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.

D uring 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 dislocates 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 TonB-related periplasmic binding protein, and an inner membrane ABC transporter, including FhuABCDE and FecABCDE, which target ferrichrome and ferric citrate, respectively (8–10).

Along with iron acquisition from siderophores, some bacterial species specifically 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 antibiotics 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 ferrisiderophore receptor FvpAI (15–17). In the case of pyocin S2, mimicry of the interactions with the receptor by the natural substrate ferrisiderophore 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 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 cytotoxic 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 clamsshell (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 clamsshell-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$^+$-tagged FusC and incubated it with His$^+$-tagged *Arabidopsis* ferredoxin (Fer$_{ArA}$) 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$_{ArA}$ in the absence of, but not in the presence of, EDTA, indicating that FusC is a metal-dependent protease and that Fer$_{ArA}$ is a FusC substrate (Fig. 1A).

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$_{ArA}$ using SDS-PAGE with high percentage gels enabled us to identify two resolvable bands corresponding to cleavage products of Fer$_{ArA}$ (Fig. 2A). 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$_{ArA}$ 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$_{ArA}$ between F64 and L65.

Further analysis by MALDI-TOF mass spectrometry of Fer$_{ArA}$ cleavage products directly from solution after incubation with FusC, indicated the presence of three peptides corresponding to cleavage products of Fer$_{ArA}$ (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$_{ArA}$ 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, coordination 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-sheet 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.

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 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.
P. carotovorum type LMG 2410, a 52% increase (relative to no FerAra addition) served for wild-type LMG 2410, but not for LMG 2410 enhancement due to the addition of purified FerAra were 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-aa polypeptide with a predicted 25-aa 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 OD₆₀₀ 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 built 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...

Fig. 3. FusC is an iron-regulated periplasmic protein. (A) FusC production is up-regulated under iron-limiting conditions. Whole-cell extracts of wild-type LMG 2410 (WT) and LMG2410 ΔfusC (ΔFusC) from cells grown in the absence (−) or presence (+) of 200 μM bipyridine were subjected to immunoblotting with αFusC antibodies. Purified FusC was run in the left lane as a marker. (B) FusC is a periplasmic protein. (Top) Wild-type LMG2410 (WT) and LMG2410 ΔfusC (ΔFusC) cells grown in the absence (−) or presence (+) of 200 μM 2,2′-bipyridine were fractionated, and whole-cell (WC), periplasmic (P), and spheroplast fractions were immunoblotted using αFusC antibodies. Purified FusC was run in the left lane as a marker. (Bottom) Periplasmic and spheroplast fractions were also subjected to αGroEL immunoblotting to determine the level of spheroplast leakage. GroEL was not identified in the periplasmic fraction. αTolB antibodies were used as a marker of periplasmic proteins, although, as previously described in E. coli, TolB is also associated with the cytoplasmic membrane and therefore with spheroplasts, presumably due to its interaction with the inner membrane TolAQR complex (36). (C) FusC is not exported to the extracellular medium. Whole-cell and precipitated proteins from the supernatant (EX) from LMG2410 (WT) cells grown in the absence (−) or presence (+) of 200 μM bipyridine or in the presence of 200 μM bipyridine and 1 μM FerAra (F) were subjected to immunoblotting with αFusC antibodies. No FusC was detected in the extracellular medium.

2410 and an isogenic ΔfusC strain after the addition of FerAra on solid media under iron-limiting conditions due to the presence of the iron chelator 2,2′-bipyridine (bipyridine). Zones of growth enhancement due to the addition of purified FerAra were observed for wild-type LMG 2410, but not for LMG 2410 ΔfusC, indicating that iron acquisition by Pectobacterium spp. from plant ferredoxin requires FusC-mediated cleavage and iron release (Fig. 2E). Enhancement of growth on the addition of FerAra in liquid media was also observed to a greater extent for wild-type P. carotovorum LMG 2410 relative to the ΔfusC strain. For wild-type LMG 2410, a 52% increase (relative to no FerAra addition) in OD₆₀₀ after 5 h of growth in the presence of 0.2 μM FerAra was observed, compared with an 11% increase for the ΔfusC strain (SI Appendix, Fig. S5).

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...
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Fig. 4.
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plasmid pFusC, which encodes FusC from
P. carotovorum
′-bipyridine until OD600 ≈
(22). Although in these cases the
substrate has not been identified, the presence of a genes
coding 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, in-
dicating that uptake and cleavage of proteins for nutrient ac-
quision 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 in-
teresting 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
protease that cleave and degrade signal peptides of
imported mitochondrial proteins (24, 25).

Materials and Methods
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 expres-
sion, 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 ad-
dition, respectively.

Materials

Arabidopsis ferredoxin (FerAra) was purified as described pre-
viously (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 Y66A Im3 and E3RNase Y66A 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
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 and 200 μM 2,2′-bipyridine were added, and after 2 h,
the cells were fractionated. Purified FerAra was run in the lane marked F.
ToB and GroEL loading controls probed with αToB 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 ferre-
doxin 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 cells (WC) and periplasmic fraction (P). M, elution from nickel beads
after incubation with filtered growth media. (Top) His-tagged FerAra is de-
tected by the αHis antibody 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
BugBuster. (Bottom) Blots reprobed with αToB 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),
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30. Hall DO, Cammack R, Rao KK (1973) The plant ferredoxins and their relationship to bacterio-ferredoxin resulting from the proteolytic activity of FusC. We measured the absorbance spectra of FeFusC at 10 μM and FusC at 1 μM. Details the MALDI-TOF analysis are provided in SI Appendix, Materials and Methods.
31. To follow the release of iron from FeFusC by Autoinduction, ferredoxin resulting from the proteolytic activity of FusC, we measured the absorbance spectra of FeFusC at 10 μM and FusC at 1 μM. 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′-CCTCTTCACACCTTGT-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.

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.

FusC Localization. FusC was localized by cell fractionation and immunoblotting using an αFusC antibody, raised to two peptides (RVOAIRHDSRYSR and LARQKANDQOSV) 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.

Ferredoxin Internalization. The ferredoxin internalization assays are described in detail in SI Appendix, Materials and Methods.

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