Bacterial iron acquisition mediated by outer membrane translocation and cleavage of a host protein

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Iron is an essential micronutrient for most bacteria and is obtained from iron-chelating siderophores or directly from iron-containing host proteins. For Gram-negative bacteria, classical iron transport systems consist of an outer membrane receptor, a periplasmic binding protein, and an inner membrane ABC transporter, which work in concert to deliver iron from the cell surface to the cytoplasm. We recently showed that Pectobacterium spp. are able to acquire iron from ferredoxin, a small and stable 2Fe-2S iron sulfur cluster containing protein and identified the ferredoxin receptor, FusA, a TonB-dependent receptor that binds ferredoxin on the cell surface. The genetic context of fusA suggests an atypical iron acquisition system, lacking a periplasmic binding protein, although the mechanism through which iron is extracted from the captured ferredoxin has remained unknown. Here we show that FusC, an M16 family protease, displays a highly targeted proteolytic activity against plant ferredoxin, and that growth enhancement of Pectobacterium due to iron acquisition from ferredoxin is FusC-dependent. The periplasmic location of FusC indicates a mechanism in which ferredoxin is imported into the periplasm via FusA before cleavage by FusC, as confirmed by the uptake and accumulation of ferredoxin in the periplasm in a strain lacking fusC. The existence of homologous uptake systems in a range of pathogenic bacteria suggests that protein uptake for nutrient acquisition may be widespread in bacteria and shows that, similar to their endosymbiotic descendants mitochondria and chloroplasts, bacteria produce dedicated protein import systems.

During pathogenesis, the ability of bacteria to acquire iron and other transition metal ions becomes a critical determining factor in the outcome of infection, because hosts invariably adopt a strategy of nutritional immunity to limit metal ion availability (1, 2). To overcome this strategy, Gram-negative bacteria produce a range of iron uptake systems that capture iron-containing substrates, such as siderophores, and host proteins at the cell surface and mediate energy-dependent iron transport to the cytoplasm (3, 4). In the case of iron-chelating siderophores, a specific TonB-dependent receptor (TBDR) binds and mediates passage of the iron-chelating compound into the periplasm, where it is bound by a cognate periplasmic binding protein and delivered to an inner membrane ABC transporter (3). TBDRs invariably consist of a 22-strand transmembrane β-barrel that is occluded by a small globular N-terminal plug domain (5). In these systems, binding of the siderophore to the TBDR triggers release of a TonB-box located at the extreme N terminus of the receptor. TonB, which forms an inner membrane energy transducing complex with ExbB, binds the exposed TonB-box and mechanically dissociates a force labile region of the plug domain from the barrel, allowing translocation of the substrate across the outer membrane (6, 7). In Escherichia coli, there are a number of well-characterized iron acquisition systems consisting of an outer membrane receptor, a periplasmic binding protein, and an inner membrane ABC transporter, including FhuABCD and FecABCDE, which target ferrichrome and ferric citrate, respectively (8–10).

Along with iron acquisition from siderophores, some bacterial species directly target host iron-containing proteins during infection. For example, pathogenic Neisseria species directly target the large iron-containing protein transferrin during human infection (4, 11). Iron acquisition from transferrin uses a bipartite receptor system, consisting of a TonB-dependent receptor, TbpA, and an outer membrane lipoprotein, TbpB, which work in concert to bind transferrin and liberate its iron at the cell surface. The apoprotein is then released from the cell surface, and the liberated iron is transported to the periplasm in a TonB-dependent process, where it is bound by a periplasmic binding protein and subsequently transported to the cytoplasm by an ABC transporter (4). Homologous systems for the acquisition of iron from lactoferrin and hemoglobin are also produced by pathogenic Neisseria species (2).

Interestingly, TonB-dependent receptors are commonly parasitized by colicin-like bacteriocins, multidomain protein antagonists that target bacteria closely related to the producing strain, presumably because they offer a potential route for large molecules to traverse the outer membrane (12–14). For example, colicin M uses FhuA on the surface of E. coli, and pyocin S2 uses the Pseudomonas aeruginosa ferrichrome receptor FvpAI (15–17). In the case of pyocin S2, mimicry of the interactions with the receptor by the natural substrate ferrichrome enables the pyocin to directly translocate through the lumen of FvpAI in an unfolded state. This process is TonB-dependent, with the pyocin possessing a TonB-box within an intrinsically disordered N-terminal region (18). However, although bacteriocins can parasitize transporters by mimicking the interactions of chemically and structurally dissimilar natural substrates, such as siderophores, dedicated receptor-mediated uptake systems in which the intended physiological substrate is a protein have not been previously described in Gram-negative bacteria.

We have previously shown that plant pathogenic Pectobacterium carotovorum and Pectobacterium atrosepticum are able to obtain iron from the small [2Fe-2S] iron-sulfur cluster containing protein ferredoxin by translocating ferredoxin across the outer membrane for intracellular processing by a highly specific protease, which induces iron release. The presence of homologous uptake and processing proteins in a range of important animal and plant pathogens suggests an exploitable route through which large molecules can penetrate the outer membrane of Gram-negative bacteria.

Significance

The outer membrane of Gram-negative bacteria is a highly impermeable barrier to a range of toxic chemicals and is responsible for the resistance of these bacteria to important classes of antibiotics. In this work, we show that plant pathogenic Pectobacterium spp. acquire iron from the small, stable, and abundant iron-containing plant protein ferredoxin by transporting ferredoxin across the outer membrane for intracellular processing by a highly specific protease, which induces iron release. The presence of homologous uptake and processing proteins in a range of important animal and plant pathogens suggests an exploitable route through which large molecules can penetrate the outer membrane of Gram-negative bacteria.

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plant ferredoxins that are highly abundant in plants (19, 20). Interestingly, some strains of *Pectobacterium* spp. produce ferredoxin-containing bacteriocins that contain an N terminal-[2Fe-2S]-containing ferredoxin domain linked to a lipid II-cleaving cytolysin domain (19, 21). These bacteriocins specifically target other strains of *P. carotovorum* and *P. atrosepticum* and share a common TBDR receptor with plant ferredoxin, FusA, which we have recently identified and structurally characterized (22). The genetic context of *fusA* revealed an operon encoding a putative iron uptake system with additional genes encoding a TonB homolog (*fusB*), an M16 protease (*fusC*), and an ABC transporter (*fusD*), indicating an atypical iron uptake system that lacks a classical periplasmic binding protein but, unusually, contains a predicted protease (22). M16 proteases have a clamshell-like structure that can exist in open or closed conformations, with the active site located in the interior of the clamshell (23). Consequently, substrate specificity is based in part on the steric exclusion of large substrates from the active site, and substrates of M16 proteases consist largely of peptides of 30–70 residues or signal peptides from larger proteins, as in the case of the mitochondrial processing peptidase (24, 25).

In this work, we show that FusC is a periplasmically located M16 protease with a highly targeted activity against plant ferredoxin. Cleavage of ferredoxin occurs at two specific peptide bonds on the ferredoxin surface, resulting in iron release from the normally stable-[2Fe-2S] ferredoxin iron sulfur cluster. Deletion of *fusC* in *P. carotovorum* results in an inability to acquire iron from plant ferredoxin, demonstrating that the Fus system is an atypical iron acquisition system. The periplasmic location of FusC shows that, in contrast to other iron acquisition systems that target host proteins, the small iron-containing ferredoxin is first transported into the periplasm before cleavage and iron release, a hypothesis supported by the accumulation of exogenous ferredoxin in the periplasm of cells lacking FusC. These data demonstrate that bacteria do in fact possess dedicated protein uptake systems, with the existence of homologous protease-containing iron acquisition systems in other Gram-negative bacteria, suggesting that the existence of bacterial protein uptake systems may be widespread.

**Results**

**FusC Is a Metal-Dependent Protease That Selectively Targets a Plant Ferredoxin.** Since FusC is a predicted metal-dependent M16 protease and is genetically linked to *fusA*, which is the outer membrane receptor for ferredoxin-containing bacteriocins and plant ferredoxin, we reasoned that a likely role of FusC is in iron acquisition, specifically to cleave plant ferredoxin to release iron from its normally stable iron sulfur cluster. Structural and functional studies of M16 proteases show that these proteins form a clamshell-like structure with the active site, which contains bound Zn$^{2+}$ located in the interior of this structure. Zinc is bound at the HXXEH zinc-binding motif, known as the inverzin motif, which is inverted relative to the classical HEXXH zinc-binding motif (26). To test this hypothesis, we purified His$_6$-tagged FusC and incubated it with His$_6$-tagged *Arabidopsis* ferredoxin (Fer$_{Ar}A$) at room temperature for 60 min in the presence and absence of the metal chelator EDTA and monitored proteolytic activity by SDS-PAGE. Under these conditions, we observed degradation of Fer$_{Ar}A$ in the absence of, but not in the presence of, EDTA, indicating that FusC is a metal-dependent protease and that Fer$_{Ar}A$ is a FusC substrate (Fig. L4).

Due to steric restrictions, the substrates of M16 proteases are generally limited to relatively small peptides, folded subdomains, and the unstructured regions of larger polypeptides that can access the active site (23, 24). To determine if FusC has a general proteolytic activity against other globular proteins or unfolded proteins of a similar molecular weight to ferredoxin (10 kDa), FusC was incubated with the small and stable colicin immunity proteins Im3 and Im6 (9 kDa), the RNase domain of colicin E3, E3 RNase (12 kDa), and its well-characterized intrinsically unfolded variant E3 RNase Y64A (12 kDa) (27, 28). Cleavage was not observed in any cases, indicating that FusC is a highly specific protease (*SI Appendix, Fig. S1*).

To test this hypothesis further, we then determined if FusC is able to cleave human ferredoxin 1 and 2, which, despite sharing limited sequence identity with *Arabidopsis* ferredoxin, possess a common core fold with plant ferredoxins and a [2Fe-2S] iron-sulfur cluster. Similarly, no activity against these distantly related ferredoxins was observed, indicating that FusC has a highly targeted proteolytic activity (Fig. 1B).

**Targeted Cleavage of Ferredoxin Results in Iron Release and Growth Under Iron-Limiting Conditions.** To further probe plant ferredoxin cleavage by FusC, we attempted to map cleavage sites of Fer$_{Ar}A$ using SDS-PAGE with high percentage gels enabled us to identify two resolvable bands corresponding to cleavage products of Fer$_{Ar}A$ (*Fig. 2*). These cleavage products were not further degraded with time and were stable as the concentration of FusC increased by 10-fold to 10 μM (*SI Appendix, Fig. S2*), indicating that proteolysis of Fer$_{Ar}A$ by FusC occurs specifically at a small number of sites. N-terminal sequencing of these cleavage products gave a sequence of LDDEQ for the upper band, with no sequence obtained for the lower band. The N-terminal sequence of this fragment indicates cleavage of Fer$_{Ar}A$ between F64 and L65.

Further analysis by MALDI-TOF mass spectrometry of Fer$_{Ar}A$ cleavage products directly from solution after incubation with FusC, indicated the presence of three peptides corresponding to cleavage products of Fer$_{Ar}A$ (*Fig. 2B*). Consistent with the results from N-terminal sequencing, the low-intensity peak corresponding to a peptide with an observed mass of 4,693.0 Da is close to the expected mass (4,690.2 Da) of the C-terminal fragment from cleavage of Fer$_{Ar}A$ before the identified LDDEQ sequence between F64 and L65. The observed peptide mass of 3,152.6
corresponds closely to the expected mass (3,153.4) of the central region of FerAra from I34 to F64 and the observed peptide mass of 3,542.2 is close to expected mass of the N-terminal region of FerAra (A2–G33) plus the mass of a sodium ion (3,541.6). We suspect that the sodium adduct is observed for this protein due to the extreme negative charge of the 32-residue peptide, which contains a total of 10 aspartic acid and glutamic acid residues. No peptides were observed on analysis after incubation of FusC or FerAra alone by MALDI-TOF mass spectrometry (SI Appendix, Fig. S3).

These data indicate that FerAra contains two FusC cleavage sites between G33/I34 and F64/L65. In plant ferredoxins, co-ordination of the [2Fe-2S] cluster is mediated by four cysteine residues arranged into a conserved CX4CXXCXnC motif with three N-terminal cysteine residues present on an extruded loop that lies between the first α-helix and third β-stand in the ferredoxin structure (29) (Fig. 2C). In FerAra [Protein Data Bank (PDB) ID code 4ZHO (22)], C40, C45, and C48 are present in the extruded loop, with C78 being the final coordinating cysteine. Interestingly, the G33/I34 cleavage site is located at the N terminus of the [2Fe-2S]-coordinating loop with the second F64/L65 site cleaving between the third and fourth [2Fe-2S]-coordinating cysteines (Fig. 2C). This suggests that cleavage at these sites will likely cause destabilization of the [2Fe-2S]-coordinating region of FerAra.

To test this hypothesis, we incubated FerAra with FusC and monitored the change in absorbance from 300 to 500 nm over time. Similar to other plant ferredoxins in their oxidized form, and due to coordination of the [2Fe-2S] cluster, the UV-vis spectrum of FerAra displays distinct maxima at 330 nm, 423 nm, and 466 nm (30, 31). On incubation of FerAra with FusC, we observed a time-dependent loss of absorption between 300 and 500 nm, and after 60 min, the complete disappearance of maxima at 330 nm, 423 nm, and 466 nm (Fig. 2D). On incubation of FerAra alone, no change in the absorbance spectrum was observed, showing that the protein is stable in the absence of FusC (SI Appendix, Fig. S4). These data show that FusC-mediated cleavage of FerAra leads to release of the [2Fe-2S] cluster.

To test growth enhancement on solid media, purified FerAra at 3.0, 1.0, and 0.33 mg/mL (left to right) was spotted (5 μL) onto a growing lawn of P. carotovorum LMG 2410 or LMG 2410 ΔfusC in the presence of 600 μM 2,2′-bipyridine. Plates were incubated for 24 h at 30 °C. The growth enhancement in WT but not in ΔfusC cells indicates that ferredoxin-enhanced growth is FusC-dependent.

**Fig. 2.** Targeted cleavage of ferredoxin results in iron release. (A) Limited proteolysis of FerAra by FusC. Purified His-tagged FusC (1 μM) was incubated with FerAra (100 μM) at room temperature in 50 mM Tris HCl and 50 mM NaCl, pH 7.5. Reactions were quenched by the addition of SDS loading buffer, and proteins were resolved by 18% SDS-PAGE and stained with Coomassie blue. Lane 1: FerAra alone, t = 0 min. Lanes 2, 3, and 4: FerAra + FusC incubated for 30, 60, and 90 min, respectively. (B) FusC cleaves two peptide bonds in FerAra. Three peptides, corresponding to N-terminal (A2–G33), central (I34–F64) and C-terminal (L65–H105) regions of FerAra were detected by MALDI-TOF MS. (C) Structure of Arabidopsis ferredoxin (PDB ID code 4ZHO) (22) highlighting cleavage sites and resulting N-terminal (red), central (green), and C-terminal (blue) peptides. The iron-coordinating cysteine residues C40, C45, C48, and C78 are highlighted in yellow.

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FusC-mediated loss of the FerAra [2Fe-2S] cluster suggests a role for FusC in the acquisition of iron by Pectobacterium spp. from plant ferredoxins, as we reported previously (19). To test this, we monitored growth enhancement of P. carotovorum LMG
observed, compared with an 11% increase for the ΔfusC strain (SI Appendix, Fig. S5).

Ferredoxin Is Cleaved by FusC After Uptake to the Periplasm. To determine whether ferredoxin cleavage occurs in the periplasm or on the external cell surface, we attempted to determine the cellular location of FusC. FusC is synthesized as a 924-amino acid polypeptide with a predicted 25-amino acid signal sequence (SignalP 4.1) (32) that is cleaved on Sec-mediated export to the periplasm, indicating a potential periplasmic location for the mature 101-kDa protein. In an attempt to confirm the cellular location of FusC, we raised antibodies to peptides from the FusC sequence and used these to probe fractionated cell extracts and precipitated proteins from *P. carotovorum* LMG 2410, as well as the isogenic ΔfusC strain by immunoblotting. Initial experiments with whole-cell extracts indicated that FusC production is strongly regulated by iron availability, with FusC production up-regulated under conditions of iron limitation due to the presence of the iron chelator bipyridine (Fig. 3A). Probing of fractionated cell extracts showed that FusC could be detected in the periplasmic extract, but not in the cytoplasmic contents or among proteins exported to the extracellular media (Fig. 3 B and C). Thus, FusC is an iron-regulated periplasmic protein.

In many bacteria, iron homeostasis is controlled by the ferric uptake regulator protein (Fur), a transcriptional regulator that binds to the Fur box, a conserved DNA sequence located within the promoter regions of iron-regulated genes. In *P. carotovorum*, fur regulates traits important to host–pathogen interactions, and its deletion results in increased siderophore production (33). The sequence of the *P. carotovorum* Fur box is not well characterized, however.

Since FusC has a periplasmic location, we reasoned that a ΔfusC strain, which could not proteolytically process ferredoxin, would accumulate FerAra in the periplasm. To test this, we grew wild-type and ΔfusC *P. carotovorum* LMG 2410 in the presence of FerAra and bipyridine, or both FerAra and bipyridine until an OD_{600} of 1 and tested for the presence of FerAra in the whole-cell and periplasmic fractions, as well as in the growth media after capture with nickel-affinity beads. In the ΔfusC strain grown in the presence of ferredoxin or with ferredoxin and bipyridine, we detected FerAra in both the whole-cell and periplasmic fractions but not in the media, indicating that the ΔfusC strain is able to translocate FerAra into the periplasm but cannot process it further. For the wild-type strain, we detected a small amount of FerAra in the media in the presence of ferredoxin alone but did not detect FerAra in the whole-cell or periplasmic fractions in any conditions, indicating that *Pectobacterium* with an intact Fus system can efficiently translocate and process ferredoxin (Fig. 4A).

To further investigate the kinetics of FerAra uptake, we grew *P. carotovorum* LMG 2410 ΔfusC in the presence of FerAra, and bipyridine and tested for the presence of FerAra in whole-cell extracts at 15, 45, and 120 min. After 15 min, FerAra was not detected in whole-cell extracts but was detected in increasing amounts at 45 and 120 min, respectively (Fig. 4B). Fractionation of cells and measurement of ferredoxin levels in spheroplasts and periplasmic extracts from cells harvested at these time points, along with levels in the growth media, show that time-dependent accumulation of FerAra in the periplasm is accompanied by a concomitant decrease in FerAra levels in the media to very low levels at 120 min (Fig. 4C and SI Appendix, Fig. S6). Plasmid-based complementation showed that reintroduction of fusC into *P. carotovorum* LMG 2410 ΔfusC was able to completely reverse periplasmic ferredoxin accumulation on isopropyl-β-D-thiogalactopyranoside (IPTG)-mediated induction of FusC production (Fig. 4D).

**Discussion**

In this work, we build on our previous identification of the ferredoxin receptor FusA and show that the periplasmic protease FusC has highly targeted activity against plant ferredoxin, and that iron acquisition by *Pectobacterium* spp. from ferredoxin is FusC-dependent. Thus, in our current model of Fus-mediated iron acquisition (Fig. 5), the substrate is bound at the cell surface by the...
A. Ferredoxin

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Bacterial Strains and Media. E. coli was grown in LB broth or plated on LB agar in the presence of ampicillin (100 μg mL⁻¹). DH5α and BL21 (DE3) strains were used as host strains for cloning and for IPTG-induced protein expression, respectively. E. coli strains were grown at 37 °C, and P. carotovorum strains were grown in LB broth or plated on LB agar at 30 °C with the addition of the iron chelator 2,2′-bipyridine where specified.

Protein Purification. FusC was expressed in E. coli BL21 (DE3) carrying the plasmid pFusC, which encodes FusC from P. atrosepticum SCR1043 with an additional LEHHHHHHH sequence for purification by nickel-affinity chromatography at the C terminus. More details are provided in SI Appendix, Materials and Methods. Arabidopsis ferredoxin (FerH₁) was purified as described previously (22), and human ferredoxin 1 (FerH₁) and human ferredoxin 2 (FerH₂) were purified with the same method from BL21 (DE3) cells harboring the plasmids pHFdx1 and pHFdx2, respectively. E3RNase, E3RNase Y64A Im₃, and E3RNase Y64A Im₆ were purified as described previously (27, 28).

Proteolytic Activity and Iron Release. Purified FusC and potential substrate proteins were incubated in 50 mM Tris·HCl and 50 mM NaCl, pH 7.5, at room temperature at the specified concentrations. Samples were removed at the

TBDR FusA and is translocated into the periplasm in a process presumably involving FusB, a TonB homolog that is conserved in the Fus operon of Pectobacterium spp. However, the exact role of FusB remains to be proven, and it can be envisaged that FusB acts in a similar manner to TonB through interaction with a TonB-box of the TBDR to mediate extraction of the FusA plug domain. Alternatively, if endogenous TonB is able to fulfill this role, then FusB may mediate the energy-dependent translocation of ferredoxin into the cell by direct contact with the substrate subsequent to extraction of the plug domain by TonB. On translocation into the periplasm, ferredoxin is then cleaved by FusC, triggering release of the [2Fe-2S] cluster, which we hypothesize is transported to the cytoplasm in a process that may be mediated by the putative ABC transporter encoded by fusD.

As we have described previously, homologs of the fus operon that minimally encode a TBDR and an M16 protease are found in the genomes of a range of pathogenic Gram-negative bacteria, including E. coli and members of the genera Neisseria and Yersinia and the Pasteurellaceae (22). Although in these cases the substrate has not been identified, the presence of a genes encoding a large (approximate 100 kDa) TBDR (TBDRs that bind siderophores are typically much smaller at 70–80 kDa) and a periplasmic M16 protease suggest a protein substrate, indicating that uptake and cleavage of proteins for nutrient acquisition may be widespread in bacteria.

The existence of dedicated and highly specific protein uptake systems in Gram-negative bacteria suggests that receptor-mediated protein uptake in mitochondria and plastids might not have evolved independently of their bacterial ancestors, although whether this indeed is the case remains to be determined. However, it is interesting to note that the processing of proteins imported into these organelles is mediated in part by M16 protease family members, such as the mitochondrial processing peptidase and presequence proteases that cleave and degrade signal peptides of imported mitochondrial proteins (24, 25).

Materials and Methods

Top His-tagged FerAra is detected in whole-cell fractions in the presence of 1 mM IPTG or 1% lactose. One of the experiment and progressively accumulated in the periplasm. (D) Plasmid based complementation of P. carotovorum LMG2410 ΔfusC with pFusC restores ferredoxin processing. WT, ΔfusC, and recomplemented ΔfusC+pFusC cells were cultured in LB media until OD₆₀₀ ≈ 0.4, after which FusC expression in two of the ΔfusC+pFusC cultures was induced by the addition of 1 mM IPTG or 1% lactose. One ΔfusC+pFusC culture labeled (+) contained 0.2 mM IPTG added at the start. At 30 min postinduction, 1 μM ferredoxin (either FerH₁ or human ferredoxin) was added. Cultures were incubated at 37 °C for 60 min and after 2 h, the cells were fractionated. Purified FerAra was run in the lane marked F. αToI and GroEL loading controls probed with αToI and αGroEL antibodies, respectively, are shown at the bottom.

Fig. 4. Ferredoxin is translocated to the periplasm for FusC-dependent processing. (A) Internalization assay showing the accumulation of ferredoxin in P. carotovorum LMG2410 ΔfusC, but not in WT cells. The cells were cultured in M9 media in the presence or absence of 1 μM ferredoxin or 100 μM 2,2′-bipyridine until OD₆₀₀ =1 and then subjected to fractionation, whole-cell (WC) and periplasmic fraction (P). M, elution from nickel beads after incubation with filtered growth media. (Top) Blots reprobed with αHis antibodies showing that FerAra can be detected in whole-cell and periplasmic fractions in the ΔfusC strain, but not in the WT strain due to proteolytic processing by FusC. Preliminary experiments showed that FerAra migrates anomalously in whole-cell fractions due to the presence of the presequence protease that cleave and degrade signal peptides of imported mitochondrial proteins (24, 25).

(Top) Blots reprobed with αHis antibodies showing that FerAra can be detected in whole-cell and periplasmic fraction (P). M, elution from nickel beads after incubation with filtered growth media. (Bottom) Blots reprobed with αTolB antibodies. (B) Time course of ferredoxin internalization in P. carotovorum LMG2410 ΔfusC. FerAra content in the whole cells (Left) together with the GroEL loading control (Right). (C) Fractionation of cells from the time course in B; spheroplast (S), periplasm (P), and media (M) fractions at each time point are shown. F, purified FerAra control. FerAra levels decreased in the media over the course of the experiment and progressively accumulated in the periplasm. (D) Plasmid based complementation of P. carotovorum LMG2410 ΔfusC with pFusC restores ferredoxin processing. WT, ΔfusC, and recomplemented ΔfusC+pFusC cells were cultured in LB media until OD₆₀₀ ≈ 0.4, after which FusC expression in two of the ΔfusC+pFusC cultures was induced by the addition of 1 mM IPTG or 1% lactose. One ΔfusC+pFusC culture labeled (+) contained 0.2 mM IPTG added at the start. At 30 min postinduction, 1 μM ferredoxin (either FerH₁ or human ferredoxin) was added. Cultures were incubated at 37 °C for 60 min and after 2 h, the cells were fractionated. Purified FerAra was run in the lane marked F. αToI and GroEL loading controls probed with αToI and αGroEL antibodies, respectively, are shown at the bottom.
SDS-PAGE. MALDI-TOF mass spectrometry was performed on FusC, FerAra, and FusD. To follow the release of iron from FusC, a novel method was used. An Arabidopsis ferroxidin resulting from the proteolytic activity of FusC, we measured the absorbance spectra of FeFus (100 μM) alone or FerFus (100 μM) mixed with FusC (1 μM) from 0 to 120 min. Experiments were performed in 50 mM Tris-HCl and 50 mM NaCl, pH 7.5, and spectra were recorded on a Shimadzu UV-1700 spectrophotometer using 1-cm path length quartz cuvettes.

Creation of P. carotovorum LMG2410 ΔfusC. The sequence of the P. carotovorum LMG2410 FusC gene was determined by amplifying and sequencing PCR products using a forward primer (5′-CGA GTT TCA ATG CCA TTG TC-3′) that anneals within the 3′ end of the FusA gene (sequence data available) and a reverse primer (5′-CAGTCTTCACCTTGGT-3′) based on sequence downstream of FusC gene that is conserved across different strains of Pectobacterium spp. The FusC gene was deleted using the lambda red protocol (34) as described in SI Appendix, Materials and Methods.

Fig. 5. Schematic representation of Fus-dependent import and processing of ferredoxin. Ferredoxin is imported via the TBDR FusA (22) presumably mediated by the TonB homolog FusB, which may play a conventional TonB-like role in removal of the plug domain from the lumen of FusA, or may directly contact the substrate. On transport to the periplasm, FusC cleaves ferredoxin, and we hypothesize this leads to the release of the [2Fe-2S] cluster, which may be transported to the cytoplasm by the putative ABC transporter FusD. The exact form of iron present in the periplasm on release from ferredoxin remains to be determined, however.

indicated time points, and the reaction was quenched by the addition of SDS loading buffer. Samples were heated to 95 °C for 2 min and then analyzed by SDS-PAGE. MALDI-TOF mass spectrometry was performed on FusC, FerAra, and FusC + FerAra, after incubation at room temperature for 1 h in 50 mM Tris-HCl and 200 mM NaCl, pH 7.5, and quenching with 1 mM EDTA with FeFus at 10 μM and FusC at 1 μM. Details the MALDI-TOF analysis are provided in SI Appendix, Materials and Methods.

FusC Localization. FusC was localized by cell fractionation and immunoblotting using an antisFusC antibody, raised to two peptides (RVIQAIRHDSRYSR and LARKQANDDQSV) in rabbits and subsequently affinity-purified (Eurogentec). Cell fractionation was achieved by the formation of spheroplasts and isolation of the periplasmic fraction. Whole-cell extracts were obtained using BugBuster Protein Extraction Reagent (Merck). To determine if FusC is exported into the extracellular medium, protein in the supernatant was precipitated with trichloroacetic acid. More information is provided in SI Appendix, Materials and Methods.

Growth Enhancement Assays. Enhancement of growth on solid media was determined using the soft agar overlay method (19, 35), as described along with the method used for growth enhancement in liquid culture in SI Appendix, Materials and Methods.

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