A key role for the periplasmic PfeE esterase in iron acquisition *via* the siderophore Enterobactin in *Pseudomonas aeruginosa*

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ABSTRACT

Enterobactin (ENT) is a siderophore (iron-chelating compound) produced by *Escherichia coli* in order to get gain access to iron, an essential nutriment for bacterial growth. ENT is used as an exosiderophore by the opportunistic human pathogen *Pseudomonas aeruginosa* with transport of ferri-ENT across the outer membrane of *P. aeruginosa* cells by the transporter PfeA. Next to *pfeA* gene on the chromosome is localized a gene encoding for an esterase, PfeE, whose transcription is regulated, as for *pfeA*, by the presence of ENT. Purified PfeE hydrolyzed ferri-ENT into three molecules of 2,3 DHBS (2,3 dihydroxybenzoylserine) still complexed with ferric iron, and complete dissociation of iron from ENT chelating groups was only possible in the presence of both PfeE and an iron reducer, such as DTT. The crystal structure of PfeE and an inactive PfeE mutant complexed with ferri-ENT or a non-hydrolysable ferri-catechol complex allowed identification of the enzyme binding site and the catalytic triad. Finally, cell fractionation and fluorescence microscopy showed periplasmic localization of PfeE in *P. aeruginosa* cells. Thus, the molecular mechanism of iron release from ENT in *P. aeruginosa* differs from that previously described in *E. coli*. In *P. aeruginosa*, siderophore hydrolysis occurs in the periplasm, with ENT never reaching the bacterial cytoplasm. In *E. coli,* ferri-ENT crosses the inner membrane via the ABC transporter FepBCD and ferri-ENT is hydrolyzed by the esterase Fes only once it is in the cytoplasm.

INTRODUCTION

Iron is a key nutriment that is often limiting for bacterial growth and virulence due to its poor bioavailability. In the host, bacteria face iron concentrations as low as 10^{-24} M[1] due to its low solubility under neutral pH aerobic conditions and sequestration by host proteins.[1] Bacteria use low-molecular-weight extremely high-affinity iron-chelating agents, called siderophores,[2,3] to obtain iron. Siderophores are synthesized by bacteria and released into their environment where they can remove iron, both from minerals and human proteins. Gram-negative bacteria take up the resulting ferri-siderophore complexes *via* specific outer membrane transporters in a process driven by the cytoplasmic membrane potential and mediated by the energy-transducing TonB-ExbB-ExbD protein complex.[4]

The three main chemical types of siderophores are catecholates, hydroxamates, and carboxylates, with the catecholates having the highest affinity for ferric iron.[2,3] Enterobactin (ENT), a macrocyclic trimer of N-(2,3-dihydrobenzoyl) serine (DHBS) is the archetype catecholate siderophore. The three catechol rings form a hexadentate ligand able to chelate Fe(III) with a stoichiometry of 1:1 with a K_d estimated to be 10^{-52} M, the strongest such complex known.[5] ENT is synthesized by several species of Enterobacteriaceae, including *E. coli*, *Klebsiella pneumonia*, and *Salmonella typhimurium*,[1,6–8] from 2,3-dihydroxybenzoic acid (DHB) and serine by non-ribosomal peptide synthetase.[1] ENT is not only used by the microorganisms that produce it but serves as an exosiderophore for many bacteria that are unable to synthesize it, such as *Pseudomonas aeruginosa*, in a strategy of siderophore piracy.[9–11]

Iron acquisition by ENT has been studied intensively in *E. coli*, in which ferri-ENT is recognized at the bacterial surface of *E. coli* cells by FepA (an outer membrane transporter) and transported into the periplasm in a TonB-dependent manner.[12] FepB, a periplasmic binding protein, delivers ferri-ENT to the inner membrane ABC transporter system, with FepDC (FepB

acting as the periplasmic binding protein with dimers of FepC and FepD. In addition, FepB proteins can form trimers by interacting with four ferri-ENT complexes when the concentration of ferri-ENT in the periplasm reaches high levels. These complexes have been visualized by dynamic light-scattering experiments and the structure solved by X-ray crystallography.[13] It has been proposed that such trimeric organization allows iron accumulation in the bacterial periplasm and reduces the leakage cycle (FepB-unbound ferri-ENT escape *via* the efflux system TolC).[13] Iron release from ferri-ENT in *E. coli* occurs in the bacterial cytoplasm in a two-step process, first by enzymatic hydrolysis of the siderophore trilactone bonds by the esterase, Fes.[14,15] Hydrolysis of all three bonds yields a much less stable ferri-$(DHBS)_3$ complex with a higher reduction potential.[16] The reduction of Fe(III) to Fe(II) by the NADPH-dependent reductase, YdjH, facilitates efficient iron release and transfer to cytoplasmic iron binding proteins.[17] In addition, *E. coli* is also able to use salmochelins (mono-, di- and tri-glucosylated ENT; MGE, DGE and TGE respectively) as siderophores. The ferri-forms of these siderophores can be hydrolyzed by cytoplasmic esterase IroD into linear trimer, dimer and monomer products.[15] A third esterase is present in *E. coli*, the periplasmic IroE, which prefers apo forms of ENT and salmochelins and only partially degrades cyclic molecules into their linear forms.[15,18]

Pseudomonas aeruginosa does not produce ENT itself but uses it as an exosiderophore,[19] although the molecular details are unclear. Only the TonB-dependent outer membrane transporter PfeA has been identified.[20] Its expression is regulated by the presence of ferri-ENT in the bacterial environment and a two-component system PfeS/PfeR that can detect ferri-ENT[21,22] (*E. coli* lacks an equivalent two-component system).

We have identified and characterized an esterase, PfeE, that hydrolyses ferri-ENT to the ferri-$(DHBS)_3$ species in *P. aeruginosa*. We show that iron reduction, by a yet unidentified reductase, is necessary for iron release from the DHBS species. The PfeE X-ray structure was

solved in a co-complex with ferri-ENT and a non-hydrolysable ferri-tri-catechol. High-resolution fluorescence microscopy localized mCherry-tagged PfeE to the periplasm in *P. aeruginosa* cells, highly suggesting that ENT hydrolysis to get iron release occurs in this cell compartment in *P. aeruginosa*.

RESULTS AND DISCUSSION

***pfeE* deletion strongly affects ^{55}Fe uptake via ENT in *P. aeruginosa* cells.** We assessed the ability of a *pfeE* deletion mutant (Δ*pfeE*) to transport and accumulate iron in the presence of ENT in an ENT-^{55}Fe uptake assay. We also used the Δ*pfeA* strain, carrying deletion, of the outer membrane transporters PfeA, as negative control. PfeA is the specific ENT-Fe outer membrane transporter in *P. aeruginosa*. [20,23] PirA has also been shown to import ENT-Fe into *P. aeruginosa* cells, but to a lower extent.[23,24] We incubated bacteria with 500 nM ENT-^{55}Fe and monitored the radioactivity incorporated into the cells over time (Figure 1A). After 30 min, 145 pmol ^{55}Fe was incorporated into wild type PAO1 cells. No ^{55}Fe accumulation occurred in the *pfeA* deletion mutants Δ*pfeA* or the PAO1 cells when they were incubated in the presence of CCCP, a proton-motive-force inhibitor that blocks all TonB-dependent transport.[25] Deletion of *pfeE* reduced both Ent-^{55}Fe uptake (by 75%) and accumulation in *P. aeruginosa* cells, consistent with it playing a key role in iron acquisition from ferri-ENT. Thus, the absence of PfeE affects the uptake of ENT-^{55}Fe across the outer membrane, by an unknown mechanism, *via* PfeA to avoid useless periplasmic accumulation of ENT-Fe.

PfeE is a periplasmic protein. In *E. coli*, Ferri-ENT is hydrolyzed in the bacterial cytoplasm by an esterase Fes.[14,15] The presence of a signal peptide at the N-terminus of *pfeE*, suggestive of periplasmic localization of PfeE, was therefore surprising. We inserted the *mcherry* gene, by allelic exchange, into wild type PAO1 to produce a strain expressing mCherry fused to the C-terminus of PfeE (*pfeEmcherry*); chromosomal insertion was chosen to maintain expression levels close to those of the wild type strain. This strain grew as well as the parental PAO1 strains (Supplementary Figure 1A) and showed ^{55}Fe uptake kinetics indistinguishable from those of the parental strain (Supplementary Figure 1B). SDS-PAGE electrophoresis and western blotting of whole-cell extracts from PAO1 and *pfeEmcherry* with anti-DsRed antibodies

showed nothing for PAO1. However, we detected a single band at approximately 60 kDa for the *pfeEmcherry* strain, (matching PfeE (32.5 kDa) fused to mCherry (28.8 KDa)) (Figure 2A).

The periplasmic localization of PfeE-mCherry was further investigated by cell fractionation and microscopy. Microscopic visualization of PfeE-mCherry showed a diffraction-limited fluorescence signal at the edge of the bacteria (Figure 2D – left and upper right panels) in cells grown in the presence of 10 µM ENT. This is consistent with periplasmic localization of the enzyme, uniformly distributed throughout the bacteria. Super-resolution images were further acquired and reconstructed using SOFI.[26,27] They showed PfeE-mCherry to be mostly localized to the periplasmic compartment of the cells (Figure 2D – middle and lower right panels). We also fractionated the bacteria expressing PfeE-mcherry, as membrane localization cannot be completely ruled out by microscopy images. Cell fractionation of *pfeEmCherry*-expressing cells showed periplasmic and cytoplasmic localization of the PfeE-mCherry protein (Figure 2B), demonstrating that this enzyme can reach the bacterial periplasm and that the lower amount of fluorescent protein found in the cytoplasm is probably due to the newly synthesized protein not having yet reached the bacterial periplasm. At last, PfeEmCherry expression was induced in the presence of ENT as previously shown for PfeA[22,23] (Figure 2C).

PfeE with DTT promotes iron dissociation from ENT in vitro. Previous studies have shown that PfeE shares high homology with IroE, a salmochelin (SAL) esterase produced by Salmonella that hydrolyzes both apo and ferric forms of ENT and SAL.[15,28] Zhu *et al.* reported that PfeE was able to linearize ENT-Fe.[28] In our hands, purified PfeE both linearized ferri-ENT and further hydrolyzed it into 2,3 DHBS, as shown by HPLC (Figure 3). Ferri-ENT exhibits absorbance at 492 nm and Ferri-$(DHBS)_3$ at 568 nm. We were thus able to directly observe the reaction spectroscopically (Figure 4A and 4B). Incubation of ferri-ENT with purified PfeE decreased the absorbance corresponding to ferri-ENT over time, while there was a parallel

increase in the absorbance of ferri-$(DHBS)_3$, indicating the formation of a ferri-$(DHBS)_3$ complex.

2,3-DHBS, with its catechol group, has been reported to function as a siderophore for both *E. coli* and *P. aeruginosa*.[29] We confirmed that *P. aeruginosa* cells grown in the presence of 100 µM 2,3-DHBS were indeed able to use 2,3-DHBS for iron uptake in a TonB-dependent manner (Figure 1B). An incorporation of 145 pmol ml^{-1} OD_{600}^{-1} was obtained.

The fact that 2,3-DHBS can act as a siderophore for *P. aeruginosa* suggests that hydrolysis of ferri-ENT alone is not sufficient to dissociate the complex. Most known siderophore-dependent iron uptake pathways involve iron reduction steps to obtain the release of iron (for a review see [30]). We thus incubated the ENT-Fe complex with DTT (reducing agent) and ferrozine (forms a stable magenta-colored complex with absorption peak at 562 nm with ferrous iron [31]), with and without purified PfeE (Figure 5). There was no color change in the absence of PfeE, but its addition resulted in an increase in absorbance at 562 nm (Figure 5).

These data (Figure 3 to 5) show that PfeE hydrolyzes ferri-ENT into ferri-$(DHBS)_3$ in *P. aeruginosa* and that this species is sensitive to the reduction necessary to release Fe(II). We have yet to identify the reductase and consequently cannot determine whether Fe(II) is released in the periplasm and transported to the cytoplasm by another carrier or whether Ferri-$(DHBS)_3$ is transported across the inner membrane into the cytoplasm and iron reduced there to release Fe(II).

PfeE is unable to hydrolyze TCV. TCV (Tris-catechol vector)[23] is a tri-catechol with the chelating groups linked by amide rather than in a trilacton cycle, as in ENT. This compound has been synthesized for use as a cargo in a Trojan-horse strategy in which antibiotics are covalently linked to siderophore analogues.[23,32] The aim of such a strategy is to use bacterial siderophore-dependent iron uptake pathways to transport efficiently antibiotics into Gram

negative bacteria. Previous studies have shown 5 to 6-fold lower ^{55}Fe uptake rates for TCV in *P. aeruginosa* cells than those observed for ENT and an uptake which occurs as for ferri-ENT via the TonB-dependent outer membrane transporter PfeA.[23] Once in *P. aeruginosa* periplasm, how does ferri-TCV interact with PfeE ? As expected, purified PfeE was unable to hydrolyze TCV (Figure 3B). Incubation of TCV with purified PfeE, ferrozine, and DTT showed no increase of absorbance at 562 nm, indicating no iron is released by TCV (Figure 5B). MICs of 512 µM and 256 nM have been determined for this compound in *P. aeruginosa* cells grown in MH and in iron restricted CAA medium, respectively. Such antibiotic activity of TCV is most likely due to its inability to release iron once it reaches the bacterial periplasm.

Structure of PfeE. The crystallographic asymmetric unit has two PfeE molecules in the asymmetric unit however analysis of the oligomerization state of the protein by PISA[33] suggests PfeE is a monomer (Figure 5A). Both gel filtration and multi-angle light scattering (SEC-MALS) analysis suggest also a monomeric species (Supplementary Figure 2A and 2B). The protein (chain A) is ordered from residue 12 to 276 (mature protein numbering) with loops 91-94 and 111-112 (inclusive) not experimentally located. PfeE adopts a classical α/β hydrolase fold with an 8-stranded central mainly parallel β-sheet surrounded by 9 α-helices[34] (Figure 6A). The SSM tool at the EBI reveals the closest structural homologue as the salmochelin hydrolase of *E. coli*, IroE (PDB ID: 2gzr)[18] with a r.m.s.d of 1.78 Å for 230 superimposed Cα atoms which is consistent with the 47% sequence similarity over 290 residues. Other matches include bacillibactin (a catechol-based siderophore) hydrolase from *Bacillus cereus* Bes (PDB ID : 2qm0 ; 43% similarity over 289 residues, r.m.s.d of 1.6 Å for 235 superimposed Cα atoms), the cytosolic enterobactin hydrolases Fes of *Shigella flexneri* (PDB ID : 2b20 ; 36% similar for 302 residues excluding the additional N-terminal domain, r.m.s.d of 2.2 Å for 213 superimposed Cα atoms) and *Salmonella typhimurium* (PDB ID : 3mga ; 35% similar for 297

residues excluding the N-terminal domain, r.m.s.d of 2.1 Å for 219 superimposed Cα atoms). There is no solved structure for E. coli Fes (36% similarity over 303 residues) but this protein shares 98% similarity with Fes of *Shigella flexneri.* PfeE has a catalytic triad arrangement comprising S157 in the short turn between β5 and α5 in a conserved GXSXXG motif hydrogen bonded to H258 which is hydrogen bonded to E217 (Figure 6B); interestingly IroE has only a diad of S189 and H287. In the current model of the mechanism, the hydroxyl side chain of the serine activated by histidine attacks the ester bond of the siderophore. This results in a trans esterification reaction breaking the siderophore ester bond and forming an acyl enzyme intermediate which is subsequently degraded by water. The catalytic glutamate stabilizes the imidazolium during the reaction. R99 is anchored by a salt link to D59, is positioned to stabilize the tetrahedral negatively charged transition state (effectively R99 is the oxyanion hole). The same Arg Asp pair is found in IroE. Mutation of PfeE H258 or S157 into Ala abolished ferri-ENT hydrolysis (Supplementary Figure 3B and 3C).

Crystals of the inactive S157A mutant of PfeE were used to obtain complexes of ferri-ENT and ferri-TCV. Both of these complexes obtained by soaking show different orientation of the ligand in subunit A and subunit B. In the ferri-ENT complex, electron density is only visible for part of the ligand in subunit A corresponding to 2,3 DHBS (Figure 6C), with four water molecules completing the coordination sphere. Either, there is some 2,3 DHBS in the sample, or ferri-ENT has been degraded during the experiment; crystallographic disorder seems very unlikely given the density for the waters. The benzene ring of 2,3 DHBS in subunit A is sandwiched in a "π stack" between P221 and the side chain of H258. The entire ferri-ENT molecule is ordered in subunit B and P221 / H258 π sandwich is seen for one catechol of ferri-ENT but the ring is flipped 180 °. Consequently, whilst in subunit A, the carboxy group of 2,3 DHBS is positioned such that a hydroxyl placed on S157A can attack the carbonyl of the ester as would be expected for the mechanism. In subunit B, the same group now points into solvent,

making such chemistry impossible (Supplementary Figure 4A). The "inactive" conformation seen in subunit B would seem to be disfavored in the native enzyme as the hydroxyl of S157 would clash with the catechol. Further in subunit B, the inactive conformer makes additional contacts due to crystal packing which we conclude stabilizes this artefactual conformation. In the final refined structure, there is evidence for electron density in subunit B that could indicate the active conformation is also present. The same two orientations are seen for the ferri-TCV complex (Supplementary Figure 4B). A low-resolution structure of the wild-type complex with ferri-TCV shows predominantly the "active" conformation but there is evidence in the electron density for the B subunit of the inactive conformation suggesting crystal packing is an important consideration.

In the active conformation of the ferri-TCV complex (Figure 6D), in addition to the striking π stacking interaction there are hydrogen bonds to the protein involving the side chains of R96, R99, S183, W185, H258 and the main chain of Y158 (Figure 6E). There are also van der Waals interactions between the ring and protein, notably L117 and S157A. The other two catechol rings of ferri-TCV make few of these contacts and are exposed to the solvent. The inactive conformation of ferri-TCV, although it preserves the π stacking interaction, makes fewer interactions with the protein than the active form but as mentioned makes several crystal contacts. The preservation of the π stack in all structures suggests this interaction drives the binding energy. The propargyl group of ferri-TCV (the site of attachment of the antibiotic) does not interact with the protein (in either conformation), it points out into solvent.

Some of residues interacting with ferri-ENT and ferri-TCV are conserved in IroE, Fes and Bes (Supplementary Figure 5 and 6) but they lack an equivalently placed residue to P221 and thus the π sandwich. The cytosolic Fes proteins have an N-terminal domain which have positively charged residues (Arg and His) pointing towards the siderophore and partly enclosing the active site. PfeE has a large binding pocket with a dominant π sandwich interacting with

only one catechol of the siderophore. This is coherent with the fact that PfeE is capable of cleaving larger molecules like Salmochelin but can only cut them once (FigX).

How iron is transported further across the inner membrane is still unknown. The mechanism of iron release from ENT, proposed above, implies that an inner membrane transporter carries ferrous iron or ferri-$(DHBS)_3$ across the inner membrane into the cytoplasm. However, no such candidate can be found near the *pfeA and pfeE* genes.

fepCBDG genes, which code for a homologue of *fepCDE*, a previously described ABC transporter for the uptake of ENT-Fe across the inner membrane in *E. coli,*[35] have been found in the *P. aeruginosa* genome. Deletion of these genes had no effect on ^{55}Fe accumulation in *P. aeruginosa* cytoplasm and periplasm when incubated in the presence of ENT (Figure 7), indicating that this ABC transporter is not involved in the ENT pathway. *fepCBDG* is certainly involved in the vibriobactin pathway, as these genes are located very close to *fvbA*, which encodes a ferri-vibriobactin outer membrane transporter (vibriobactin is a catechol siderophore produced by *Vibrio cholera*).[36]

Previous studies have shown that PVD and citrate, two siderophores used by *P. aeruginosa*, both release iron into the bacterial periplasm *via* a mechanism involving iron reduction, with the iron reduction step occurring in the periplasm or inner membrane. In the pyoverdine pathway, iron release from this siderophore involves a reduction step by the inner membrane reductase, FpvG, and chelation of the formed ferrous iron by a periplasmic binding protein called FpvC.[37,38] Ferrous iron is then transported further across the inner membrane by the ABC transporter, FpvDE.[37] In the citrate pathway, the Fe^{2+} FeoABC transporter has been strongly suggested to be involved in the transport of the metal across the inner membrane into the cytoplasm.[39] Deletion of both *fpvCD* and *feoABC* had no effect on ^{55}Fe accumulation in *P. aeruginosa* cells in the presence of ENT (Figure 7), indicating that these two transporters are

not involved in the ENT pathway.

Further studies will be necessary to identify the inner membrane transporter of iron of the ENT pathway.

Conclusion. We report the first X-ray structure of the complex between a siderophore esterase and its substrate. The structure of PfeE reveals very close structural homology with other esterases, especially the periplasmic salmochelin hydrolase of *E. coli*, IroE, but also bacillibactin hydrolase from *Bacillus cereus*, Bes, and the cytosolic ENT hydrolases, Fes, of *S. flexneri* and S. *typhimurium*. These enzymes, from different bacterial species, all have in common the ability to hydrolyze ferri-ENT or analogues like salmochelins, but they are not necessarily localized to the same cell compartment: PfeE and IroE are periplasmic, whereas Bes and Fes are cytoplasmic.

Identification and characterization of PfeE has revealed novel features of iron acquisition by ENT in *P. aeruginosa*. In this pathogen, iron-loaded ENT, after import into the periplasm by the TonB-dependent outer membrane transporter PfeA, is hydrolyzed by PfeE into three molecules of 2,3 DHBS still in complex with the ferric iron ion. Iron reduction is necessary to obtain complete release of the metal from 2,3 DHBS. This step may occur in the bacterial periplasm immediately after the action of PfeE, with subsequent transport of catechol-free ferrous iron across the inner membrane toward the cytoplasm (Scheme 1). Another possible scenario is the hydrolysis of ENT-Fe by PfeE in the periplasm, followed by transport of the formed ferri-$(DHBS)_3$ complex though the inner membrane and an iron reduction step with iron release only once it is in the cytoplasm. We were not able to identify the iron reducer involved in the ENT pathway in *P. aeruginosa*, nor the periplasmic or cytoplasmic protein, which shuttles the resulting ferrous iron ion. The periplasmic localization of PfeE in the ENT-dependent iron uptake pathway of *P. aeruginosa* indicates that the siderophore is hydrolyzed

into 2,3 DHBS in the bacterial periplasm and not in the cytoplasm, as in the *E. coli* pathway, a major difference between the two pathways. However, iron release from ENT in *P. aeruginosa* seems to be similar to what has been described for *Campylobacter*, where a periplasmic trilactone esterase, Cee, also hydrolyses ferri-ENT in the bacterial periplasm.[40] Our data also show that PfeE is an essential player of the ENT-dependent iron uptake pathway in *P. aeruginosa*: its deletion abolishes ferri-ENT uptake across the outer membrane via PfeA and any ferri-tri-catechol entering bacteria by this pathway and being unable to be hydrolysed by PfeE will slow down bacterial growth (MIC of 256 nM for TCV in iron-restricted growth conditions).

Many studies have highlighted that siderophore-dependent bacterial iron-uptake pathways can be used to promote the transport of antibiotics into bacteria using a Trojan horse approach.[32,41,42] The antibiotic is covalently linked to the siderophore and each time a ferric ion is transported into the target bacteria by the siderophore, an antibiotic molecule is transported as well. Recently, IroD esterase was shown to be capable of restoring the activity of the antibiotic (ciprofloxacin) in bacteria by hydrolyzing the ENT linked to it and then being used for the uptake of the drug across the bacterial membranes.[43] The *P. aeruginosa* PfeE esterase may also be a good candidate in Trojan horse strategies involving siderophore hydrolysis to promote antibiotic release in the bacterial periplasm.

METHODS

Chemicals, growth media, and siderophores. The protonophore CCCP was purchased from Sigma. $^{55}FeCl_3$ was obtained from Perkin Elmer Life and Analytical Sciences, in solution, at a concentration of 71.1 mM, with a specific activity of 10.18 Ci g^{-1}. ENT was obtained from Sigma-Aldrich, 2,3 DHBS was a gift from GlaxoSmithKline; TCV was prepared as described previously,[44] ferrozine, and DTT was obtained from Sigma Aldrich. LB broth and LB broth agar medium were purchased from Difco and were used as nutriment-rich medium in all experiments.

Bacterial strains, plasmids, and growth conditions. The *P. aeruginosa* and *Escherichia coli* strains and plasmids used throughout this study are shown in Table 1 and Table 1SM (in the supplemental material), respectively. *Escherichia coli* strains were routinely grown in LB broth at 37 °C. For cultures of *P. aeruginosa* strains in iron-limited media, bacteria were first grown in LB broth overnight at 30 °C. The bacteria were then washed in CAA medium (casamino acid medium, composition: 5 g L^{-1} low-iron CAA (Difco), 1.46 g L^{-1} K_2HPO_4 $3H_2O$, 0.25 g L^{-1} $MgSO_4$ $7H_2O$), diluted by a factor of two and incubated for 24 h at 30°C. CAA is a highly iron-restricted medium with iron concentrations of 20 nM.[45] The iron concentration in LB is approximately 4 μM.[45]

Plasmid and strain construction. All enzymes for deoxyribonucleic acid (DNA) manipulation were purchased from ThermoFisher Scientific and used according to the manufacturer's instructions. *Escherichia coli* strain TOP10 (Invitrogen) and NEB 5-alpha (NEB) were used as the host strain for the plasmids. The DNA fragments from *P. aeruginosa* used for cloning were amplified from the genomic DNA of strain PAO1 with Phusion High-Fidelity DNA polymerase (ThermoFisher Scientific). The primers used are listed in Table 1 in the supplemental material.

The general procedure for the construction of the pEXG2 *pfeEmcherry* plasmid involved insertion of the *mcherry* gene flanked by upstream and downstream regions of 700 bp, relative to the insertion site, into the pEXG2 vector, as previously described [46]. For construction of the pEXG2 Δ*pfeE* plasmid, the 700 bp flanking sequences of the gene to be deleted were cloned into the pEXG2 vector using the NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs), according to the manufacturer's instructions. Mutations in the chromosomal genome of *P. aeruginosa* were generated by transferring the allelic exchange vectors from the *E. coli* strain (NEB 5-alpha or TOP10) into the PAO1 strain by triparental mating and allowing the plasmid to integrate into the chromosome, with selection for gentamicin resistance.[47] Deletion mutants were selected by streaking the gentamicin resistant clones on no-salt LB agar containing 5% (w/v) sucrose. The sucrose-resistant clones were verified by PCR and sequencing.

The *pfeA* deletion in the chromosomal genome of *P. aeruginosa* was generated by transferring the pME3088 Δ*pfeA* vector from the *E. coli* TOP10 strain into the PAO1 strain by triparental mating, allowing the plasmid to integrate into the chromosome, with selection for tetracycline resistance. A second crossing-over event, to excise the vector, was achieved by enrichment for tetracycline-sensitive cells to generate the corresponding mutants.[48] The mutants were verified by PCR and sequencing.

For the over-expression of PfeE in E coli, the position of the cleavage of the signal peptide of the proteins was predicted using Signal P4.0.[50] The coding sequence of the mature PfeE protein (PA2689) was amplified from genomic PAO1 DNA using PA2689F and PA2689R primers (Supplementary Table 1) and KOD Hot Start DNA polymerase (Novagen). The PCR product was digested by BspHI and BamHI restriction enzymes and the gene cloned in the pBADHISTEV vector using NcoI and BamHI restriction sites. Mutations S157A and H258A were constructed with a modified protocol of the QuikChange method[51] using the primers

PA2689-S157A-F, PA2689-S157A-R, PA2689-H258A-F and PA2689-H258A-R (Table 1).

Iron uptake assays and cell fractionation. Bacteria were grown in CAA medium, as described above. The cells were pelleted by centrifugation and re-suspended in fresh CAA medium. The resulting suspension was diluted to an optical density at 600 nm (OD_{600}) of 0.1 units, in the presence of 10 µM ENT or 100 µM DHBS, and incubated 24 h at 30°C. The ENT-^{55}Fe and $(DHBS)_3$-^{55}Fe complexes were prepared at ^{55}Fe concentrations of 50 µM, with a siderophore:iron (mol:mol) ratio of 20:1 or 400:1. Bacteria were grown in CAA medium, as described above, washed with 50 mM Tris-HCl pH 8.0, and diluted to an OD_{600} of 1. Transport assays were initiated by adding 500 nM ENT-^{55}Fe or $(DHBS)_3$-^{55}Fe to the bacteria grown in the presence of ENT or DHBS, respectively. The incorporation of radioactivity into the bacteria was monitored immediately after the addition of ENT-^{55}Fe at various incubation times by centrifugation, as previously described for PVD.[23] The experiments were repeated with cells pretreated with 200 µM CCCP. This compound inhibits the proton-motive force across bacterial cell membranes, inhibiting TonB-dependent iron uptake.[25]

Periplasm and cytoplasm fractions were prepared as previously described.[49]

Over-expression in E coli, purification and crystallisation of PfeE.

Proteins were over-expressed in *E.coli* C43 (DE3) cells. Cells were grown at 37 °C in LB medium containing 100 µg.mL^{-1} ampicillin until an OD_{600} of 0.6 and then induced with 0.2 % arabinose at 25°C overnight.

Cells were resuspended in lysis buffer (20 mM sodium phosphate pH 7.5, 500 mM NaCl, 10 mM imidazole, 10% glycerol with the addition of complete EDTA-free protease inhibitor tablets (Roche), DNase (0.2 mg per 10 mL buffer) and lysozyme (1 mg per 10 mL buffer,) and lysed with a cell disruptor at 30 kPSI (Constant Systems Ltd). The crude lysate was cleared by

centrifugation (40,000 *g*, 4 °C, 30 min) before been loaded onto a 5ml His-trap (GE Healthcare) column. The column was washed with the lysis buffer containing 25 mM imidazol. The protein was eluted by step gradient of imidazole (25, 40, 150 and 300 mM). Eluted proteins were dialysed against buffer 50 mM Tris-HCl pH 8, 250 mM NaCl, 2 mM β-mercaptoethanol, 10% glycerol before adding the tobacco etch virus protease to remove the His_6-tag. The uncleaved protein and protease were then removed by passage through a second HisTrap column in lysis buffer containing 20 mM imidazole. Finally, the PfeE was loaded onto a gel filtration Superdex 75 column (GE Healthcare) in 10 mM Tris-HCl pH 8.0, 150 mM NaCl.

Crystals appeared at 20°C after few weeks from a hanging drop of 1 μL of protein solution (23 $mg.mL^{-1}$) with 1 μL of reservoir solution containing 17% PEG MME 2000, 0.1 M Sodium acetate pH 5.5, 0.16 M potassium nitrate in vapor diffusion with reservoir. Crystals were frozen with the same solution containing 25% ethylene glycol. Complex structures by were obtained by soaking apo crystals 30 minutes with mother liquor containing 5 mM Fe^{3+}-enterobactin or Fe^{3+}-TCV before cryoprotection. Data were collected at 100 K in house using a Rigaku Micromax™-007HF Cu anode with VariMax optics and Rigaku Saturn 944+ CCD detector and at the Diamond light source Oxfordshire, beamlines I24. Data were processed with XIA2[52–56] or autoproc[57]. Structure has been solved using the coordinate of iroE (Pdb code 2gzr) and the structure refined with REFMAC and adjusted with COOT[58] and Autobuild program of PHENIX.[59] Subsequent structures were solved using the coordinates from the native structure and were refined was carried out using REFMAC in the CCP4 program suite with NCS restraints and TLS parameters.[60] The same “dimeric” arrangement of monomers was seen in all crystal structures despite changes in space-group. In 6GI5, this ‘dimeric arrangement’ can be observed by generating the symmetry related molecules in the lattice. However, biophysical data (gel filtration and SEC-MALS) (Supplementary Figure 2A and 2B) do not support the dimer as the relevant solution species. Final refinement statistics are given in Table 1. Atomic

coordinates and structure factors have been deposited in the Protein Data Bank (6GI0, 6GI1, 6GI2, 6GI5). Coordinates and topologies of ligands were generated by PRODRG.[61] The quality of all structures was checked with MOLPROBITY.[62] The Ramachandran statistics are as followed: 98 % favored and 2 % allowed for the apo structure and TCV complex; 97.7 % favored and 2.3 % allowed for the ENT complex. Figures were drawn using PYMOL.[63]

Determination of PfeE and PfeE mutant activities. ENT and iron chelator ferric complexes were prepared by incubating one equivalent of Fe(III) with one equivalent of siderophore or iron chelator in 50 mM HEPES buffer pH 7.4, at 5 mM for 10 min. For the HPLC analyses, 1 mM of the siderophore-Fe solutions were incubated with or without 10 µM purified PfeE (or purified PfeE mutants) in 50 mM HEPES buffer (pH 7.4) at 37°C. Aliquots were removed at various times for HPLC analysis or conserved at -80 °C if not immediately analyzed. Samples were analyzed and the reaction products purified by HPLC (Gilson) on a C18 reverse-phase column (Agilent ZORBAX SB-C18 4.6 x 250 mm) using a 50-min long gradient from 0 to 99% acetonitrile in water with detection at 254 nm (flow rates 1 $mL.min^{-1}$).

For the enzymatic kinetics, 500 µM siderophore-iron complex was incubated at 37°C in 50 mM HEPES buffer at pH 7.4 with or without 1 µM purified PfeE (or purified PfeE mutants). UV absorbance spectra were measured every 12 min using a TECAN M200 plate reader. When the enzymatic activity of PfeE was followed in the presence of ferrozine, 50 µM siderophore-iron complex was incubated at 37°C for 5 min in 50 mM HEPES buffer at pH 7.4, with or without 100 mM DTT and/or 20 mM ferrozine, and 500 nM PfeE (or purified PfeE mutants) were added just before the first measure. The absorbance at 562 nm was measured every minute using a TECAN M200 plate reader.

Immunoblot analysis. Immunoblot analysis was carried out as previously described for the labeling of proteins of the PVD pathway.

Growth and quantification of fluorescence intensity. The cells were cultured overnight in CAA medium as described above, pelleted by centrifugation, and re-suspended in fresh CAA medium. The resulting suspension was diluted to an OD_{600} of 0.01 units. We dispensed 200 µl of the suspension per well into a 96-well plate (Greiner, U-bottomed microplate) in the presence of 100 µM ENT. The plate was incubated at 30 °C, with shaking, in a Tecan microplate reader (Infinite M200, Tecan) for measurements of OD_{600} and mCherry (excitation/emission wavelengths: 570 nm/610 nm) fluorescence, at 30 min intervals, for 40 h. We calculated the mean of three replicates for each measurement.

Fluorescence microscopy imaging. Bacteria were grown in CAA medium as described above. The cells were pelleted by centrifugation and re-suspended in fresh CAA medium. The resulting suspension was diluted to an OD_{600} of 0.1 units in the presence of 10 µM ENT and incubated overnight at 30 °C. The overnight culture was washed twice with CAA medium and spotted on 1 % agarose pads containing CAA medium. TIRF and super resolution images were performed on a home-built Micro-Manager-controlled[64] inverted microscope based on a Nikon Eclipse II equipped with a 100X 1.49 objective (Nikon - Japan) and a drift focus compensator (Perfect Focus System - Nikon -Japan). A 561nm diode laser (Oxxius -France) was used to excite mCherry and it was reactivated with a 405nm laser diode (Spectra Physics - Germany) when appropriate, selected by an acousto-optic tunable filter (AOTFnC 400-650 TN, AA Otpo Electronic- France). The fluorescence emission of mCherry was collected through a quadri-band dichroic filter (FF405/496/560/651-Di01-25x36 - Semrock - USA) and further filtered with a 607/70-nm band-pass filter (Chroma - Germany). The wavefront distortions of the

emission signal were corrected using an adaptive optics device (MicAO, Imaging Optics - France) before being imaged on a 512 x 512-pixel EM-CCD camera operating at -65 °C with an ADU-to-photon conversion factor of 62 (ImagEM - Hamamatsu Photonics - Japan). Approximately 2,000 frames, with an exposure time of 50 ms, were typically recorded for super-resolution optical fluctuation imaging (SOFI) using a home-written Beanshell acquisition script. Images were reconstructed using Localizer, an open source software package[65] running within Igor Pro 7 (Wavemetrics).

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REFERENCES

(1) Raymond, K. N.; Dertz, E. A.; Kim, S. S. Enterobactin: An Archetype for Microbial Iron Transport. *Proc Natl Acad Sci U A* **2003**, *100* (7), 3584–3588.
(2) Boukhalfa, H.; Crumbliss, A. L. Chemical Aspects Od Siderophore Mediated Iron Transport. *Biometals* **2002**, *15* (4), 325–339.
(3) Hider, R. C.; Kong, X. Chemistry and Biology of Siderophores. *Nat Prod Rep* **2011**, *27* (5), 637–657.
(4) Schalk, I. J.; Mislin, G. L.; Brillet, K. Structure, Function and Binding Selectivity and Stereoselectivity of Siderophore-Iron Outer Membrane Transporters. *Curr Top Membr* **2012**, *69*, 37–66.
(5) Harris, W. R.; Carrano, C. J.; Cooper, S. R.; Sofen, S. R.; Avdeef, A. E.; McArdle, J. V.; Raymond, K. N. Coordination Chemistry of Microbial Iron Transport Compounds. 19. Stability Constants and Electrochemical Behavior of Ferric Enterobactin and Model Complexes. *J. Am. Chem. Soc.* **1979**, *101* (20), 6097–6104.
(6) O'Brien, I. G.; Gibson, F. The Structure of Enterochelin and Related 2,3-Dihydroxy-N-Benzoylserine Conjugates from Escherichia Coli. *Biochim Biophys Acta* **1970**, *215* (2), 393–402.
(7) Pollack, J. R.; Ames, B. N.; Neilands, J. B. Iron Transport in Salmonella Typhimurium: Mutants Blocked in the Biosynthesis of Enterobactin. *J. Bacteriol.* **1970**, *104* (2), 635–639.
(8) Perry, R. D.; San Clemente, C. L. Siderophore Synthesis in Klebsiella Pneumoniae and Shigella Sonnei during Iron Deficiency. *J. Bacteriol.* **1979**, *140* (3), 1129–1132.
(9) Schubert, S.; Fischer, D.; Heesemann, J. Ferric Enterochelin Transport in Yersinia Enterocolitica: Molecular and Evolutionary Aspects. *J. Bacteriol.* **1999**, *181* (20), 6387–6395.
(10) Cornelis, P.; Dingemans, J. Pseudomonas Aeruginosa Adapts Its Iron Uptake Strategies in Function of the Type of Infections. *Front Cell Infect Microbiol* **2013**, *3*, 75.
(11) Barber, M. F.; Elde, N. C. Buried Treasure: Evolutionary Perspectives on Microbial Iron Piracy. *Trends Genet. TIG* **2015**, *31* (11), 627–636.
(12) Lundrigan, M. D.; Kadner, R. J. Nucleotide Sequence of the Gene for the Ferrienterochelin Receptor FepA in Escherichia Coli. Homology among Outer Membrane Receptors That Interact with TonB. *J. Biol. Chem.* **1986**, *261* (23), 10797–10801.
(13) Li, B.; Li, N.; Yue, Y.; Liu, X.; Huang, Y.; Gu, L.; Xu, S. An Unusual Crystal Structure of Ferric-Enterobactin Bound FepB Suggests Novel Functions of FepB in Microbial Iron Uptake. *Biochem. Biophys. Res. Commun.* **2016**, *478* (3), 1049–1053.
(14) Brickman, T. J.; McIntosh, M. A. Overexpression and Purification of Ferric Enterobactin Esterase from Escherichia Coli. Demonstration of Enzymatic Hydrolysis of Enterobactin and Its Iron Complex. *J Biol Chem* **1992**, *267* (17), 12350–12355.
(15) Lin, H.; Fischbach, M. A.; Liu, D. R.; Walsh, C. T. In Vitro Characterization of Salmochelin and Enterobactin Trilactone Hydrolases IroD, IroE, and Fes. *J Am Chem Soc* **2005**, *127* (31), 11075–11084.
(16) Cooper, S. R.; McArdle, J. V.; Raymond, K. N. Siderophore Electrochemistry: Relation to Intracellular Iron Release Mechanism. *Proc. Natl. Acad. Sci. U. S. A.* **1978**, *75* (8), 3551–3554.
(17) Miethke, M.; Hou, J.; Marahiel, M. A. The Siderophore-Interacting Protein YqjH Acts as a Ferric Reductase in Different Iron Assimilation Pathways of Escherichia Coli. *Biochemistry (Mosc.)* **2011**, *50* (50), 10951–10964.
(18) Larsen, N. A.; Lin, H.; Wei, R.; Fischbach, M. A.; Walsh, C. T. Structural Characterization of Enterobactin Hydrolase IroE. *Biochemistry (Mosc.)* **2006**, *45* (34), 10184–10190.

(19) Poole, K.; Young, L.; Neshat, S. Enterobactin-Mediated Iron Transport in Pseudomonas Aeruginosa. *J Bacteriol* **1990**, *172* (12), 6991–6996.
(20) Dean, C. R.; Poole, K. Cloning and Characterization of the Ferric Enterobactin Receptor Gene (PfeA) of Pseudomonas Aeruginosa. *J. Bacteriol.* **1993**, *175* (2), 317–324.
(21) Dean, C. R.; Poole, K. Expression of the Ferric Enterobactin Receptor (PfeA) of Pseudomonas Aeruginosa: Involvement of a Two-Component Regulatory System. *Mol Microbiol* **1993**, *8* (6), 1095–1103.
(22) Dean, C. R.; Neshat, S.; Poole, K. PfeR, an Enterobactin-Responsive Activator of Ferric Enterobactin Receptor Gene Expression in Pseudomonas Aeruginosa. *J. Bacteriol.* **1996**, *178* (18), 5361–5369.
(23) Gasser, V.; Baco, E.; Cunrath, O.; August, P. S.; Perraud, Q.; Zill, N.; Schleberger, C.; Schmidt, A.; Paulen, A.; Bumann, D.; et al. Catechol Siderophores Repress the Pyochelin Pathway and Activate the Enterobactin Pathway in Pseudomonas Aeruginosa: An Opportunity for Siderophore-Antibiotic Conjugates Development. *Environ. Microbiol.* **2016**, *18* (3), 819–832.
(24) Ghysels, B.; Ochsner, U.; Mollman, U.; Heinisch, L.; Vasil, M.; Cornelis, P.; Matthijs, S. The Pseudomonas Aeruginosa PirA Gene Encodes a Second Receptor for Ferrienterobactin and Synthetic Catecholate Analogues. *FEMS Microbiol Lett* **2005**, *246* (2), 167–174.
(25) Clément, E.; Mesini, P. J.; Pattus, F.; Abdallah, M. A.; Schalk, I. J. The Binding Mechanism of Pyoverdin with the Outer Membrane Receptor FpvA in Pseudomonas Aeruginosa Is Dependent on Its Iron-Loaded Status. *Biochemistry (Mosc.)* **2004**, *43*, 7954–7965.
(26) Dertinger, T.; Colyer, R.; Iyer, G.; Weiss, S.; Enderlein, J. Fast, Background-Free, 3D Super-Resolution Optical Fluctuation Imaging (SOFI). *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (52), 22287–22292.
(27) Dertinger, T.; Colyer, R.; Vogel, R.; Enderlein, J.; Weiss, S. Achieving Increased Resolution and More Pixels with Superresolution Optical Fluctuation Imaging (SOFI). *Opt. Express* **2010**, *18* (18), 18875–18885.
(28) Zhu, M.; Valdebenito, M.; Winkelmann, G.; Hantke, K. Functions of the Siderophore Esterases IroD and IroE in Iron-Salmochelin Utilization. *Microbiology* **2005**, *151* (Pt 7), 2363–2372.
(29) Hantke, K. Dihydroxybenzoylserine--a Siderophore for E. Coli. *FEMS Microbiol. Lett.* **1990**, *55* (1–2), 5–8.
(30) Schalk, I. J.; Guillon, L. Fate of Ferrisiderophores after Import across Bacterial Outer Membranes: Different Iron Release Strategies Are Observed in the Cytoplasm or Periplasm Depending on the Siderophore Pathways. *Amino Acids* **2013**, *44* (5), 1267–1277.
(31) Stookey, L. L. Ferrozine-A New Spectrophotometric Reagent for Iron. *Anal. Chem.* **1970**, *42* (7), 779–781.
(32) Mislin, G. L.; Schalk, I. J. Siderophore-Dependent Iron Uptake Systems as Gates for Antibiotic Trojan Horse Strategies against Pseudomonas Aeruginosa. *Metallomics* **2014**, *6* (3), 408–420.
(33) Krissinel, E.; Henrick, K. Inference of Macromolecular Assemblies from Crystalline State. *J. Mol. Biol.* **2007**, *372* (3), 774–797.
(34) Nardini, M.; Dijkstra, B. W. Alpha/Beta Hydrolase Fold Enzymes: The Family Keeps Growing. *Curr. Opin. Struct. Biol.* **1999**, *9* (6), 732–737.
(35) Chu, B. C. H.; Otten, R.; Krewulak, K. D.; Mulder, F. A. A.; Vogel, H. J. The Solution Structure, Binding Properties, and Dynamics of the Bacterial Siderophore-Binding Protein FepB. *J. Biol. Chem.* **2014**, *289* (42), 29219–29234.
(36) Elias, S.; Degtyar, E.; Banin, E. FvbA Is Required for Vibriobactin Utilization in Pseudomonas Aeruginosa. *Microbiology* **2011**, *157* (Pt 7), 2172–2180.

(37) Brillet, K.; Ruffenach, F.; Adams, H.; Journet, L.; Gasser, V.; Hoegy, F.; Guillon, L.; Hannauer, M.; Page, A.; Schalk, I. J. An ABC Transporter with Two Periplasmic Binding Proteins Involved in Iron Acquisition in Pseudomonas Aeruginosa. *ACS Chem Biol* **2012**, *7* (12), 2036–2045.
(38) Ganne, G.; Brillet, K.; Basta, B.; Roche, B.; Hoegy, F.; Gasser, V.; Schalk, I. J. Iron Release from the Siderophore Pyoverdine in Pseudomonas Aeruginosa Involves Three New Actors: FpvC, FpvG, and FpvH. *ACS Chem. Biol.* **2017**, *12* (4), 1056–1065.
(39) Marshall, B.; Stintzi, A.; Gilmour, C.; Meyer, J.-M.; Poole, K. Citrate-Mediated Iron Uptake in Pseudomonas Aeruginosa: Involvement of the Citrate-Inducible FecA Receptor and the FeoB Ferrous Iron Transporter. *Microbiol. Read. Engl.* **2009**, *155* (Pt 1), 305–315.
(40) Zeng, X.; Mo, Y.; Xu, F.; Lin, J. Identification and Characterization of a Periplasmic Trilactone Esterase, Cee, Revealed Unique Features of Ferric Enterobactin Acquisition in Campylobacter. *Mol. Microbiol.* **2013**, *87* (3), 594–608.
(41) Tillotson, G. S. Trojan Horse Antibiotics-A Novel Way to Circumvent Gram-Negative Bacterial Resistance? *Infect. Dis.* **2016**, *9*, 45–52.
(42) Klahn, P.; Brönstrup, M. Bifunctional Antimicrobial Conjugates and Hybrid Antimicrobials. *Nat. Prod. Rep.* **2017**, *34* (7), 832–885.
(43) Neumann, W.; Sassone-Corsi, M.; Raffatellu, M.; Nolan, E. M. Esterase-Catalyzed Siderophore Hydrolysis Activates an Enterobactin-Ciprofloxacin Conjugate and Confers Targeted Antibacterial Activity. *J. Am. Chem. Soc.* **2018**, *140* (15), 5193–5201.
(44) Baco, E.; Hoegy, F.; Schalk, I. J.; Mislin, G. L. Diphenyl-Benzo[1,3]Dioxole-4-Carboxylic Acid Pentafluorophenyl Ester: A Convenient Catechol Precursor in the Synthesis of Siderophore Vectors Suitable for Antibiotic Trojan Horse Strategies. *Org Biomol Chem* **2014**, *12* (5), 749–757.
(45) Cunrath, O.; Geoffroy, V. A.; Schalk, I. J. Metallome of Pseudomonas Aeruginosa: A Role for Siderophores. *Env. Microbiol* **2015**.
(46) Guillon, L.; El Mecherki, M.; Altenburger, S.; Graumann, P. L.; Schalk, I. J. High Cellular Organisation of Pyoverdine Biosynthesis in Pseudomonas Aeruginosa: Localization of PvdA at the Old Cell Pole. *Env. Microbiol* **2012**, *14*, 1982–1994.
(47) Hmelo, L. R.; Borlee, B. R.; Almblad, H.; Love, M. E.; Randall, T. E.; Tseng, B. S.; Lin, C.; Irie, Y.; Storek, K. M.; Yang, J. J.; et al. Precision-Engineering the Pseudomonas Aeruginosa Genome with Two-Step Allelic Exchange. *Nat. Protoc.* **2015**, *10* (11), 1820–1841.
(48) Ye, R. W.; Haas, D.; Ka, J. O.; Krishnapillai, V.; Zimmermann, A.; Baird, C.; Tiedje, J. M. Anaerobic Activation of the Entire Denitrification Pathway in Pseudomonas Aeruginosa Requires Anr, an Analog of Fnr. *J Bacteriol* **1995**, *177* (12), 3606–3609.
(49) Gasser, V.; Guillon, L.; Cunrath, O.; Schalk, I. J. Cellular Organization of Siderophore Biosynthesis in Pseudomonas Aeruginosa: Evidence for Siderosomes. *J Inorg Biochem* **2015**, *148*, 27–34.
(50) Petersen, T. N.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 4.0: Discriminating Signal Peptides from Transmembrane Regions. *Nat. Methods* **2011**, *8* (10), 785–786.
(51) Liu, H.; Naismith, J. H. An Efficient One-Step Site-Directed Deletion, Insertion, Single and Multiple-Site Plasmid Mutagenesis Protocol. *BMC Biotechnol.* **2008**, *8*, 91.
(52) Kabsch, W. Automatic Processing of Rotation Diffraction Data from Crystals of Initially Unknown Symmetry and Cell Constants. *J Appl Cryst* **1993**, *26*, 795–800.
(53) Sauter, N. K.; Grosse-Kunstleve, R. W.; Adams, P. D. Robust Indexing for Automatic Data Collection. *J. Appl. Crystallogr.* **2004**, *37* (3), 399–409.
(54) Evans, P. Scaling and Assessment of Data Quality. *Acta Crystallogr. D Biol. Crystallogr.* **2006**, *62* (Pt 1), 72–82.
(55) Zhang, Z.; Sauter, N. K.; van den Bedem, H.; Snell, G.; Deacon, A. M. Automated

Diffraction Image Analysis and Spot Searching for High-Throughput Crystal Screening. *J. Appl. Crystallogr.* **2006**, *39* (1), 112–119.
(56) Winter, G. Xia2: An Expert System for Macromolecular Crystallography Data Reduction. *J. Appl. Crystallogr.* **2010**, *43* (1), 186–190.
(57) Vonrhein, C.; Flensburg, C.; Keller, P.; Sharff, A.; Smart, O.; Paciorek, W.; Womack, T.; Bricogne, G. Data Processing and Analysis with the AutoPROC Toolbox. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, *67* (Pt 4), 293–302.
(58) Emsley, P.; Cowtan, K. Coot: Model-Building Tools for Molecular Graphics. *Acta Cryst D* **2004**, *60*, 2126–2132.
(59) Adams, P. D.; Grosse-Kunstleve, R. W.; Hung, L. W.; Loerger, T. R.; McCoy, A. J.; Moriarty, N. W.; Read, R. J.; Sacchettini, J. C.; Sauter, N. K.; Terwilliger, T. C. PHENIX: Building New Software for Automated Crystallographic Structure Determination. *Acta Cryst D* **2002**, *58*, 1948–1954.
(60) Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. Refinement of Macromolecular Structures by the Maximum-Likelihood Method. *Acta Crystallogr. D Biol. Crystallogr.* **1997**, *53* (Pt 3), 240–255.
(61) Schüttelkopf, A. W.; van Aalten, D. M. F. PRODRG: A Tool for High-Throughput Crystallography of Protein-Ligand Complexes. *Acta Crystallogr. D Biol. Crystallogr.* **2004**, *60* (Pt 8), 1355–1363.
(62) Chen, V. B.; Arendall, W. B. 3rd; Headd, J. J.; Keedy, D. A.; Immormino, R. M.; Kapral, G. J.; Murray, L. W.; Richardson, J. S.; Richardson, D. C. MolProbity: All-Atom Structure Validation for Macromolecular Crystallography. *Acta Cryst D* **2010**, *66*, 12–21.
(63) Schrodinger. *The PyMOL Molecular Graphics System.*
(64) Edelstein, A. D.; Tsuchida, M. A.; Amodaj, N.; Pinkard, H.; Vale, R. D.; Stuurman, N. Advanced Methods of Microscope Control Using MManager Software. *J. Biol. Methods* **2014**, *1* (2).
(65) Dedecker, P.; Duwé, S.; Neely, R. K.; Zhang, J. Localizer: Fast, Accurate, Open-Source, and Modular Software Package for Superresolution Microscopy. *J. Biomed. Opt.* **2012**, *17* (12), 126008.
(66) Stover, C. K.; Pham, X. Q.; Erwin, A. L.; Mizoguchi, S. D.; Warrener, P.; Hickey, M. J.; Brinkman, F. S.; Hufnagle, W. O.; Kowalik, D. J.; Lagrou, M.; et al. Complete Genome Sequence of Pseudomonas Aeruginosa PAO1, an Opportunistic Pathogen. *Nature* **2000**, *406* (6799), 959–964.
(67) Rietsch, A.; Vallet-Gely, I.; Dove, S. L.; Mekalanos, J. J. ExsE, a Secreted Regulator of Type III Secretion Genes in Pseudomonas Aeruginosa. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102* (22), 8006–8011.

TABLES

Table 1: Strains and plasmids used in this study.

Strains and plasmids	Collection ID	Relevant characteristics	Source or references
Pseudomonas aeruginosa			
PAO1	PAO1	Wild-type strain	[66]
pvdAmcherry	PAS159	PAO1; *pvdAmcherry* chromosomally integrated	[49]
Δ*pvdF*Δ*pchA*	PAS283	PAO1; *pvdF* and *pchA* chromosomally deleted	[23]
Δ*pfeA*	PAS292	PAO1; *pfeA* chromosomally deleted	This study
Δ*pfeE*	PAS345	PAO1; *pfeE* chromosomally deleted	This study
Δ*feoCBA*	PAS354	PAO1; *feoCBA* chromosomally deleted	This study
pfeEmcherry	PAS355	PAO1; *pfeEmcherry* chromosomally integrated	This study
Δ*fepCBDG*	PAS356	PAO1; *fepCBDG* chromosomally deleted	This study
Escherichia coli			
TOP10		*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-*	Invitrogen
C43(DE3)		*F – ompT hsdSB (rB- mB-) gal dcm (DE3)*	Lucigen
NEB® 5-alpha		*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
Plasmids			
pEXG2	pEXG2	Allelic exchange vector with pBR origin, gentamicin resistance, sacB	[67]
pME3088 Δ*pfeA*	pVEGA18	*pME3088 carrying the sequence to delete pfeA*	[23]
pEXG2 Δ*pfeE*	pVEGA23	*pEXG2 carrying the sequence to delete pfeE*	This study
pEXG2 Δ*feoCBA*	pVEGA25	*pEXG2 carrying the sequence to delete feoCBA*	This study
pEXG2 *pfeEmcherry*	pVEGA26	*pEXG2 carrying the sequence to insert a mcherry tag in Cter of pfeE*	This study
pEXG2 Δ*fepCBDG*	pVEGA27	*pEXG2 carrying the sequence to delete fepCBDG*	This study
pBADHISTEV	pBADHISTEV	*Bacterial expression vector with Ampicillin resistance, pBR origin, araBAD promoter and an N-terminal tobacco etch virus (TEV) protease cleavable His_6-tag*	Courtesy of Dr. Huanting Liu, University of St Andrews
pBADHISTEV PfeE	pBADHISTEV_PfeE	*PBADHISTEV carrying pFeE for over-expression of PfeE in E. coli.*	This study

Table 2: Crystallographic data and refinement statistics

	WT	S157A_ferri-ENT	S157A_ ferri-TCV	WT_ ferri-TCV
Pdb code	6GI0	6GI1	6GI2	6GI5
Data collection				
Space group	P 1 21 1	$P2_12_12_1$	$P2_12_12_1$	$I4_122$
Cell dimensions				
a, *b*, *c* (Å)	45.34, 111.10, 48.50	58.55, 76.43, 130.67	57.79, 76.60, 129.87	126.61, 126.61, 293.71
α, β, γ (°)	90.0, 105.47, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å)	37.03-2.0 (2.05-2.0)	58.55-1.66 (1.69-1.66) *	129.87-1.48 (1.51-1.48) *	89.53-3.11 (3.16-3.11)
R_{sym} or R_{merge}	0.050 (0.209)	0.1291 (1.901)	0.058 (0.796)	0.193 (1.046)
$I / \sigma I$	17.3 (4.6)	11.60 (1.1)	16.8 (2.2)	12.2 (2.9)
Completeness (%)	96.6 (75.9)	100 (99.9)	100 (99.3)	99.95 (97.01)
Redundancy	3.6 (2.4)	12.61 (7.3)	7.2 (7.1)	25.2 (25.8)
CC half	0.999 (0.93)	0.998 (0.525)	0.999 (0.863)	0.998 (0.373)
Refinement				
Resolution (Å)	37.03-2.0	58.55-1.66	65.98-1.49	89.53-3.11
No. reflections	28603	66478	90838	20163
R_{work} / R_{free}	0.18/0.22	0.17/0.20	0.17/0.20	0.24/0.28
No. atoms				
Protein	3976	4087	4088	4200
Ligand/ion	8	95	126	102
Water	123	254	260	-
B-factors				
Protein	37.14	37.26	26.19	103.65
Ligand/ion	31.44	36.15	26.42	163.97
Water	31.69	40.17	29.76	-
R.m.s deviations				
Bond lengths (Å)	0.011	0.012	0.013	0.014
Bond angles (°)	1.334	1.340	1.423	1.112

Each dataset was collected from a single crystal. *Values in parentheses are for highest-resolution shell.

Figure legends:

Figure 1. Time-dependent uptake of ^{55}Fe in *P. aeruginosa* strains. A. ^{55}Fe uptake in the presence of ENT. The PAO1 strain and its corresponding deletion mutants Δ*pfeE* and Δ*pfeA* (grown in CAA medium) were incubated for 15 min in 50 mM Tris-HCl (pH 8.0) before the initiation of transport assays by the addition of 500 nM ENT-^{55}Fe (PAO1, black; Δ*pfeA*, blue and Δ*pfeE*, red). Samples (100 µL) from the suspension were removed at various time points, centrifuged, and the radioactivity accumulated in the bacteria counted. The results are expressed as pmol of ENT-^{55}Fe transported per mL of cells at an OD_{600} of 1. The experiment was repeated with the cells incubated in the presence of the CCCP protonophore at a concentration of 200 µM (PAO1, green; data not shown for Δ*pfeA* and Δ*pfeE* incubated in the presence of CCCP, but the results were equivalent to those for PAO1 incubated with CCCP). **B. ^{55}Fe uptake in the presence of 2,3 DHBS.** The experiment was carried out with the Δ*pvdF*Δ*pchA* strain (in order to avoid any ^{55}Fe uptake via pyoverdine and pyochelin, 2,3 DHBS having a lower affinity for iron compared to ENT) in the absence (purple) or presence of CCCP (orange) incubated in the presence of 500 nM $(2,3\ DHBS)_3$-^{55}Fe, prepared as described in Materials and Methods. The experiments for both 1A and 1B were repeated three times and equivalent kinetics were observed.

Figure 2. A. Immunoblot analysis of PAO1 and *pfeEmcherry* strain by western blotting with anti-DsRed antibodies. The equivalent of 0.25 OD_{600} units of each strain were lysed in loading buffer, the DNA digested by benzonase (1 U), and loaded onto SDS-PAGE gels for protein separation. Proteins were blotted onto nitrocellulose membrane and the PfeEmCherry protein detected using anti-mCherry antibodies. Molecular weight (MW) marker bands are indicated on the left.

B. Cellular fractionation of the *pfeEmcherry* strain. Bacteria (*pfeEmcherry and pvdAmcherry* strains) were fractionated as described in Materials and Methods. *pvdAmcherry* (strain expressing fluorescent tagged PvdA, an enzyme involved in pyoverdine biosynthesis) was used as a control to show the fractionation profile of a cytoplasmic fluorescent protein. The fluorescence of pyoverdine and mCHERRY were measured for equal volumes of the cytoplasmic, periplasmic, and cell-membrane fractions. The data are presented as the means of three independent experiments, shown as the percentages of the signals normalized to that of total fluorophore fluorescence. Black and gray columns represent pyoverdine and mCHERRY fluorescence, respectively. Pyoverdine fluorescence was detected only in the periplasmic fraction of *P. aeruginosa* cells.

C. Monitoring of PfeEmCherry fluorescence during bacterial growth in the presence and absence of ENT. Fresh CAA medium was inoculated with *pfeEmcherry* cells grown in the same medium and the resulting suspension dispensed into the wells of a 96-well plate. ENT (200 µM) (○) was added and a control experiment with no siderophore addition was carried out in parallel (●). OD_{600} measurements were used to assess growth over time. The fluorescence of mCherry was measured by excitation at 570 nm, with monitoring of the emission of the fluorescence at 610 nm. The measurements were performed at 30 min intervals in a Tecan microplate reader at 30 °C, with shaking. Each curve corresponds to the mean of three replicates.

D. Representative microscopy images of *pfeEmcherry P. aeruginosa* cells. Cells were cultured in the presence of 10 µM ENT and imaged using diffraction limited TIRF (greyscale image left panel) or SOFI (redscale image middle panel) (scale bar = 5µm). Scale bar for the images of the left panel = 2 µm. The uniformly distributed fluorescence of *pfeEmCherry* at the edge of the bacteria is consistent with a periplasmic location of the enzyme.

Figure 3. Separation of the PfeE reaction product by HPLC. A. Ferri-ENT at 1 mM was incubated in the presence of 10 µM purified PfeE in 50 mM HEPES buffer at pH 7.4. After a 30-min incubation, aliquots were analyzed by HPLC on an Agilent ZORBAX SB-C18 column and eluted with an H_2O/acetonitrile gradient (chromatogram in black). Assignment of the hydrolysis products is based on MS data. The retention times of ENT and 2,3-DHBS were 26 and 16 min, respectively. The experiment was repeated in the absence of PfeE (chromatogram with dashed lines). **B.** The experiment was repeated with Ferri-TCV incubated with (chromatogram with dashed lines) or without (chromatogram in grey) purified PfeE. The retention time of TCV was 23 min.

Figure 4. A. UV spectra of ENT and 2,3-DHBS in their apo and ferric forms, Fe(III), and PfeE. The spectra were measured in 50 mM HEPES buffer at pH 7.4. **B. UV spectra of ENT-Fe hydrolyzed by PfeE.** ENT-Fe (1 mM) was incubated in the presence of 1 µM PfeE in 50 mM HEPES buffer at pH 7.4. The UV spectrum was measured at T_0 (spectrum in black) and every 12 minutes for 2 h (last spectrum in light green).

Figure 5. Kinetics of ferrozine-Fe(II) formation. ENT-Fe (50 µM) was incubated with or without 500 nM PfeE, 100 mM DTT, and 20 mM ferrozine in 50 mM HEPES buffer at pH 7.4. The formation of ferrozine-Fe(II) was followed at 562 nm every 60 seconds.

Figure 6: Structural biology of PfeE. For A to E, the orange sphere corresponds to ferric iron. **A. Overall structure of PfeE.** The TCV molecule is shown as sticks (carbon in cyan, oxygen in red and nitrogen in blue). Secondary structure elements of the protein are labeled and colored red (α-helices), yellow (β-sheets) and green (loops).

B. Catalytic triad of PfeE. Carbon atoms of the DHBS are salmon and active site residues of the protein are in green. The S157A has been mutated back.

C. Fo – Fc electron density omit map at 3 σ around the DHS molecule present in chain A of the complex. DHBS is shown as sticks, with carbon atoms colored salmon; other atoms colored as in A.

D. Fo – Fc electron density omit map at 3 σ around the TCV molecule in chain A. TCV is shown as sticks, with carbon atoms colored in cyan; other atoms colored as in A.

E. Binding site of the TCV in chain A. Residues within 4.0 Å of the TCV are displayed. Carbon atoms of the TCV are cyan and residues of the protein are light blue. Active site residues are colored in green.

Figure 7: Effect of *feoCBA* and *fepCBDG* deletions on ENT-^{55}Fe uptake. ^{55}Fe repartition in the cell compartments of *P. aeruginosa* PAO1, *feoCBA*, and *fepCBDG* strains after incubation with ENT-^{55}Fe. PAO1 and its corresponding deletion mutants, *feoCBA* and *fepCBDG*, were first grown in CAA medium and then incubated with 500 nM ENT-^{55}Fe for 1 h. The cells were pelleted, the periplasmic and cytoplasmic fractions isolated for each strain, as described in Materials and Methods, and the amount of ^{55}Fe present measured (radioactivity in the extracellular medium, black bars; in the periplasm, grey bars; and in the cytoplasm, white bars). The results are expressed as the percentage of pmol of ^{55}Fe incubated with the strains.

Scheme 1: Proposed model of ENT-dependent iron uptake pathway in *P. aeruginosa*. The model indicates that PfeE is a periplasmic enzyme that hydrolysis ferri-ENT into ferri-$(DHBS)_3$. In periplasm, ferri-$(DHBS)_3$ may follow two different journeys. One possibility is an iron reduction step in this same cell compartment to get iron release and transport of ferrous iron into the cytoplasm. The second possibility is a transport of ferri-$(DHBS)_3$ into the cytoplasm

with an iron reduction step there to get iron release. In both scenario, the ferric iron reductase stays uncharacterized.