is primarily a pathogen of the mammalian respiratory system it might frequently encounter oxidative stress. Thus, it is also possible that *ctpD* might be induced in response to $H_2O_2$ stress. Future work to monitor the expression of *ctpD* in vitro in response to specific stresses and in vivo during the course of infection will be needed to elucidate the physiological role of CtpD during the infection process.

## PmtA in group A *Streptococcus*

Group A *Streptococcus* (GAS), a human pathogen, is the causative agent of a wide range of diseases, from mild skin infection to life-threatening diseases such as necrotizing fasciitis[69]. GAS encodes a $P_{1B4}$-type ATPase under regulation of PerR, and was therefore named a PerR-regulated metal transporter (PmtA). In a *perR* null mutant, high level expression of *pmtA* is associated with derepression of genes normally responsive to cellular zinc status due to repression by AdcR[70], a $Zn^{2+}$-dependent repressor. This simplest interpretation of this result is that PmtA may function as a $Zn^{2+}$ efflux transporter. Consistent with this notion, a *perR* null mutant has an increased resistance to $Zn^{2+}$, and this depends on PmtA[70]. However, it is unclear why cells would efflux $Zn^{2+}$ in response to $H_2O_2$ stress, nor is there any evidence that PmtA is important for $Zn^{2+}$ resistance in wild-type cells, which presumably relies on the $Zn^{2+}$-inducible CzcD efflux pump to ameliorate $Zn^{2+}$-toxicity.

By analogy with PfeT and its orthologs, an alternative interpretation is that the primary role of PmtA is as a $H_2O_2$-inducible $Fe^{2+}$-efflux pump and that activity with $Zn^{2+}$ may only be revealed when it is constitutively overexpressed in a *perR* null mutant. Two recent studies have confirmed the primary role of PmtA as an $Fe^{2+}$-efflux pump[38, 39]. PmtA is important for resistance to iron intoxication, and a *pmtA* null mutant accumulates elevated levels of intracellular iron. As expected, expression of *pmtA* is strongly induced by $Fe^{2+}$. Although a *pmtA* null mutant shows similar sensitivity to peroxide stress as a wild type strain in the

absence of excess $Fe^{2+}$, it exhibits significantly increased susceptibility to peroxide stress when treated with $Fe^{2+}$. Since GAS is catalase negative, PmtA might be a frontline defense against peroxide stress. PmtA is also a critical virulence factor and is required for survival during infection in both intramuscular and subcutaneous mouse models[38], which again links iron efflux and peroxide resistance to pathogen virulence.

### Nia in *Sinorhizobium meliloti*

In addition to the $P_{1B4}$-ATPases featured above, it is possible that $P_{1B}$-ATPases of other groups may also have physiologically relevant activity with iron. One example is Nia, a $P_{1B5}$-ATPase with a C-terminal hemerythrin domain. Since hemerythrin domains bind $O_2$ via a diiron active site, this suggests a possible role in $O_2$-sensing[71, 72]. Nia is encoded by the symbiotic plasmid A of *Sinorhizobium meliloti*, a nitrogen fixing microbe in the *Rhizobiales* lineage that has a symbiotic relationship with legumes in which it establishes nodules associated with roots.

Consistent with a possible role in $Fe^{2+}$ efflux, a *nia* null mutant accumulates $Fe^{2+}$ under excess metal conditions[73]. However, Nia also functions with $Ni^{2+}$ and a *nia* null mutant accumulates $Ni^{2+}$ when in excess. The precise physiological role of Nia is not yet resolved. Biochemical assays suggest that Nia interacts with both $Fe^{2+}$ and $Ni^{2+}$ (but not $Co^{2+}$). However, a *nia* null mutant showed moderate sensitivity to $Ni^{2+}$, but not to $Fe^{2+}$, under the conditions tested[73]. Expression of *nia* was moderately induced by $Fe^{2+}$ (3-fold), $Ni^{2+}$ (3-fold), and $Co^{2+}$ (2-fold), but not by other metals. Interestingly, *nia* was most strongly induced (20-fold) in root nodules, thought to be a microaerobic, iron-rich environment[74]. These results lead to a model in which Nia is expressed in nitrogen-fixing root nodules, in response to either iron excess or microaerobic conditions. The C-terminal hemerythrin domain may also participate in or regulate transport activity, perhaps in response to $O_2$[73]. More work needs to be done to characterize the details of *nia* gene regulation and to more clearly define the physiological role of Nia during the *S. meliloti* -plant symbiosis.

## Cation diffusion facilitator (CDF) proteins

Cation diffusion facilitators (CDFs) are a family of membrane-bound proteins that export and thereby confer tolerance to heavy metal ions[75, 76]. CDF proteins are ubiquitous in bacteria, archaea, and eukaryotes[77]. Collectively, bacterial CDF proteins have been implicated in transport of a wide range of metal ions ($Zn^{2+}$, $Cd^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Fe^{2+}$ and $Mn^{2+}$) with some transporters able to transport multiple metals[78–82]. Phylogenetic analysis of the CDF transporters defines three major groups corresponding to substrate specificity: 1) manganese efflux, 2) iron/zinc efflux, 3) zinc and other metals (but not manganese or iron) efflux[83].

A typical bacterial CDF contains an N-terminal domain (NTD), 6 transmembrane helices (TM), a histidine-rich interconnecting loop (IL) between TM4 and TM5, and a C-terminal cytoplasmic domain (CTD)[75] (Fig 2). However, the detailed mechanisms of metal selectivity are unknown. Some studies suggest the cytoplasmic domain or the IL loop is important for metal specificity[84–86], but other studies highlight the role of residues in the TM3 helix on metal selectivity[87]. For the *E. coli* FieF transporter, evidence supports a role for a tetrahedral

metal-binding site formed between TM2 and TM5 in metal selectivity[88]. So far, there is no unifying model that can account for metal selectivity of CDF proteins.

### FieF in *E. coli*: $Zn^{2+}$ vs. $Fe^{2+}$ efflux

There are two CDF transporters in *E. coli*: ZitB and FieF (also named as YiiP). ZitB is the secondary zinc efflux system that is critical for maintaining zinc homeostasis only when the zinc efflux ATPase ZntA is absent[89]. FieF has been studied for more than a decade, but its physiological function has been controversial. In 2004, the first two reports of its structural analysis were built on the assumption that FieF acts as a zinc efflux protein[90, 91]. In fact, prior studies had demonstrated that *fieF* is induced by either zinc or iron[89]. However, ectopic expression of FieF does not restore zinc tolerance in a zinc-sensitive strain, suggesting it might not play a role in zinc homeostasis[89].

Physiological studies suggest that the major physiological role of FieF may be in iron tolerance. Indeed, FieF is important for full resistance to iron intoxication in a *fur* null mutant, where iron homeostasis is disrupted and iron uptake systems are constitutively expressed[92]. Ectopic expression of FieF leads to reduced accumulation of iron in a *fieF* null mutant. Moreover, reconstitution of FieF in proteoliposomes showed that it mediates iron transport in vitro[92]. These results all support the assignment of FieF (ferrous iron efflux) as an iron efflux transporter. However, this notion has been challenged by others. For example, FieF was shown to selectively bind zinc and cadmium with high affinity, but not iron or other metals tested[93]. Based on the site-directed fluorescence resonance energy transfer (FRET) measurements, Lu *et al.* proposed an autoregulation model of transport activity in response to intracellular zinc levels[94]. Currently, FieF (YiiP) is referred to as a $Zn^{2+}$ transporter in most published papers.

Ever since its structure was solved in 2007[79], FieF has been considered as a prototype for bacterial CDF proteins, which makes it more frustrating that its physiological role has remained controversial. The regulation of *fieF* expression has not been well defined, but it does not appear to be regulated by Fur[92]. The physiological studies of FieF are certainly supportive of a role in Fe(II) efflux. This inference is further supported by the observation that the FieF homologs MamM and MamB form a heterodimeric CDF protein required for Fe(II) import into vesicles in support of magnetosome formation in the magnetotatic bacterium *Magnetospirillum gryphiswaldense*[95, 96].

### AitP in *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is Gram-negative, opportunistic pathogen that is highly antibiotic resistant. *P. aeruginosa* encodes three paralogous CDF efflux systems: CzcD (PA0397), AitP (PA1297), and YiiP (PA3963). Of these, the alternative iron transport protein (AitP) most likely functions physiologically in $Fe^{2+}$ efflux. Deletion of *aitP* leads to an increased sensitivity to both $Fe^{2+}$ and $Co^{2+}$, increased intracellular accumulation of both ions, and decreased survival in presence of $H_2O_2$[97]. The observed sensitivity to $H_2O_2$ is most consistent with a role in $Fe^{2+}$ efflux, as noted above for P-type ATPases. In contrast with AitP, the CzcD and YiiP proteins were inferred to function physiologically in $Zn^{2+}$ resistance, although this role is largely masked in wild-type cells by the activity of the $Zn^{2+}$

efflux P-type ATPase, ZntA[98]. All the three transporters are critical for virulence in a plant infection model[97]. However, it remains unclear why this organism requires multiple classes of $Zn^{2+}$ efflux proteins or under what conditions the three proteins are physiologically important during the infection process.

### FeoE in *Shewanella oneidensis* MR-1

*Shewanella oneidensis* MR-1 is a facultative anaerobe in the γ-proteobacterium family that is capable of respiration using metals (e.g. manganese, lead, uranium and ferric iron) as electron acceptors[99]. *S. oneidensis* cells are usually pink or red, reflective of a high iron content in hemoproteins and cytochromes[100]. When $Fe^{3+}$ is used as a terminal electron acceptor, cells generate a large amount of soluble $Fe^{2+}$ which could potentially lead to iron intoxication. FeoE, a CDF protein, is required for cell growth during anaerobic iron respiration, and deletion of *feoE* increased susceptibility to $Fe^{2+}$ intoxication, consistent with a physiological role in $Fe^{2+}$ efflux[101]. Further work is required to understand how *feoE* expression is regulated. It is unclear, for example, whether *feoE* is induced in response to excess iron. Fur is the primary regulator that modulates iron acquisition in *S. oneidenis*[102], and is a candidate for an iron-responsive transcription factor that could be involved.

## Major facilitator superfamily (MFS)

The major facilitator superfamily (MFS) of membrane transporters function with a wide scope of small molecules such as ions, nucleosides, amino acids, small peptides, and lipids[103]. They can be categorized into three groups: uniporters that transport a single substrate, symporters that transport a substrate coupled with another ion (generally a proton), and antiporters that transport two substrates in opposite directions[104, 105]. All the MFS transporters share a canonical structural fold composed of two distinct domains [Fig. 2], each consisting of six transmembrane helices. The substrate binding site is located at the interface between these two domains[103].

The mechanism of transport by MFS proteins is not clear, but several related models have been proposed. The first, an alternate-access model, was proposed more than five decades ago[106]. This model speculates that the transporters undergo a conformational change that alternates between a form where substrate can bind from one side of the membrane to one where it can only bind from the other side. This has been validated by many structural studies such as the xylose/$H^+$ symporter XylE and for LacY[107–109]. The second, a rocker-switch model, postulates that conformational changes are accomplished through rocker-switch-type rotation between the N and C domain. This model is supported by some open-conformation structures[110] but not by the structures in occluded states[111–114]. A third, clamp-and-switch model, provides a two-step transport mechanism: a clamping step that mediates occlusion of the binding site and a switching step that mediates the exposure of the binding site. This model postulates four conformational states: inward open, outward open, inward-facing occlusion, and outward-facing occlusion[105]. This model is in a good agreement with studies of some MFS transporters[115, 116], but more structural analyses combined with biochemical and computational analyses are needed to further understand the transport mechanism of MFS transporters.

### IceT (iron and citrate efflux transporter) in *Salmonella* Typhimurium

*Salmonella* Typhimurium is a Gram-negative pathogen commonly found in the gastrointestinal tract. IceT (MdtD) is a member of the MFS superfamily in *S.* Typhimurium. The mdtABCD baeSR operon encodes IceT and two other systems: a RND (resistance-nodulation-division) drug efflux system MdtABC and a two-component regulatory system BaeSR that regulates antibiotic resistance and efflux[117–119]. IceT is proposed to be an iron-citrate efflux transporter and it can export either iron citrate or citrate alone[120]. The *iceT* null mutant shows increased susceptibility to the antibiotic streptonigrin (SN), the activity of which is modulated by the level of intracellular free iron[121]. This result suggests that the mutation of *iceT* leads to an increase in intracellular labile iron pools. Consistent with this result, induction of IceT expression leads to reduced levels of intracellular iron[120].

Although the mdtABCD baeSR operon is not induced directly by high $Fe^{2+}$ [122], it is induced by disruption of iron homeostasis in a *fur* null mutant where iron uptake systems are constitutively expressed, supportive of a physiological role for IceT in iron efflux. Although IceT confers resistance to peroxide stress in a *fur* null mutant, the mdtABCD baeSR operon is not induced by $H_2O_2$ or superoxide-generating reagents such as paraquat[120]. However, it is induced by nitric oxide, which is also known to interact with the labile iron pool[120]. The significance of the regulation of IceT, together with its co-transcribed ABC transporter, by the BaeSR two-component system is not understood, nor is it yet clear whether or not IceT is important for pathogenesis.

## Membrane bound ferritin A (MbfA) in *Agrobacterium tumefaciens* and *Bradyrhizobium japonicum*

*Agrobacterium tumefaciens* belongs to the *Rhizobiales* lineage and is the causative agent of the economically important plant disease, crown gall. MbfA was originally described as membrane-bound ferritin A, and is a member of the erythrin-vacuolar iron transport (Er-VIT1) ferritin-like superfamily. MbfA has two major domains: an N-terminal ferritin-like or Er domain (Er) and a C-terminal membrane-embedded vacuolar iron transporter domain (VIT1) (Fig. 2). The Er domain has a di-iron binding site and the VIT1 domain shows sequence homology to *Arabidopsis* VIT1, which is responsible for transferring iron into vacuoles[123]. Ferritin is a cytosolic iron storage protein ubiquitous in prokaryotes and eukaryotes[124], however, MbfA is not a *bona fide* ferritin and its physiological function was not immediately apparent.

Plant hosts often produce reactive oxygen species as a defense mechanism in response to microbial infection. Initial studies revealed that MbfA confers resistance to $H_2O_2$ stress, suggesting that it may play an important role in plant-pathogen interaction[125]. Moreover, *mbfA* expression was induced in response to high iron conditions as sensed by the iron response regulator protein, Irr[125]. However, these results could not distinguish between a role for MbfA in sequestration of iron (through its ferritin domain) or iron efflux. A follow up study revealed that MbfA is important for resistance to iron intoxication under acidic conditions (pH 5.5), which enhances iron solubility thereby promoting toxicity[126]. Compared to wild-type, an *mbfA* null mutant had a modest increase in intracellular total iron

as well as labile iron[125]. Since its expression is induced by high iron under acidic conditions[125], and leads to reduced intracellular iron levels, MbfA was postulated to function as an iron efflux transporter[125].

*Bradyrhizobium japonicum* also encodes an MbfA protein implicated in iron efflux[127]. *B. japonicum* is a nitrogen-fixing endosymbiotic microbe that, like *A. tumefaciens*, belongs to the *Rhizobiales* lineage. As in *A. tumefaciens*, iron homeostasis in *B. japonicum* is also under control of Irr[128], which regulates iron uptake, storage, and utilization[129]. MbfA in *B. japonicum* is specifically induced by high iron and confers resistance to iron intoxication and $H_2O_2$ stress. Moreover, an *mbfA* null mutant accumulates significantly high levels of iron.

Collectively, these data support the idea that MbfA functions physiologically as an iron efflux transporter[127]. Interestingly, the N-terminal ferritin-like domain located on the cytoplasmic side of inner membrane is required for iron transport activity and stress resistance. The purified ferritin domain forms a dimer in solution, which suggests that MbfA may dimerize to form a functional channel[127]. By mediating the efflux of $Fe^{2+}$, MbfA functions cooperatively with bacterioferritin (Bfr), which functions in iron sequestration, to prevent iron intoxication[130]. Mutation of either *mbfA* or *bfr* increases $Fe^{2+}$ sensitivity, but a double *mbfA bfr* mutant is extremely sensitive to iron[130].

## Conclusions

Efflux systems play a central role in the resistance of bacteria to heavy metals, but their role in iron homeostasis has been relatively slow to emerge. This is perhaps a reflection of the fact that iron limitation is a far more prevalent challenge for bacteria than iron intoxication[131], due in part to the very low solubility of iron under aerobic conditions of near neutral pH. Recent results, however, have greatly expanded our appreciation of the central importance of iron efflux systems and their contribution to virulence in human pathogens[27, 37, 38]. This implies that iron intoxication imposes a selective pressure during infection, although how this arises is not yet clear. For example, iron intoxication may arise from an uncontrolled influx of iron into the cell from the outside. Indeed, it is thought that macrophages impose $Zn^{2+}$ and $Cu^{+}$ toxicity on engulfed bacteria by import of metals into the phagolysosome[132]. However, iron is not known to be imported into the phagocytic vacuole. Iron overload may also result when bacteria exposed to an iron limited environment, and therefore expressing high affinity uptake systems, transition to an iron-rich environment. The sudden influx of iron may then be best accommodated by storage or efflux. Alternatively, or in addition, iron intoxication may arise from within the cell. For example, oxidative stress may lead to the release of iron from abundant iron-sulfur and mononuclear iron enzymes, thereby leading to an increase in cytosolic iron levels.

Iron intoxication may also be present in specific environments. For example, acidophilic bacteria grow in low pH environments where iron concentrations may be $10^{18}$ times higher than that found in pH neutral environments[133]. In the case of iron-respiring bacteria, high local concentrations of $Fe^{2+}$ may be produced by reduction of $Fe^{3+}$-containing minerals[101].

Further work is needed to better define the prevalence of iron intoxication in natural environment settings and the role of iron efflux in these environments.

With the identification of the several families of iron efflux systems noted here, the stage is now set for further structural, biochemical and genetic studies to address their mechanisms of metal selectivity. It is presently unclear how these efflux transporters discriminate $Fe^{2+}$ from competing substrates and how, at a structural level, efflux is coupled to substrate binding and energy consumption. It is also unclear why some cells rely on ATP-dependent P-type transporters and others utilize CDF proteins, which are coupled to the proton motive force. It is notable that in several cases efflux pumps were initially assigned a role for substrates others than $Fe^{2+}$ (PfeT, FrvA, CtpD), and in other cases (FieF, Nia) the most relevant physiological substrate is still unclear. This highlights the fact that metal selectivity cannot be easily predicted from protein sequence alone, and biochemical assays need to be interpreted in context of the physiology of the organisms. In several of the cases described, the most compelling evidence to assign function has emerged from a careful analysis of mutant phenotypes combined with detailed analysis of regulation to infer those conditions that specifically induce expression.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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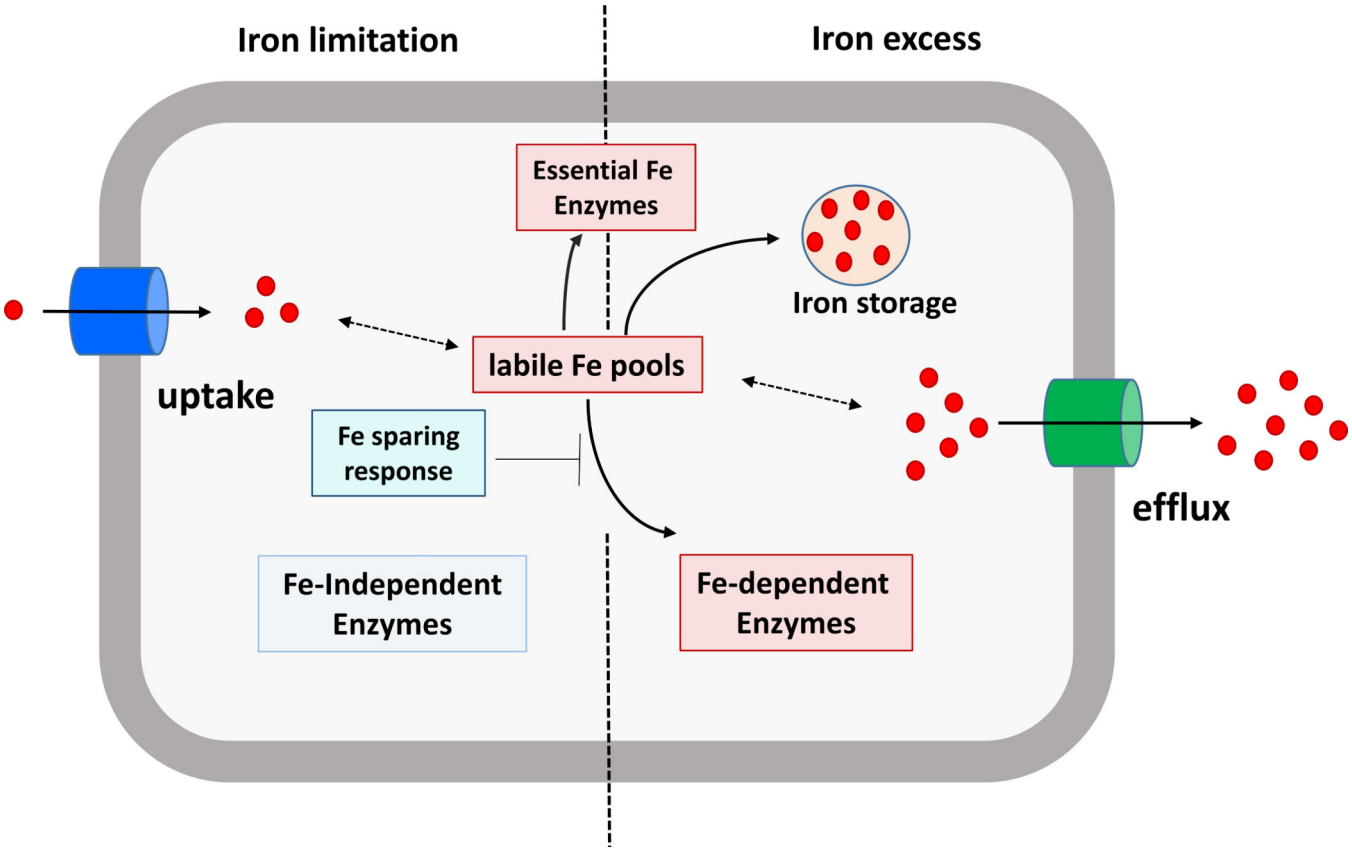


**fig. 1. Iron homeostasis in bacteria**

Under iron deficient conditions (left), high affinity iron uptake systems are induced to scavenge iron from the surroundings to maintain the cell's labile iron pool. when iron is limiting, it is selectively partitioned to the most essential functions and incorporation into lower priority iron enzymes is translationally inhibited as part of an iron sparing response. in many cases, iron-independent enzymes may be derepressed to replace functions that would otherwise depend on iron. under iron excess conditions, the cell will have a full complement of iron-requiring enzymes, and iron in excess of immediate needs will be either stored for future use or exported by $fe^{2+}$ efflux transporters to prevent iron overload.

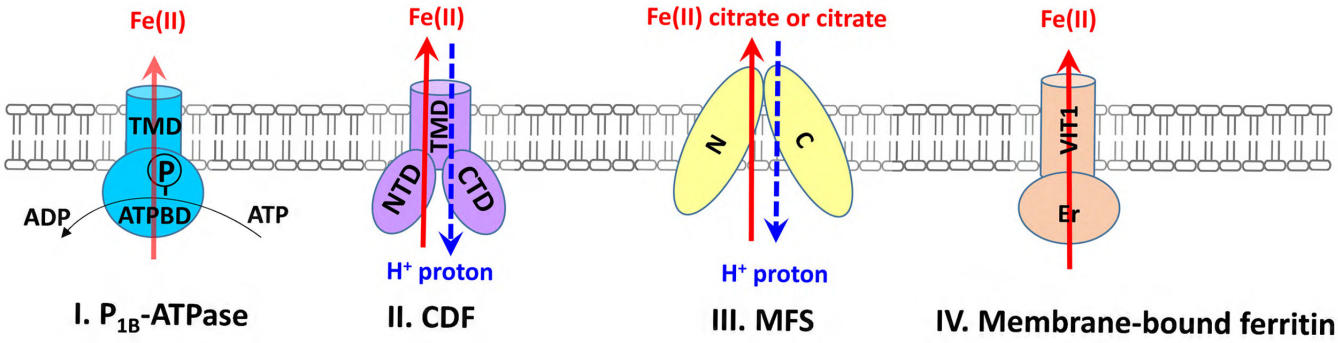


**fig. 2. Ferrous iron efflux systems in bacteria**

Four different groups of transporters can function as $fe^{2+}$ efflux pumps. i. $p_{1b}$-atpase; ii. cation diffusion facilitator (cdf); iii. major facilitator superfamily (mfs); iv. membrane-bound ferritin. a typical $p_{1b}$-atpase consists of a transmembrane domain (tmd) that has 6–8 helices, a soluble actuator domain (not shown), and an atp-binding domain (atp-bd)[52]. a cdf transporter contains a n-terminal domain (ntd), a transmembrane domain (tmd) that has 6 helices, a histine-rich interconnecting loop (il) between tm4 and tm5 (not shown), and a c-terminal cytoplasmic domain (ctd)[75]. the common structural fold (mfs fold) of a mfs transporter is composed of two distinct domains, n domain and c domain. each domain has six consecutive transmembrane helices[103]. a membrane-bound ferritin transporter has two major domains, n-terminal ferritin-like or er domain (er) and c-terminal membrane-embedded vacuolar iron transporter domain (vit1).

**Table 1**

$Fe^{2+}$ efflux transporters in bacteria.

| Protein | Organism | Function | Category | Substrate specificity* | Transcription regulation | References |
|---|---|---|---|---|---|---|
| PfeT | *Bacillus subtilis* | $Fe^{2+}$ efflux | $P_{1B4}$-type ATPase | $Fe^{2+}$ *a, b*, $Co^{2+}$ *a* | PerR & Fur | 36 |
| FrvA | *Listeria monocytogenes* | $Fe^{2+}$ efflux | $P_{1B4}$-type ATPase | $Fe^{2+}$ *a, b*, $Co^{2+}$ *a*, $Zn^{2+}$ *a* | PerR & Fur | 27 |
| CtpD | *Mycobacterium tuberculosis* | $Fe^{2+}$ efflux | $P_{1B4}$-type ATPase | $Fe^{2+}$ *a, b*, $Co^{2+}$ *a* | Unknown | 37 |
| PmtA | group A *Streptococcus* | $Fe^{2+}$ efflux | $P_{1B4}$-type ATPase | $Fe^{2+}$ *b* | PerR | 38, 39 |
| Nia | *Sinorhizobium meliloti* | $Fe^{2+}$ or $Ni^{2+}$ efflux | $P_{1B5}$-type ATPase | $Fe^{2+}$ *a, b*, $Ni^{2+}$ *a, b* | Unknown | 73 |
| FieF (YiiP) | *Escherichia coli* | $Fe^{2+}$ or $Zn^{2+}$ efflux | CDF family | $Fe^{2+}$ *b*, $Zn^{2+}$ *a*, $Cd^{2+}$ *a* | Unknown | 92–94 |
| AitP | *Pseudomonas aeruginosa* | $Fe^{2+}$ or $Co^{2+}$ efflux | CDF family | $Fe^{2+}$ *b* | Unknown | 97 |
| FeoE | *Shewanella oneidensis* | $Fe^{2+}$ efflux | CDF family | $Fe^{2+}$ *b* | Unknown | 101 |
| IceT | *Salmonella typhimurium* | $Fe^{2+}$ citrate or citrate efflux | MFS family | $Fe^{2+}$ *b* | BaeSR | 120 |
| MbfA | *Agrobacterium tumefaciens* | $Fe^{2+}$ efflux | Membrane bound ferritin | $Fe^{2+}$ *b* | Irr | 125 |
| MbfA | *Bradyrhizobium japonicum* | $Fe^{2+}$ efflux | Membrane bound ferritin | $Fe^{2+}$ *b* | Irr | 127 |

*Note: the substrate specificity of the transporters is either based on biochemical measurements (a), inferred from physiology studies (b), or both (a, b).