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PfeT, a P_{1B4}-type ATPase, effluxes ferrous iron and protects *Bacillus subtilis* against iron intoxication

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Summary

Iron is an essential element for nearly all cells and limited iron availability often restricts growth. However, excess iron can also be deleterious, particularly when cells expressing high affinity iron uptake systems transition to iron rich environments. *Bacillus subtilis* expresses numerous iron importers, but iron efflux has not been reported. Here, we describe the *B. subtilis* PfeT protein (formerly YkvW/ZosA) as a P_{1B4}-type ATPase in the PerR regulon that serves as an Fe(II) efflux pump and protects cells against iron intoxication. Iron and manganese homeostasis in *B. subtilis* are closely intertwined: a *pfeT* mutant is iron sensitive, and this sensitivity can be suppressed by low levels of Mn(II). Conversely, a *pfeT* mutant is more resistant to Mn(II) overload. *In vitro*, the PfeT ATPase is activated by both Fe(II) and Co(II), although only Fe(II) efflux is physiologically relevant in wild-type cells, and null mutants accumulate elevated levels of intracellular iron. Genetic studies indicate that PfeT together with the ferric uptake repressor (Fur) cooperate to prevent iron intoxication, with iron sequestration by the MrgA mini-ferritin playing a secondary role. Protection against iron toxicity may also be a key role for related P_{1B4}-type ATPases previously implicated in bacterial pathogenesis.

Introduction

Iron homeostasis is a highly regulated process controlled both by iron availability and by reactive oxygen species such as hydrogen peroxide (H₂O₂). In *B. subtilis*, the ferric uptake repressor (Fur) protein is the primary sensor of intracellular iron levels and directly represses several operons encoding iron import functions (Bsat *et al.*, 1998, Ollinger *et al.*, 2006). In addition, Fur also indirectly activates the expression of some abundant iron-containing enzymes under conditions of iron sufficiency (Smaldone *et al.*, 2012a, Smaldone *et al.*, 2012b). *B. subtilis* also exhibits a complex adaptive response to peroxide stress that is coordinated by three transcription factors, PerR, OhrR and σ^B (Helmann *et al.*, 2003). Low levels of H₂O₂ inactivate the iron-containing repressor protein PerR leading to the

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derepression of enzymes for peroxide detoxification as well as iron storage. Higher levels of H₂O₂ additionally induce the general stress σ^B regulon, whereas the OhrR-regulated *ohrA* gene responds selectively to organic peroxides.

PerR is a member of the Fur family of metalloregulatory proteins and requires a metal cofactor to bind with high affinity to its operator sites (Herbig & Helmann, 2001, Lee & Helmann, 2007). Both structural and biochemical studies support a model in which PerR functions as a dimer with each monomer containing a structural Zn(II) ion and a second, regulatory metal ion (Lee & Helmann, 2006a, Ma *et al.*, 2011). PerR can be activated to bind DNA by either Fe(II) or Mn(II), which lead to the PerR:Zn,Fe and PerR:Zn,Mn forms of the repressor, respectively. Importantly, only the PerR:Zn,Fe form responds to H₂O₂ (Lee & Helmann, 2006b). Exposure to H₂O₂ leads to oxidation of the bound Fe(II) atom and results in the oxidation of one of two histidine ligands (H37 or H91) that serve to coordinate the iron (Lee & Helmann, 2006b, Traore *et al.*, 2009). The resulting conformational change leads to derepression of the PerR regulon.

PerR functions in the adaptive response to H₂O₂ in which low levels of H₂O₂ induce a regulon including those enzymes (the KatA catalase and AhpCF alkylhydroperoxide reductase) that directly detoxify H₂O₂ (Bsat *et al.*, 1998, Chen *et al.*, 1995). This response thereby protects cells against challenge with higher doses of H₂O₂ (Duarte & Latour, 2010, Zuber, 2009). In addition to its role as the primary regulator of KatA and AhpCF, PerR also regulates expression of proteins that have direct impacts on cellular iron levels (Faulkner & Helmann, 2011, Faulkner *et al.*, 2012). These include the MrgA mini-ferritin, Fur, KatA, and heme biosynthesis enzymes. MrgA is a dodecameric mini-ferritin that sequesters Fe(II), and simultaneously consumes H₂O₂, by oxidation of Fe(II) to form a ferric-hydroxide core inside the spherical protein shell (Chen & Helmann, 1995, Chiancone & Ceci, 2010). Finally, PerR regulates enzymes for heme biosynthesis that are co-induced with the heme-requiring catalase (KatA) and are needed to support high levels of catalase activity.

The impact of the PerR regulon on iron homeostasis is highlighted by the severe growth defects of a *perR* null mutant, which have been ascribed to iron deficiency (Ma *et al.*, 2012, Faulkner *et al.*, 2012). The two main contributors to iron deficiency in the absence of PerR are the very high levels of catalase protein, which creates a high demand for its iron-containing heme cofactor, and increased expression of the Fur repressor. When iron is deficient, Fur is expected to be inactive as a repressor, and therefore derepress iron uptake functions. However, in the *perR* mutant the increased Fur protein levels result in Fur acting as a repressor that is now cofactored by the ambient levels of Mn(II) in the cell (Ma *et al.*, 2012). This results in the inappropriate repression of iron uptake, despite iron deficiency.

We previously reported that PerR also regulates ZosA/YkvW (Gaballa & Helmann, 2002), a P_{1B4}-type ATPase, here renamed as PfeT. Unlike other PerR regulon members, the *pfeT* regulatory region is notable for the presence of both Fur and PerR boxes, suggesting that this gene may be regulated by both available Fe(II) and H₂O₂ (Fuangthong & Helmann, 2003). In addition, like some other members of the PerR regulon, repression of *pfeT* by PerR is primarily mediated by PerR:Zn,Mn and not PerR:Zn,Fe (Fuangthong *et al.*, 2002). Based on our prior studies, we proposed that YkvW might function in Zn(II) import in response to

oxidative stress, and we therefore renamed the protein as ZosA (Gaballa & Helmann, 2002). Indeed, null mutants do display increased growth under zinc excess conditions (as confirmed here). However, whether or not this was due to alterations in Zn(II) transport was not clear. Moreover, P_{1B}-type ATPases are most frequently implicated in efflux rather than import, and the few characterized members of the P_{1B4} subfamily of ATPases are implicated in efflux of Co(II) (Argüello *et al.*, 2011). We therefore set out to reexamine the role of YkvW/ZosA in metal ion homeostasis in *B. subtilis*. As reported herein, this P_{1B4}-ATPase effluxes ferrous iron and is therefore now renamed as a peroxide-induced ferrous efflux transporter, PfeT.

Results and Discussion

A *pfeT* mutation increases sensitivity to Fe(II) and Fe(III) salts

B. subtilis PfeT is a member of the P_{1B4}-subfamily of P-type ATPases, which are generally classified as Co(II)-efflux pumps (Argüello, 2003). Typically, mutations in metal ion efflux systems lead to an increased sensitivity to the transported metal ions. Metal ion sensitivity can be conveniently assayed using a disk diffusion (zone of inhibition) assay, which is a reflection of the maximal permissive concentration (MPC) for growth. Compared to wild-type (WT), the *pfeT* null mutant strain displayed a significantly increased sensitivity to Fe(II) and Fe(III) (Fig. 1A). In contrast, there was no apparent change in sensitivity to Zn(II) or Co(II), and a small but significant decrease in sensitivity to Mn(II), as monitored by this assay. The elevated sensitivity to iron salts is apparent both as an increased zone of growth inhibition (Fig. 1A) and, in the case of Fe(II), as a large zone of reduced cell density that is often observed after 24-72 h of incubation (Fig. 1B). Inspection of cell density over time suggests that this secondary zone is largely a result of increased cell lysis. These results indicate that the *pfeT* null mutant has reduced fitness under conditions of iron excess.

The sensitivity of the *pfeT* null mutant to iron excess is also apparent in an efficiency of plating (EOP) assay in which mid-logarithmic phase cells (OD₆₀₀ ~0.4) are serially diluted and spotted on LB medium amended with Fe(II) (Fig. 1C). Both WT and the *pfeT* null mutant form colonies with high efficiency up to a concentration of 2 mM Fe(II), but the *pfeT* null mutant strain forms very small colonies, indicative of a reduced growth rate, under this condition. With 3 mM Fe(II), the EOP of WT is reduced and the *pfeT* null strain is unable to form colonies.

A *pfeT* mutation increases sensitivity to the Fe(II)-activated antibiotic streptonigrin

Streptonigrin (SN) is a quinone antibiotic whose activity is correlated with intracellular iron availability (Yeowell & White, 1982). In order to compare the levels of intracellular free iron between WT and *pfeT*, the sensitivity to SN was tested by both disk diffusion and growth curve assays. The *pfeT* mutant displayed a significantly increased SN sensitivity on LB plates amended with 100 µM FeSO₄. In contrast, only a slight increase in SN sensitivity was noted on LB plates, and the *pfeT* mutation had no effect on sensitivity on LB plates amended with 2, 2'-dipyridyl (DP), a cell membrane-permeable iron chelator (Fig. 2). These results suggest that deletion of *pfeT* led to an increase in intracellular iron levels, particularly in an iron-rich medium. Consistent results were noted in liquid culture growth experiments:

the *pfeT* mutant displayed increased SN sensitivity, and this was enhanced in medium amended with FeSO₄ (**Fig. S1**).

Cells lacking PfeT accumulate elevated levels of intracellular iron

To test directly the effects of PfeT on intracellular metal ion levels, we challenged cells in LBC medium with 4 mM FeSO₄, and monitored metal ion levels by inductively coupled plasma mass spectrometry (ICP-MS). For this experiment we used a *pfeT* null mutant complemented with an IPTG-inducible copy of the *pfeT* gene. In samples taken between 1 and 15 min after iron addition there was a large increase in intracellular iron in the absence of IPTG. However, when the same strain was grown in the presence of 1 mM IPTG there was relatively little iron accumulation (**Fig. 3**). Note that prior to iron addition, the levels of Mn(II) were ~5-fold lower than iron under these conditions and were largely unchanged over the course of the experiment.

PfeT is an Fe(II)- and Co(II)-activated ATPase

The above results are most simply explained by postulating that PfeT functions physiologically to efflux iron from cells. Since the dominant form of iron in the reducing environment of the cytoplasm is Fe(II), we anticipated that PfeT might function to efflux Fe(II). P-type ATPases couple ATP hydrolysis to metal ion transport. Consequently, monitoring metal ion activated ATPase activity provides a convenient method to survey substrate selectivity (Argüello *et al.*, 2007). The *B. subtilis* PfeT protein was overproduced in *E. coli*, solubilized from the membrane fraction, purified by affinity chromatography, and reconstituted in micellar form as described previously for other P-type ATPases (Raimunda *et al.*, 2014, Raimunda *et al.*, 2012). The resulting protein was >90% pure (**Fig. S2**).

When the ability of various metal ions to activate the PfeT ATPase was surveyed, the highest activity was observed with Fe(II), with lower activity noted for Co(II). No activation was observed with Fe(III), Ni(II), Zn(II), Mn(II), Cu(II) or Cu(I) (**Fig. 4A**). Fe(II) activated the ATPase most strongly, albeit with a relatively low apparent affinity ($K_{1/2}$ for activation 0.52 ± 0.12 mM). The $V_{\max}$ observed with Fe(II) was 3.25 ± 0.21 $\mu\text{mol mg}^{-1} \text{h}^{-1}$ (**Fig. 4B**), which compares favorably to the reported values for several other metal activated P-type ATPases. For example, a $14 \mu\text{mol mg}^{-1} \text{h}^{-1}$ activity was observed in ZntA, the *E. coli* Zn²⁺-ATPase (Mitra & Sharma, 2001), and P_{1B1}-family Cu(I) efflux ATPases have reported values of 3-4 $\mu\text{mol mg}^{-1} \text{h}^{-1}$ as measured for *Archaeoglobus fulgidus* CopA when activated by Cu(I) (Mandal *et al.*, 2002). In particular, PfeT Co²⁺-ATPase activity is similar to CtpD and CtpJ, P_{1B4}-family ATPases. These had $V_{\max}$ values for Co(II) in the range of ~0.5-1.5 $\mu\text{mol mg}^{-1} \text{h}^{-1}$ (Raimunda *et al.*, 2012, Raimunda *et al.*, 2014). The maximal ATPase activation of PfeT by Co(II) ($V_{\max}$ of 0.70 ± 0.07 $\mu\text{mol mg}^{-1} \text{h}^{-1}$) is substantially lower than for Fe(II) (0.7 vs. 3.3 $\mu\text{mol mg}^{-1} \text{h}^{-1}$). However, the apparent affinity for Co(II) is higher than for Fe(II) with a $K_{1/2}$ of 42 ± 16 μM for Co(II) vs. 520 μM Fe(II) (**Fig. 4B and 4C**).

In our initial survey, we had failed to detect a significant role for PfeT in resistance to Co(II) (**Fig. 1A**). We reasoned that the ability of PfeT to efflux Co(II) may have been largely masked by the presence of other efflux mechanisms, including the CzcD protein. CzcD is a broad specificity cation diffusion facilitator (CDF) protein known to be the primary

determinant of Co(II) resistance in *B. subtilis* (Moore *et al.*, 2005, Guffanti *et al.*, 2002). As expected, deletion of *czcD* significantly increased Co(II) sensitivity, but there was no increase in sensitivity when *pfeT* was additionally deleted (**Fig. 5**). However, when PfeT was expressed from an IPTG-inducible promoter in the *czcD* null mutant strain, resistance to Co(II) was largely restored. Indeed, increased resistance was noted even in the absence of IPTG induction, likely due to leaky expression from the P_{spac} promoter. This indicates that PfeT can serve as an efflux pump for Co(II) *in vivo*, but that this activity is unlikely to be significant under physiological conditions due to (i) the much greater activity of the CzcD transporter and (ii) poor expression of the *pfeT* gene under these conditions. The *pfeT* gene is known to be repressed by PerR (Gaballa & Helmann, 2002). Although, PerR-regulated genes are often repressed in response to Mn(II) and Fe(II), they can also be repressed by Co(II) (Chen *et al.*, 1993).

A *pfeT* mutation modestly improves growth under high Zn(II) stress

Previously, PfeT was reported to affect Zn(II) homeostasis as judged by an increase in cell growth in the presence of excess Zn(II) in strains containing a *pfeT* mutation (Gaballa & Helmann, 2002). In addition, the *pfeT* mutation was reported to reduce intracellular Zn(II) accumulation, consistent with a postulated role in Zn(II) import (Gaballa & Helmann, 2002). However, Zn(II) does not activate the PfeT ATPase (**Fig. 4A**). We therefore re-investigated the effects of the *pfeT* mutation on Zn(II) sensitivity, both in WT and in efflux defective strains, using both disk diffusion (**Fig. S3A**) and growth assays (**Fig. S3B-F**). A *pfeT* null mutation did not lead to a significant change in the MPC for Zn(II) (**Fig. 1A**), and similar results were seen in strains defective for either or both Zn(II) efflux systems (*pfeT cadA*, *pfeT czcD*, and *pfeT cadA czcD*; **Fig. S3A**). However, the *pfeT* mutant did grow better under high Zn(II) stress conditions in liquid medium. This effect was most notable when comparing a *perR* null strain, in which *pfeT* is expressed constitutively, against a *perR pfeT* double mutant (**Fig. S3B**). However, effects were also noted in the WT (**Fig. S3C**) and *cadA* mutant backgrounds (**Fig. S3D**). The exception is a *cadA czcD* double mutant (defective for Zn(II) efflux) (**Fig. S3E-S3F**), which displayed a greatly increased Zn(II) sensitivity (**Fig. S1A**) (Ma *et al.*, 2014).

We conclude that mutation of *pfeT* does influence Zn(II) tolerance (Gaballa & Helmann, 2002), but in light of the results reported herein this is likely an indirect effect of alterations in intracellular Fe(II) levels. The molecular basis of Zn(II) toxicity in bacteria is poorly understood, but under conditions of oxidative stress Zn(II) may competitively inhibit binding of Fe(II) to mononuclear enzymes and inhibit their activity (Imlay, 2014, Sobota *et al.*, 2014). We can therefore speculate that elevated cytosolic iron, as a consequence of the *pfeT* mutation, may help prevent enzyme mismetallation by Zn(II). This mechanism may suffice to partially suppress Zn(II) toxicity in cells that are also competent for Zn(II) efflux, but may be insufficient in efflux defective cells.

PfeT reduces Fe(II)-dependent cell killing and facilitates adaptation to Fe(II) excess

To further characterize the effects of *pfeT* on iron intoxication we sought to develop a robust and reproducible growth assay using a Bioscreen growth analyzer. To reduce the precipitation of insoluble ferric hydroxides (which can interfere with optical density

measurements of the Bioscreen growth analyzer), we amended LB medium (with a basal level of ~8-10 μM Fe; Ollinger *et al.*, 2006) with 1 g per liter of citrate trisodium dihydrate (3.4 mM). This is the same concentration of citrate used in metal-limiting minimal medium in previous studies of iron physiology (Chen *et al.*, 1993, Gaballa *et al.*, 2008, Ollinger *et al.*, 2006, Smaldone *et al.*, 2012b), and was shown previously to allow increased iron availability (as judged by repression of the Fur regulon) when added to M9 medium (Smaldone *et al.*, 2012b). In this medium (designated LBC), cells tolerate high levels of FeSO_4 (up to 3 mM), but when the Fe(II) concentration surpasses that of citrate, iron toxicity results and WT cells only resume growth after a long lag phase (**Fig. 6A**). In this medium, *pfeT* mutant cells are unable to grow in the presence of 4 mM FeSO_4 (**Fig. 6B**).

The long lag phase in this assay is due, in part, to a significant loss of viability upon dilution of mid-logarithmic phase ($\text{OD}_{600} \sim 0.4$) cells into the LBC + 4 mM Fe(II) medium. To estimate the extent of killing under these conditions, we estimated the concentration of viable cells by spot dilution onto LB medium prior to and 30 and 60 min after amendment with 4 mM Fe(II) (**Fig. S4**). We estimate that the viable cells counts for WT decrease by $\sim 10^4$ to 10^5 -fold under these conditions, and there is an even larger decrease for the *pfeT* null mutant. Significantly, cells in which *pfeT* was induced from an IPTG-inducible promoter prior to Fe(II) shock are able to survive this treatment with relatively little loss of viability.

To monitor cell recovery after Fe(II) shock under conditions directly comparable to the Bioscreen experiments, we diluted cells 100-fold into LBC medium with and without 4 mM Fe(II) and monitored viable cell counts over time. As expected from the Bioscreen results (**Fig. 6**) and the cell killing assay (**Fig. S4**), there was a large decrease in viable cell counts after dilution into the Fe(II)-amended medium with cell density recovering after 9 h (**Fig. 7**). No recovery was seen for the *pfeT* null mutant up to 15 h. In contrast, a *perR* null strain (constitutively expressing *pfeT*) experienced comparatively little cell killing (~ 100 -fold) and resumed growth immediately. The ability of the *perR* strain to survive Fe(II) intoxication was dependent on PfeT: the *perR pfeT* double mutant was unable to adapt and failed to resume growth within 15 h (**Fig. 7B**). We suggest that the resumption of growth under these conditions of Fe(II) intoxication requires efflux of Fe(II) from the cytosol, rather than modification of the growth medium by the cells. In cultures inoculated with both WT and *pfeT* mutant cells, only the WT cells are recovered after outgrowth (data not shown).

Sensitivity of cells to Fe(II) intoxication is pH dependent

In a series of further experiments (see **Fig. S5-S7**), we systematically investigated the effects of variations in FeSO_4 , citrate, and pH on cell growth as monitored using the Bioscreen assay. In LB medium amended with 4 mM FeSO_4 neither WT nor the *pfeT* null mutant are able to grow in the absence of citrate (**Fig. S5**). With citrate amended at 1 g l^{-1} , WT resumes growth after an extended (~ 10 h) lag, whereas the *pfeT* null mutant is unable to grow (consistent with **Fig. 6 and 7**).

One key finding from these studies is that iron intoxication is highly sensitive to pH: the observed toxicity (**Figs. 6 and 7**) is due to both the high iron concentration (added from a

100 mM FeSO₄ stock in 0.1 N HCl) and a reduction of the pH of the growth medium to ~5.7 upon addition of FeSO₄ to 4 mM. We serendipitously observed that if Fe(II) is added from a 1 M stock (and therefore less HCl is added), there is comparatively little growth inhibition (**Fig. S6**). This motivated studies to define the pH dependence of iron intoxication. In LBC medium amended with 4 mM FeSO₄ and buffered using 60 mM 2-(N-morpholino)ethanesulfonic acid (MES; pKa 6.1), iron intoxication increases substantially as the pH is reduced below 7, and the effect of PfeT is most notable when the pH is less than 6.5 (**Fig 8A, 8B**). Indeed, when the pH is 5.7, growth is largely dependent on PfeT. Presumably, the increased solubility of Fe(II) at low pH, and perhaps increased uptake, increase Fe(II) intoxication. Indeed, in LB medium buffered to pH 5.7, PfeT contributes significantly to Fe(II) tolerance even in the absence of citrate (**Fig. S7**).

Mn(II) suppresses Fe(II) intoxication

As noted above (**Fig. 6**), the *pfeT* null mutant cannot grow under conditions of Fe(II) intoxication (4 mM FeSO₄ in LBC medium) whereas WT grows, but only after an extended lag phase. The molecular origins of the growth inhibition are not yet understood, but presumably reflect high intracellular levels of Fe(II) that interfere with essential cell processes. One likely target of Fe(II) toxicity is interference with the proper metallation of enzymes requiring other metals as cofactors (Imlay, 2014, Cotruvo & Stubbe, 2012). In general, Fe(II) and Mn(II) are both maintained at homeostatic levels of ~10⁻⁵ to 10⁻⁴ M in the cytoplasm, as judged by the metal-binding affinity of their cognate uptake regulators (Fur and MntR), and both ions compete for metallation of many mononuclear metalloenzymes (Helmann, 2014). Thus, we hypothesized that inhibition of one or more Mn(II)-dependent enzymes could contribute to Fe(II) toxicity. Consistent with this notion, supplementation of LBC medium with as little as 2.5 μM Mn(II) enabled growth of the *pfeT* mutant strain with a lag phase only slightly longer than for WT under comparable conditions, and both the WT and the *pfeT* mutant strains responded to elevated Mn(II) with a substantial reduction in growth lag (**Fig. 8C, 8D**).

A *pfeT* null mutant is more resistant to Mn(II) intoxication

In our initial survey of metal sensitivity (**Fig. 1A**), we noted that a *pfeT* null mutant was slightly more resistant to MnCl₂ as measured in a disk diffusion assay. Consistent with this observation, in LB medium supplemented with 2 mM MnCl₂ WT is unable to grow, while the *pfeT* null mutant does grow, albeit with a lag phase of ~8 h. Similarly, the lag phase for growth in LB supplemented with 1 mM MnCl₂ is reduced ~2-fold in a *pfeT* mutant strain (**Fig. 9A vs. 9B**). As demonstrated previously, an *mntR* mutant strain constitutively expresses both the MntH and the MntABC Mn(II) uptake systems and is highly sensitive to Mn(II) intoxication (Que & Helmann, 2000). In an *mntR* mutant background, deletion of the gene encoding PfeT also improves fitness (**Fig. 9C vs. 9D**). These phenotypes suggest that deletion of *pfeT* protects against Mn(II) intoxication, presumably by leading to an elevation of intracellular Fe(II) levels even in LB medium lacking iron supplementation. This is consistent with the increased SN sensitivity observed in unsupplemented LB medium (**Figs. 2 and S1A**). Further, this supports a model in which Mn(II) intoxication results from the competitive inhibition of one or more Fe(II)-dependent processes or enzymes in the cell.

Indeed, recent results in *E. coli* have identified ferrochelatase as a target of Mn(II) toxicity in that organism (Martin *et al.*, 2015).

PerR regulated genes critical for protection against Fe(II) intoxication

To determine if other PerR regulon members also play a role in resistance to Fe(II) intoxication we analyzed the epistatic interactions between *perR*, *pfeT*, and other PerR regulon members (Fig. 10). As expected, under conditions of Fe(II) intoxication (LBC medium with 4 mM FeSO₄), WT grows (albeit after a long lag), the *pfeT* mutant is unable to grow, and this defect is complemented by ectopic expression of *pfeT*. Note that in this experiment, IPTG was not included in the preculture, so the induction of *pfeT* occurs concomitant with exposure to high Fe(II) and this eventually enables adaptation of the surviving cells and outgrowth of the culture. This is comparable to what happens in the WT strain, where *pfeT* expression is also induced by high Fe(II) conditions (data not shown).

Remarkably, the *perR* null mutant grows very well under these conditions with only a ~1 h lag (Fig. 10A). We conclude that derepression of the PerR regulon greatly enhances resistance to Fe(II) intoxication, consistent with the results observed by spot dilution (Fig 7C). The resistance of the *perR* null mutant to Fe(II) intoxication is due almost entirely to derepression of PfeT as evidenced by comparison of the *perR* null strain and the *perR pfeT* double mutant (Fig. 10A and 7B). We infer that the relatively high level of survival and rapid adaptation of the *perR* mutant strain is likely due to the presence of comparatively high levels of PfeT in the precultures. This is consistent with the observation that induction of PfeT from an IPTG-inducible promoter prior to challenge also greatly reduces cell killing (Fig. S4).

In addition to PfeT, derepression of other members of the PerR regulon also provides some advantage to cells experiencing Fe(II) intoxication. The lag phase of the *perR* null mutant (~1 h) is increased in strains additionally containing mutations in *mrgA* (~1.5 h), *kata* (~3 h), or both *kata* and *ahpCF* (~6 h), but in all cases these strains still grow better than WT (~10 h). These results indicate that PfeT plays the primary role in protection against Fe(II) intoxication, whereas other PerR regulon members play a secondary role. This is further apparent from comparison of the *pfeT* strain (unable to grow) and the *pfeT perR* double mutant (growth after a lag of >13 h). This rescue of the *pfeT* growth defect is lost in the *perR pfeT mrgA* triple mutant (Fig. 10A) and in the *perR pfeT kata ahpCF* quadruple mutant (Fig. 10B). In the absence of the PfeT efflux ATPase, the MrgA mini-ferritin (Dps family protein) presumably contributes to fitness by virtue of its ability to sequester iron.

PerR regulated genes critical for protection against H₂O₂ stress

The PerR regulon is widely appreciated for its role in inducible resistance to H₂O₂ (Faulkner & Helmann, 2011, Zuber, 2009). We considered the possibility that adding high concentrations of Fe(II) to aerobic LBC medium might lead to the generation of reactive oxygen species (ROS) (King *et al.*, 1995), and that Fe(II) intoxication might simply be a manifestation of stress imposed by ROS. However, direct measurements of H₂O₂ levels in our LBC medium with and without amendment with 4 mM FeSO₄ failed to reveal a significant increase; under all measured conditions, the ambient levels of H₂O₂ were in the

range of 2-3 μM , consistent with prior measurements of aerobic LB medium (Seaver & Imlay, 2001). Under these conditions, excess Fe(II) likely catalyzes the decomposition of H_2O_2 (Fenton reaction) with formation of hydroxyl radicals which are quenched by the organic-rich LBC medium.

Bacterial killing by elevated levels of H_2O_2 is exacerbated by an increase in intracellular labile iron pools (Imlay, 2013). We therefore anticipated that PfeT, which is known to be induced by H_2O_2 (Gaballa & Helmann, 2002; Helmann *et al.*, 2003; Mostertz, *et al.*, 2004), might be an important resistance determinant for H_2O_2 . To determine if PfeT plays a significant role in H_2O_2 resistance, we measured sensitivity using a disk diffusion assay in a series of mutant strains lacking PfeT or other members of the PerR regulon, as well as the σ^{B} -regulated Dps protein which functions, like MrgA, as an Fe-sequestration protein (**Fig. S8**). The results indicate that PfeT plays a comparatively minor role in H_2O_2 resistance, with small effects noted only in the *dps* null mutant and the *katA ahpCF mrgA* triple mutant backgrounds. The data further highlight the importance of PerR-regulated genes in inducible H_2O_2 resistance and demonstrate, as previously reported (Gaballa & Helmann, 2002), that catalase is the primary determinant for resistance to high levels of H_2O_2 . Together, these results indicate that Fe(II) intoxication and H_2O_2 intoxication, although physiologically linked, are distinct stresses with different proteins playing the most central roles in adaptation. Indeed, PfeT can efficiently protect cells against Fe(II) intoxication even in cells lacking the major H_2O_2 detoxification enzymes, KatA and AhpCF (**Fig. 10B**; e.g. compare *perR katA ahpCF* vs. *perR katA ahpCF pfeT*).

Genetic interactions between PfeT and the Fur regulon: PfeT and Fur cooperate to prevent Fe intoxication

Next, we investigated the genetic interactions between PfeT, a PerR-regulated iron efflux system, and the Fur-regulated iron uptake systems. Since we anticipated that the *fur* mutation would exacerbate the growth defect of the *pfeT* null mutant, we reduced the level of iron intoxication by using LBC medium amended with 3.5 mM FeSO_4 (**Fig. 6**). Whereas WT grows well in this medium, both the *pfeT* and *fur* single mutants grow after an ~5 h lag (**Fig. 11**). These effects are additive, with the *pfeT fur* double mutant growing after a lag of over 10 h.

To test the hypothesis that the *fur* mutation was acting by derepressing iron uptake functions, we mutated the genes encoding the major iron-citrate uptake system (encoded by the *yfmC* operon) and the elemental iron uptake system (encoded by the *ywbL* operon; also known as *efeU*; Miethke *et al.*, 2013), shown previously to be the major iron uptake pathways in LB medium (Faulkner *et al.*, 2012). Note that *B. subtilis* 168 strains do not make a functional bacillibactin siderophore due to the presence of an *sfp*⁰ mutation (Quadri *et al.*, 1998). The growth results indicate that elimination, either individually or in combination, of the iron-citrate and the elemental iron uptake pathways improves growth, but these two systems do not completely account for the effect of the *fur* mutation on iron sensitivity (**Fig. 11**).

The roles of the PerR and Fur regulators in iron homeostasis are also apparent using spot dilution assays (**Fig. S9**). In this assay, the *fur* and *pfeT* null mutants both form very small colonies on LB medium amended with 2 mM FeSO₄, whereas the *pfeT fur* double mutant has a low EOP under these conditions, and forms small colonies on plates containing 1 mM FeSO₄. These additive effects are consistent with the notion that iron intoxication is most severe in cells that are unable to repress iron uptake, due to the *fur* mutation, and also lack PfeT. In contrast, the *perR* null mutant (but not the *perR pfeT* double mutant) has a high EOP even on plates with 3 mM FeSO₄, a condition that restricts the growth of WT.

It is notable that the *perR* null mutant displays a small colony size on the unamended LB medium, and better growth on medium amended with 1 mM FeSO₄. This is consistent with prior results demonstrating that this strain is growth-restricted due to iron sequestration by the abundant catalase hemoprotein and repression of iron uptake by the Fur protein which, under these conditions, is mismetallated by Mn(II) (Faulkner *et al.*, 2012, Ma *et al.*, 2012). Although derepression of PfeT, in a *perR* null mutant, increases fitness in high iron (e.g. 3 mM, **Fig. S9**), it does not contribute to the iron limitation that characterizes the *perR* mutant strain. As shown previously, both the *perR* and *perR pfeT* mutants are very slow growing on standard LB medium (containing ~10 μ M iron) (Faulkner *et al.*, 2012). In contrast, deletion of either or both of *kataA* and *fur* dramatically relieve the iron limitation phenotype (Faulkner *et al.*, 2012). We conclude that derepression of PfeT does not contribute significantly to iron deprivation, even when it is constitutively expressed in a *perR* null mutant. This is likely due to the relatively low affinity of this efflux pump for its substrate ($K_{1/2}$ ~0.52 mM, **Fig 4B**), Fe(II), which is calibrated to efficiently pump iron out of the cell under conditions of excess, but is of sufficiently weak affinity so as not to deplete the cytosol of iron.

PfeT homologs are implicated in bacterial virulence

PfeT is the first member of the P_{1B}-type ATPases implicated in iron efflux. Within P_{1B}-type ATPases, it has been possible to predict metal selectivity based on primary amino acid sequence and, in particular, the presence of specific metal-coordinating residues (Argüello, 2003). PfeT resides within the P_{1B4} subgroup of transporters which have been generally considered to function as Co(II) efflux pumps. Indeed, as shown here, PfeT can be activated by Co(II) *in vitro* (**Fig. 4C**) and can confer Co(II) resistance when expression is artificially induced (**Fig. 5**). However, this does not appear to be its normal physiological role. Several other P_{1B4} family ATPases have been characterized and representatives of this sub-family have been implicated in virulence for some bacterial pathogens. In *Listeria monocytogenes*, a PfeT homolog (FrvA) was suggested to enhance resistance to heme and shown to be important for virulence in mice (McLaughlin *et al.*, 2012). In this same study, however, it was also noted that an *frvA* deletion strain had increased sensitivity to FeSO₄ in a zone of inhibition assay. PfeT homologs have also been studied in *Mycobacterium* spp. In this case, one homolog (CtpJ) is inducible by and confers resistance to Co(II) (Raimunda *et al.*, 2014). However, the other homolog, and likely PfeT ortholog, CtpD was induced by redox stress and was shown to be important for survival in the mouse lung and for virulence (Raimunda *et al.*, 2014). We suggest, based on the results herein, that CtpD may function physiologically as an iron exporter, a hypothesis that is currently under investigation.

Conclusions

Although iron uptake mechanisms have been studied since the 1930s, as recently as 1991 Joe Neilands could write that “There seems to be no known biological mechanism for the excretion of iron, uptake of the element being regulated at the membrane level in all species studied” (Neilands, 1991). Iron excretion mechanisms were first appreciated in mammals where ferroportin was identified as a key iron exporter (Donovan *et al.*, 2005). In *E. coli*, the FieF cation diffusion facilitator has been implicated physiologically in iron efflux (Grass *et al.*, 2005), but most detailed characterization has focused on the ability of this system to transport Zn(II) (Gupta *et al.*, 2014, Lu & Fu, 2007). In addition, an ABC transporter has been proposed to function in iron efflux (Nicolaou *et al.*, 2013). In *Salmonella* Typhimurium, a major facilitator superfamily pump designated IceT was shown to efflux iron citrate and contributes to resistance under iron stress conditions (Frawley *et al.*, 2013, Frawley & Fang, 2014). Finally, MbfA proteins containing both a ferritin like domain and a membrane-localized domain related to CCC1 family eukaryotic vacuolar metal transporters have been shown to efflux iron in *Agrobacterium tumefaciens* (Bhubhanil *et al.*, 2014) and *Bradyrhizobium japonicum* (Sankari & O'Brian, 2014). Thus, there is an emerging realization that many bacteria likely have mechanisms to efflux excess iron, analogous to the long-known mechanisms for resistance to other metals.

The studies described herein demonstrate that *B. subtilis* PfeT can be added to the list of transporters that function physiologically to efflux iron from bacterial cells. Our results demonstrate that Fur, by regulation of iron import, and PfeT, required for iron export, cooperate to allow cells to survive and ultimately grow under iron intoxication conditions (Fig. 12). MrgA, a mini-ferritin that sequesters iron, plays a secondary role as revealed in a *perR* null strain that is derepressed for *mrgA* expression. Our results also highlight the complex interactions between iron and manganese homeostasis: increased intracellular iron (in a *pfeT* null strain) leads to greater tolerance to Mn(II) and, conversely, even micromolar levels of Mn(II) protect cells against the effects of iron intoxication. In ongoing studies, we seek to better understand how iron intoxication perturbs cell physiology and ultimately limits growth.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Bacillus subtilis strains used are derivatives of strain CU1065 and are shown in Table S1. *E. coli* strain DH5 α was used for standard cloning procedures. Bacteria were grown in LB medium (10 g tryptone, 5 g yeast extract and 5 g l⁻¹ NaCl) at 37°C with vigorous shaking or on solid LB containing 1.5% Bacto agar with appropriate selection. Where indicated, LBC medium (LB medium amended with 1 g l⁻¹ of citrate trisodium dihydrate; 3.4 mM) was used. Ampicillin (*amp*; 100 μ g ml⁻¹) was used to select *E. coli* transformants. For *B. subtilis*, antibiotics used for selection were: spectinomycin (*spc*; 100 μ g ml⁻¹), kanamycin (*kan*; 15 μ g ml⁻¹), chloramphenicol (*cat*; 10 μ g ml⁻¹), tetracycline (*tet*; 5 μ g ml⁻¹), neomycin (*neo*; 10 μ g ml⁻¹), and macrolide lincosamide-streptogramin B (MLS; contains 1 μ g ml⁻¹ erythromycin and 25 μ g ml⁻¹ lincomycin). For iron intoxication experiments, 100 mM FeSO₄ stocks (except where indicated) were prepared in 0.1 N HCl and iron was added to

the indicated concentrations. OD₆₀₀ readings were taken on a Spectronic 21 spectrophotometer. H₂O₂ concentrations in the growth medium were determined using the Amplex red/horseradish peroxidase method as described (Seaver & Imlay, 2001).

Strain constructions

Gene deletions were generated by replacing the coding region with an antibiotic resistance cassette using long flanking homology PCR (LFH-PCR) followed by DNA transformation as previously described (Mascher *et al.*, 2003). Chromosomal DNA was used for transformation as described previously (Harwood & Cutting, 1990). The IPTG-inducible constructs were generated using vector pPL82 (Quisel *et al.*, 2001). PCR products were amplified from *B. subtilis* CU1065 chromosomal DNA, digested with endonucleases, and cloned into pPL82. pPL82 contains a chloramphenicol resistance cassette, a multiple cloning site downstream of the P_{spac(hy)} promoter, and the *lacI* gene between the up- and downstream fragments of the *amyE* gene. Primer pairs used for PCR amplification are 6170/6171 for *pfeT* (Table S3). The sequences of the inserts were verified by DNA sequencing (Cornell DNA sequencing facility). Plasmids were linearized by ScaI and used to transform *B. subtilis*, where they integrated into the *amyE* locus.

Measurement of maximal permissive concentrations for growth using disk diffusion assays

Disk diffusion assays were performed as described (Mascher *et al.*, 2007). Briefly, strains were grown to an OD₆₀₀ of 0.4. A 100 µl aliquot of these cultures was mixed with 4 ml of 0.75% LB soft agar (kept at 50°C) and directly poured onto LB plates (containing 15 ml of 1.5% LB agar). The plates were dried for 10 min in a laminar airflow hood. Filter paper disks containing 10 µl of the chemicals to be tested were placed on the top of the agar and the plates were incubated at 37°C overnight. The overall diameter of the inhibition zones was measured along two orthogonal lines. Plates were imaged using a Chemi Doc™ MP Imaging System (BIO-RAD) with white trans illumination. For IPTG -treated cells, IPTG was added to both the soft agar and the plates to a concentration of 0.1 mM. Unless otherwise noted, 10 µl of the following chemicals were used in the disk diffusion assays: 100 mM ZnCl₂, 100 mM MnCl₂, 10 mM CdCl₂, 1 M FeSO₄, 1 M FeCl₃, 50 mM CoCl₂. For streptonigrin (SN) sensitivity tests, FeSO₄ or 2, 2'-dipyridyl (DP) was added to both the soft agar and the plates to a concentration of 0.1 mM, 5 mg ml⁻¹ streptonigrin solution in dimethyl sulfoxide (DMSO) was added to the filter paper disks.

Measurement of growth using Bioscreen growth analyzer

Strains were grown to an OD₆₀₀ of 0.4 in LB media. 2 µl culture was inoculated to 200 µl LB or LBC containing FeSO₄ and/or MnCl₂ in a Bioscreen 100-well microtiter plate. Since Fe(II) rapidly oxidizes to Fe(III) in aerobic solutions generating insoluble ferric hydroxides, unless otherwise noted, 1 g l⁻¹ citrate trisodium dihydrate was added to LB media containing FeSO₄ (LBC medium). Growth was measured spectrophotometrically (OD₆₀₀) every 15 min for 24 h using a Bioscreen C incubator (Growth Curves USA, Piscataway, NJ) at 37°C with continuous shaking. For streptonigrin susceptibility studies, WT (CU1065) and

pfeT were grown in LB medium, LB containing 0.1 mM FeSO₄, or LB containing 0.1 mM DP.

Measurement of efficiency of plating and growth (colony size) by spot dilution

Strains were grown to an OD₆₀₀ of 0.4 in LB media. For Figs. 1C and S9, serial ten-fold dilutions were made from the original culture for each strain and 3 µl aliquots were spotted on LB plates containing 0, 1, 2 or 3 mM FeSO₄. The plates were incubated at 37°C overnight. In this assay, an inability to grow at the highest dilutions indicates a reduced efficiency of plating (EOP). When cells can grow, they may grow at a slower rate, as is apparent from those strains and conditions where the EOP is high, but the colony size (as observed at the highest dilutions) is reduced.

To monitor the extent of cell killing upon exposure to Fe(II) intoxication conditions (Fig. S4), cells were grown in 5 ml LBC medium with 0, 1, or 10 mM IPTG as indicated. The WT, *pfeT*, *perR*, *perR pfeT*, and *pfeT* complemented strains were shocked by addition of FeSO₄ to a final concentration of 4 mM Fe(II) (from a 100 mM stock in 0.1 N HCl) when cells reached an OD₆₀₀ ~0.4. 1 ml samples were taken before shock (0 min) and 30 min and 60 min after shock, washed by centrifugation (2 min at 13,000 rpm) and gentle resuspension in 1 ml LB, and serially diluted in LB pre-warmed to 37 °C. 3 µl of cells were spotted in each quadrant on 30 ml LB plates and left to dry before incubating at 37 °C overnight. The columns are (left to right), undiluted, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵ fold dilutions of the washed cells.

To monitor cell killing and outgrowth under conditions comparable to the Bioscreen growth analyzer experiments, cells were grown in LB to an OD₆₀₀ of ~0.4 and 0.5 ml of culture was inoculated into either 50 ml of LBC or LBC+4 mM Fe(II). 1 ml samples were removed at time 0 (right after inoculation), 0.5, 1, 3, 6, 9, 12, and 15 h. The cells were harvested (5 min at 13,000 rpm) and resuspended in LB. Viable cell counts were estimated by spotting (3 µl) of serial dilutions onto LB plates.

Protein expression and purification

DNA encoding *B. subtilis pfeT* was amplified using genomic DNA as template and primers that introduced a Tobacco etch virus (TEV) protease site coding sequence at the amplicon 3' ends. The PCR product was cloned into the pBADTOPO/His vector (Invitrogen) that introduces a (His)₆-tag at the carboxyl end of the protein. For PfeT-TEV-(His)₆ expression the construct was introduced into *E. coli* LMG194 *copA* cells (Rensing *et al.*, 2000). Cells were grown at 37°C in Terrific broth (TB) media (Sambrook *et al.*, 1990) supplemented with 0.1% arabinose, 100 µg ml⁻¹ ampicillin, and 20 µg ml⁻¹ kanamycin. Affinity purification of membrane proteins and removal of the (His)₆-tag was performed as previously described (Raimunda *et al.*, 2014). Solubilized lipid/detergent micellar forms of PfeT proteins were stored at -20°C in buffer C containing 25 mM Tris, pH8.0, 50 mM NaCl, 0.01% n-dodecyl-β-d-maltopyranoside, 0.01% asolectin, and 20% glycerol until use. The (His)₆-tag was removed from the PfeT-(His)₆ fusion protein by treatment with (His)₆-tagged TEV protease (Rosadini *et al.*, 2011) at 5:1 PfeT:TEV weight ratio for 1 h at 22°C in buffer C plus 1 mM Tris(2-carboxyethyl)phosphine (TCEP) and 0.5 mM EDTA. TEV-

(His)₆ was removed by affinity purification with Ni-NTA resin. Protein determinations were performed in accordance to Bradford (Bradford, 1976). Protein purity was assessed by Coomassie brilliant blue staining of overloaded SDS-PAGE gels and by immunostaining of Western blots with rabbit anti-(His)₆ polyclonal primary antibody (GenScript) and goat anti-rabbit IgG secondary antibody coupled to horseradish peroxidase. Prior to ATPase activity determinations, proteins (1 mg ml⁻¹) were treated with 0.5 mM EDTA and 0.5 mM tetrathiomolybdate for 45 min at room temperature. Chelators were removed using Ultra-30 Centricon (Millipore) filtration devices.

ATPase assays

These were performed at 37°C in a medium containing 50 mM Tris (pH7.4), 50 mM NaCl, 3 mM MgCl₂, 3 mM ATP, 0.01% asolectin, 0.01% n-dodecyl-β-d-maltopyranoside, 2.5 mM TCEP, 40 μg ml⁻¹ purified protein, and freshly prepared transition metal ions at the desired concentrations. Fe³⁺ was added as FeCl₃, Cu²⁺ as CuSO₄ and in both cases TCEP was not included in the assay media. Cu⁺ was obtained by including TCEP with CuSO₄ salt. Fe²⁺ and Zn²⁺ were included in the assay media as the sulfate salts while Co²⁺, Ni²⁺ and Mn²⁺ were included as their chloride salts. ATPase activity was stopped after 20 min incubation and released P_i determined (Lanzetta et al., 1979). ATPase activity measured in the absence of transition metals was subtracted from plotted values. Curves of ATPase activity versus metal concentrations were fit to $v = V_{\max}[\text{metal}]/([\text{metal}] + K_{1/2})$. The reported standard errors for V_{max} and K_{1/2} are asymptotic standard errors reported by the fitting software KaleidaGraph (Synergy).

Measurements of intracellular metal ion concentrations by ICP-MS

Cells were grown in LBC medium to an OD₆₀₀ of 0.4 with or without 1 mM IPTG and then shocked with 4 mM FeSO₄. 4 ml samples were taken before shock and at several time points after shock. Samples were harvested and washed twice with phosphate buffered saline (PBS) buffer containing 0.1 M EDTA followed by two chelex-treated PBS buffer only washes. Cells were resuspended in 400 μl of chelex-treated PBS buffer containing 75 mM NaN₃ to induce autolysis (Jolliffe *et al.*, 1981) and 1% Triton X-100, and incubated at 37°C for 90 min. Samples were centrifuged and a Bradford assay was carried out with 10 μl of the sample. Then, 600 μl of 5% HNO₃ with 0.1% (v/v) Triton X-100 was added to the samples which were boiled at 95°C for 30 min. After centrifuging the samples again, the supernatant was diluted for inductively coupled plasma mass spectrometry (ICP-MS) measurements. All samples were analyzed using a Perkin Elmer ELAN DRC II ICP-MS, equipped with a Microflow PFA-ST nebulizer. The DRC mode was used with ammonia as the reaction gas. Gallium was added at 50 ppb as an internal standard by an in-line mixing block. The total concentration of ions was calculated relative to the protein content of the sample to determine the mean metal concentration (expressed as ppm by weight).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A *pfeT* mutant is sensitive to iron intoxication.

A. Sensitivity of wild-type (WT; CU1065; black bars) and an isogenic *pfeT* null mutant (white bars) to metal ion stress as monitored using a disk diffusion (zone of inhibition) assay. The results are expressed as the diameter of the inhibition zone (mm) minus the diameter of the filter paper disk (6.5 mm). The mean±SE from at least three biological replicates are reported. Significant differences from WT as determined by two-tailed *t* test are indicated: *, *p* < 0.01.

B. Representative photograph (from at least six replicates) of a disk diffusion assay with WT and *pfeT* mutant cells on LBC plates. The disks were spotted with 10 µl 1 M FeSO₄ or 1 M FeCl₃ as indicated.

C. Efficiency of plating of WT and an isogenic *pfeT* null mutant under Fe(II) intoxication conditions as monitored using a spot dilution assay. Mid-logarithmic phase cultures were sequentially diluted by 10-fold and 3 µl spotted on LB medium amended with the indicated concentration of FeSO₄. The columns are (left to right), undiluted, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵ fold dilutions.

Fig 2.

A *pfeT* mutation increases sensitivity to streptonigrin (SN).

Representative photographs (from at least six replicates) of a disk diffusion assay with WT and *pfeT* mutant cells on LB plates containing either no supplement, 100 μM FeSO₄, or 100 μM dipyritydyl. Each disk was spotted with 5 μl of 5 mg ml⁻¹ (25 μg) SN.

Fig. 3.

The *pfeT* mutant accumulates high levels of intracellular iron.

Levels of intracellular Fe (mean $\pm$ SE of duplicate measurements) were monitored by inductively coupled plasma mass spectrometry (ICP-MS) for the *pfeT* mutant strain complemented with an IPTG-inducible copy of *pfeT* (HB17852; CU1065 *pfeT::spc amyE::P_{spac}-pfeT*) before and after addition of 4 mM FeSO₄ to LBC medium. In uninduced cells (■), Fe accumulates to a high level (~144 ppm) within 15 min. In cells grown with IPTG (and therefore expressing PfeT), the accumulation of Fe is much reduced (●). Prior to Fe addition (time 0), the basal level of Fe (~5.7 ppm) was ~5-fold higher than Mn (~1.1 ppm) under these conditions. Over the course of the experiment, levels of Mn (▲, ▼) and Zn (not shown) were largely unchanged with values averaged over the 8 measurements ($\pm$ SD) of 0.81 $\pm$ 0.16 ppm and 4.2 $\pm$ 0.8 ppm, respectively.

Fig. 4.

The PfeT ATPase is activated by Fe(II) and Co(II).

A. ATPase activity ($\mu\text{mol mg}^{-1} \text{h}^{-1}$) was measured in vitro for purified PfeT in the presence of 0.1 mM (white bars) or 1.0 mM (grey bars) of the indicated metal ions. Data are the mean and SE of three independent measurements.

B. Kinetic characterization of the PfeT ATPase in the presence of Fe(II). Data are the mean and SE of three independent measurements.

C. Kinetic characterization of the PfeT ATPase in the presence of Co(II). Data are the mean and SE of three independent measurements.

Fig. 5.

PfeT can confer Co(II) resistance if artificially expressed in an efflux defective mutant. The effect of a *pfeT* mutation on sensitivity to Co(II) was monitored using a zone of inhibition assay on LB plates. The WT, *pfeT*, *czcD*, and *czcD pfeT* double mutant were plated on LB with a filter disk containing 100 mM CoCl₂. In the final columns, the *czcD* mutant strain contained an inducible P_{spac}-*pfeT* construct and cells were grown on plates with (grey) and without (black) 0.1 mM IPTG. The zone of inhibition was measured as the total diameter of the clearance zone minus the diameter of filter paper disk (6.5 mm). The mean and SE from at least three biological replicates are reported. Significant differences were determined by two-tailed t test. *, $p < 0.01$.

Fig. 6.

Role of PfeT under iron intoxication conditions by monitoring cell growth in liquid culture.

A. Iron concentration dependence of growth inhibition for WT in LBC medium amended with various concentrations of FeSO₄ (added from a 100 mM stock prepared in 0.1 N HCl).

B. Iron concentration of growth inhibition for the *pfeT* mutant in LBC medium amended with FeSO₄. Growth inhibition is most apparent with 3.5 mM Fe(II) (●) and 4 mM Fe(II) (■).

Fig. 7.

Role of PfeT under iron intoxication conditions: cell viability and growth in liquid culture.

To determine whether the long lag phase observed under Fe(II) intoxication was due to cell stasis or cell killing, mid-logarithmic phase cultures were diluted 100-fold into either LBC (A) or LBC + 4 mM Fe(II) (B) and samples were taken as a function of time (time 0 is immediately after dilution). Cell viability (colony forming units) was estimated using a spot dilution assay with washed cells (spots represent undiluted on the left to 10⁻⁵ on the right in 10-fold decrements). Under these conditions, only the *perR* null mutant strain recovers rapidly (an ~3 h lag relative to the unstressed cells) and this recovery requires PfeT.

Fig. 8.

Iron intoxication of the *pfeT* null strain is exacerbated at low pH and abrogated by Mn(II).

A. pH dependence of growth inhibition for WT in LBC medium amended with 4 mM FeSO₄ (added from a 100 mM stock prepared in 0.1 N HCl). LBC medium was either unbuffered (No Bf; ►) leading to a final pH of 5.7, or buffered with 60 mM MES to final pHs ranging from 5.2 (■, longest lag) to ~7 (●, shortest lag).

B. pH dependence of growth inhibition for the *pfeT* mutant in LBC medium (as for panel A).

C. WT cells were grown in LBC medium with 4 mM FeSO₄ with additional Mn(II) added (no addition, *; 2.5 μM, ▲; 25 μM, ●; 250 μM, ■). Growth curves are an average of four cultures monitored in parallel (technical replicates) and the results are representative of experiments performed at least three times.

D. *pfeT* mutant cells were grown in LBC medium with 4 mM FeSO₄ and Mn(II) added at the concentrations indicated (as for panel C).

Fig. 9.

Deletion of *pfeT* increases resistance to Mn(II) intoxication.

A and B. Comparison of growth of WT (A) and a *pfeT* null mutant (B) in LB medium amended with additional Mn(II) (no addition, *; 0.5 mM, ▲; 1 mM, ●, or 2 mM, ■). Growth curves are an average of at least six cultures.

C and D. Comparison of an *mntR* mutant (C) and an *mntR pfeT* double mutant (D) in the presence of additional Mn(II) (no addition, ▲; 62.5 μM, ●, or 125 μM, ■). Growth curves are an average of at least six cultures.

Fig. 10.

Multiple PerR-regulated genes contribute to growth under iron intoxication.

A. WT (*), *pfeT* (●), *perR* (▽), *perR pfeT* (▼), *perR mrgA* (□), and *perR mrgA pfeT* (■) mutants and a complemented *pfeT* mutant (HB17852; ★) were grown in LBC containing 4 mM FeSO₄. The *perR* null mutant is highly resistant to iron intoxication due primarily to PfeT, and secondarily to MrgA. Growth curves are an average of at least four cultures and the results shown are representative of at least three biological replicates.

B. PerR regulated genes involved in H₂O₂ detoxification play a comparatively minor role in resistance to iron intoxication. WT (*), *pfeT* (★), *perR* (▽), *perR katA* (○), *perR katA pfeT* (●), *perR katA ahpCF* (◇), *perR katA ahpCF pfeT* (◆), and *perR pfeT* (▼) mutants were grown in LBC containing 4 mM FeSO₄. The increased fitness of the *perR* mutant (relative to WT) is not eliminated even in strains lacking both catalase (*katA*) and alkyl hydroperoxide reductase (*ahpCF*). Growth curves are an average of three cultures.

Fig. 11.

The Fur repressor cooperates with PfeT to prevent Fe intoxication.

WT and mutant strains were grown in LBC amended with 3.5 mM FeSO₄, a concentration that is growth inhibitory but still allows growth of the *pfeT* mutant. WT (■), *pfeT* (●), *fur* (▲), *fur pfeT* (▼), *fur pfeT ywbL* (◆), *fur pfeT yfmC* (◄), and *fur pfeT yfmC ywbL* (►).

Fig. 12.

Schematic summary of the contributions of the PerR and Fur regulons to growth under iron intoxication. The holo-form of PerR (PDB code: 3F8N) is shown with Fe(II) bound at its metal sensing site (PerR:Zn,Fe). In this form, PerR binds to DNA, repressing its target genes. H₂O₂ oxidizes PerR:Zn,Fe leading to derepression of the PerR regulon. PerR regulated genes encode proteins whose primary role relates to H₂O₂ detoxification (KatA, AhpCF, and heme biosynthesis functions; orange) and those that directly impact iron homeostasis (PfeT and MrgA; purple). PfeT is induced both by H₂O₂ stress and, independently, by high Fe(II) (not shown). MrgA (here represented using the structure of the ortholog from *E. coli*, Dps, PDB code: 1DPS) is a dodecameric mini-ferritin that sequesters iron in the presence of H₂O₂. PfeT (here represented by the structure of the *Legionella pneumophila* copper P1B-type ATPase, PDB code: 4BBJ) is a P_{1B4}-type ATPase that pumps out excess intracellular iron, thus protecting the cell from its toxic effects. PerR also regulates the expression of the ferric uptake repressor, Fur, which in its holoform (here modeled on *Streptomyces coelicolor* Zur, PDB code: 3MWM), represses the expression of iron import functions (namely, YwbLMN and YfmCDEF). With respect to Fe(II) intoxication, PfeT is the primary resistance determinant (1st place medal), whereas MrgA places a secondary role (2nd place).