Structure of the bacterial plant-ferredoxin receptor FusA

Iron is a limiting nutrient in bacterial infection putting it at the centre of an evolutionary arms race between host and pathogen. Gram-negative bacteria utilize TonB-dependent outer membrane receptors to obtain iron during infection. These receptors acquire iron either in concert with soluble iron-scavenging siderophores or through direct interaction and extraction from host proteins. Characterization of these receptors provides invaluable insight into pathogenesis. However, only a subset of virulence-related TonB-dependent receptors have been currently described. Here we report the discovery of FusA, a new class of TonB-dependent receptor, which is utilized by phytopathogenic Pectobacterium spp. to obtain iron from plant ferredoxin. Through the crystal structure of FusA we show that binding of ferredoxin occurs through specialized extracellular loops that form extensive interactions with ferredoxin. The function of FusA and the presence of homologues in clinically important pathogens suggests that small iron-containing proteins represent an iron source for bacterial pathogens.
The central role of iron in the electron transfer reactions of cellular redox chemistry and its insolubility under oxygenic conditions makes it a generally limiting nutrient for microbial growth. Eukaryotic organisms exploit this limited availability, via a mechanism termed ‘nutritional immunity’, hindering the growth of pathogenic microbes by tightly sequestering iron within specialized proteins. To counter this, microbes have developed specialized systems for liberating and importing iron from host proteins. In Gram-negative bacteria, outer membrane receptors of the TonB-dependent receptor (TBDR) family fulfill this role by binding microbial iron-scavenging siderophores and iron-containing host proteins such as lactoferrin, transferrin and haemoglobin. TBDRs interact with their substrates through a highly specialized extracellular structure, formed by the outer loops of a 22-stranded transmembrane β-barrel. After these initial interactions, this barrel provides a conduit for the iron or iron-siderophore complex to cross the outer membrane. As illustrated by structural and evolutionary analysis of the TonB-dependent transferrin receptor from the genus Neisseria, these systems play an important role in pathogenesis and represent part of the evolutionary arms race between host and pathogen. In contrast to our understanding of protein-binding TBDRs from mammalian pathogens, there have been no reports of specialist receptors utilized by plant pathogens to obtain iron from host proteins during infection.

Previously, we reported the discovery and subsequent structural characterization of the pectocins, an unusual class of colicin-like bacteriocins. Colicin-like bacteriocins are protein toxins produced by Gram-negative bacteria mostly for intraspecies or intragenus competition and often parasitize TbDRs to gain entry to their target cell. Pectocins M1 and M2, which are produced by phytopathogenic Pectobacterium spp. for intra-specific competition, contain a cytotoxic domain that is active against the cell wall precursor lipid II in the periplasm, fused to an iron-containing plant-like ferredoxin that acts as a receptor-binding domain. During our characterization of the pectocins, it became apparent that in addition to being susceptible to a ferredoxin-containing bacteriocin, Pectobacterium spp. are also able to utilize plant ferredoxins as an iron source under iron-limiting conditions. Moreover, competition experiments showed that both the pectocins and ferredoxin are bound by the same receptor during cell entry.

In our current work we have identified the outer membrane receptor responsible for ferredoxin and pectocin import in Pectobacterium spp., which we designate FusA. To understand the mechanism of ferredoxin import we have solved the crystal structure of FusA and two of its plant ferredoxin substrates, and using nuclear magnetic resonance (NMR)-driven molecular docking we have modelled the FusA-ferredoxin complex. In addition, through bioinformatic analysis, we show that FusA homologues are widespread in members of Enterobacteriaceae that form commensal or pathogenic associations with mammalian hosts. This suggests that this family of TBDRs also plays role in iron acquisition from the mammalian host.

Results

Identification of the pectocin M1 receptor in Pectobacterium. To identify the outer membrane receptor of the ferredoxin uptake system, which we designated FusA, we isolated proteins from the outer membrane of the pectocin M1-sensitive Pectobacterium strain Pectobacterium atrosepticum LMG2386 and applied them to a nickel affinity column pre-loaded with His6-tagged pectocin M1. After elution of bound protein we observed a protein on SDS-PAGE at ~100 kDa, which co-purified with pectocin M1 (Supplementary Fig. 1A). Peptide mass fingerprinting identified a 97 kDa TBDR PCC21_007820 from Pectobacterium carotovorum (Pbc) subsp. carotovorum PCC21 as the closest match (Supplementary Fig. 1B). To confirm this protein interacts with the pectocins, we repeated this experiment with the outer membrane fraction from Escherichia coli recombinantly expressing FusA (PCC21_007820), showing that recombinant FusA also interacts with pectocin M1 and M2 (Supplementary Fig. 1C). We then constructed a ΔfusA mutant using P. atrosepticum LMG2386 and determined its sensitivity to pectocin M1. In contrast to the parent strain, the ΔfusA mutant shows complete resistance to pectocin M1, with complementation of fusA restoring sensitivity (Fig. 1a). Thus, FusA is the receptor for the ferredoxin domain containing bacteriocin pectocin M1. As we have previously shown that pectocin M1 and spinach ferredoxin compete for binding to the same receptor, we also propose that FusA is also a plant ferredoxin receptor.

Bioinformatic analysis of this newly identified receptor shows that closely related homologues of fusA are found in all other sequenced strains of P. atrosepticum and P. carotovorum (>75% amino-acid sequence identity) and strains of the related soft rot pathogens of Dickeya species (>60% identity). In all cases, fusA is found in a putative operon with three additional genes that encode a TonB-like protein, a predicted periplasmic M16 protease.

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**Figure 1** FusA from Pectobacterium is responsible for susceptibility to the ferredoxin domain containing bacteriocin pectocin M1. (a) Purified pectocin M1 spotted onto iron-limiting Lysogeny broth (LB) agar plus IPTG overlaid with a lawn of soft agar seeded with P. atrosepticum LMG2386, Wt, ΔfusA or ΔfusA complemented with a plasmid derived copy of fusA with an IPTG inducible promoter. Genetic knockout of fusA totally abolishes susceptibility of the strain to pectocin M1, while complementation of the knockout strain with plasmid encoded fusA restores the pectocin M1 susceptible phenotype (experiment repeated >6 times). (b) A schematic of the proteins of the Fus operon showing their predicted cellular localization and function. (c) The genetic organization of the putative Fus operon in Pectobacterium spp. and homologous genes from a subset of human pathogens.
and a fused ABC transporter that we refer to as fusB, fusC and fusD, respectively (Fig. 1b). In Dickeya dadantii, homologues of fusA and fusC are among the most highly upregulated genes during plant infection. Interestingly, more distantly related homologues of the fus operon were also identified in bacteria that colonize and cause disease in mammalian hosts. These species include E. coli and members of the genera Neisseria, Shigella, Yersinia, Pasteurella, as well as more distantly related members of β, δ and ε-proteobacteria (Fig. 1c, Supplementary Fig. 2 and Supplementary Table 1). Many of these species renowned for their utilization of specialized TBDRs and associated proteins to obtain iron from their hosts. In Pasteurella multocida, fusC is upregulated in response to iron limitation and during infection, and in uropathogenic E. coli, homologues of FusA (YddB) and FusC (PqqL) are important for fitness in systemic infection. The presence of genes encoding FusA homologues in bacteria associated with infection of diverse eu-karyotic hosts suggests that the use of small iron-containing proteins as an iron source during infection extends beyond Pectobacterium spp.

The crystal structure of the ferredoxin receptor FusA. To determine the structural basis of ferredoxin binding by FusA we solved the structure of FusA by X-ray crystallography. Recombinant FusA (derived from P. atrosepticum SCR1043) was expressed in E. coli and purified and refolded from inclusion bodies to yield stable, monodisperse FusA, which was used to undertake crystallization trials (Supplementary Fig. 3). Sparse matrix screening yielded crystals in a number of conditions and undertaking crystallization trials (Supplementary Fig. 3). Sparse bodies to yield stable, monodisperse FusA, which was used to

| Table 1 | FusA and ferredoxin crystallographic data collection and structural solution statistics. |

|                     | FusA native (4ZGV) | FusA PCl4 | Arabidopsis ferredoxin (4ZHO) | Potato ferredoxin (4ZHP) |
|---------------------|--------------------|-----------|-------------------------------|--------------------------|
| **Data collection** |                    |           |                               |                          |
| Space group         | P2_1               | P2_1      | P4_22,2                       | I222                     |
| Cell dimensions     | a, b, c (Å)        |           |                               |                          |
|                     | 137.27, 79.89, 137.90 | 136.97, 78.44, 136.86 | 60.73, 60.73, 154.73 | 50.66, 71.69, 79.32 |
|                     | a, b, c (°)        |           |                               |                          |
|                     | 90.0, 90.7, 90.0   | 90.0, 90.56, 90.0 | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| Peak wavelength     | –                  | 0.88      |                               |                          |
| Resolution (Å)*     | 48.95–3.20 (3.31–3.20) | 48.64–4.20 (4.54–4.20) | 60.73–2.34 (2.40–2.34) | 42.70–2.46 (2.52–2.46) |
| Rmerge               | 30.5 (173.4)       | 16.2 (58.8) | 6.6 (59.8) | 12.4 (59.6) |
| Rwork               | 13.7 (80.1)        | 7.5 (26.3) | 2.7 (25.7) | 5.2 (24.9) |
| I/σ(I)              | 7.0 (1.3)          | 9.9 (3.7) | 23.2 (4.2) | 13.9 (4.3) |
| CC1/2               | 58.7 (56.7)        | 99.7 (91.0) | NA | NA |
| Completeness (%)    | 100.0 (100.0)      | 99.9 (100.0) | 99.4 (98.4) | 99.7 (99.4) |
| Redundancy          | 6.6 (6.4)          | 6.7 (6.8) | 12.3 (11.8) | 12.3 (12.8) |
| **Refinement statistics** |                    |           |                               |                          |
| Resolution (Å)      | 48.95–3.20 (3.31–3.20) | 48.64–4.20 (4.54–4.20) | 60.73–2.34 (2.40–2.34) | 42.70–2.46 (2.52–2.46) |
| No. of reflections  | 49734 (4517)       | 21,631 (4,438) | 12,894 (904) | 5,483 (382) |
| Rwork/Rfree         | 219.27/1.1         | 19.51/23.79 | 17.75/21.68 |                          |
| No. of atoms        | 12,895             | 1462      | 740                           |                          |
| Protein             | (β-OG, LDAO) 129   | (2Fe-2S cluster) 4 | (2Fe-2S cluster) 4 |                          |
| Water               | 0                  | 21        | 16                            |                          |
| r.m.s. deviations   | Bond lengths (Å)   | 0.011     | 0.017                         | 0.016                     |
|                      | Bond angles (°)    | 1.66      | 1.96                          | 1.98                      |

NA, not applicable; r.m.s., root mean squared.

*Values in parentheses are for highest-resolution shell.

Data from one crystal were collected for each structure.

See Supplementary Fig. 13 for representative electron density maps for each structure.
greatest sequence variation between FusA homologues, the charged surface this region presents is largely conserved (Supplementary Fig. 8C,D).

**FusA forms extensive interactions with ferredoxin.** To probe the interactions between FusA and its ferredoxin substrate, using NMR, we expressed and purified $^{15}$N-labelled, ferredoxin domain from pectocin M1 (PM1$_{fer}$), and ferredoxin isoform 2 from *Arabidopsis thaliana* (NP_176291) (Ferara). Titration of purified FusA into $^{15}$N-labelled Ferara resulted in a decrease in the intensity of the $^{15}$N heteronuclear single quantum correlation (HSQC) spectral peaks that was proportional to FusA concentration, suggesting formation of a 1:1 complex in an intermediate to slow exchange regime (Supplementary Fig. 9). In contrast, titration of $^{15}$N-labelled PM1$_{fer}$ with FusA gave HSQC...
spectra in which some peaks were relatively unperturbed while others became broadened and shifted with increasing FusA concentration. At a 1:1 stoichiometry, the majority of PM1fer peaks were still visible (Fig. 4a and Supplementary Fig. 10), indicating that formation of the complex between PM1fer and FusA occurs in the fast exchange regime. The FusA-binding surface of ferredoxin could therefore be mapped based on chemical shift perturbation (CSP) analysis. Using $^{13}$C,$^{15}$N-labelled PM1fer 56 backbone amides (of a total of 70 observed) were assigned, of which 34 display a CSP of $>0.01$ ppm on addition of FusA (Fig. 4b and Supplementary Fig. 10). Residues displaying a CSP of $>0.02$ ppm form a contiguous binding surface on the ferredoxin, predominantly localized to the ferredoxin β-sheet. This binding surface is composed of charged (K7, E14, D56, D64 and K86), polar (S54, Q67 and N80) and non-polar side chains (G11, L50, I51, Y70 and V84) (Fig. 4c).

NMR-driven docking of ferredoxin to FusA. Using the CSP data obtained from the FusA–PM1fer NMR experiments, we modelled the FusA–ferredoxin complex. We solved the crystal structures of *Arabidopsis* ferredoxin isoform 2 and potato (*Solanum tuberosum*) ferredoxin isoform 1 (CAC38395) (Fer pot) and performed docking with extracellular portion of the FusA structure using the programme HADDOCK2. Docking solutions formed two major clusters in which the ferredoxins docked with the extracellular pocket of FusA, with the C terminus either pointing up and away from the FusA barrel or at right angles to it (Supplementary Fig. 11A and Supplementary Data Set 1–4). The C-terminal up solution was favoured for both ferredoxins (Supplementary Table 2). Superimposition of the crystal structures of pectocin M2 with these solutions, based on their common ferredoxin domains, showed that only in the C-terminal up orientation was the cytotoxic domain of these structures accommodated without significant clashes with FusA, strongly suggesting it is the correct solution (Supplementary Fig. 11B). In this solution, the ferredoxin molecule is positioned directly over the pore of the FusA barrel and forms extensive interactions with the β-wall formed by loops 4, 5 and 7, and the plug domain loop of FusA (Fig. 5a, b). These interactions correlate well with the FusA-binding surface predicted by our NMR experiments, suggesting that these features are responsible for the initial stages of ferredoxin binding (Fig. 5c). When normal mode analysis was repeated on this docked complex, FusA underwent analogous conformational changes to unliganded FusA, indicating closure of the outer loops around the ferredoxin in a glove-like fashion (Supplementary Fig. 12 and Supplementary Movie 1). These conformational changes possibly represent those that occur upon ferredoxin binding and import by FusA.

Growth enhancement of *Pectobacterium* by plant ferredoxin. The FusA–ferredoxin docking simulations indicate that a discrete FusA-binding surface of ferredoxin mediates initial binding to the...
receptor. As such, it would be expected that sequence variation at this interface would affect the interaction of FusA with its ferredoxin substrate. To investigate this we tested purified leaf ferredoxins from potato isoform 1, maize (Zea mays) isoform 1 (ACG46956) and Arabidopsis isoform 2, which share 70–75% sequence identity, for their ability to enhance the growth of a panel of Pectobacterium isolates. Interestingly, these proteins enhanced growth to widely varying degrees (Supplementary Table 3 and Fig. 6a). Arabidopsis ferredoxin enhanced the growth of 14/17 strains, while potato ferredoxin enhanced 9/17 strains, with a noticeably weaker overall effect. Maize ferredoxin only enhanced one strain weakly, demonstrating that the discrete amino-acid sequence differences between the ferredoxins have a large effect on the ability of FusA to utilize the protein as a substrate (Fig. 6b,c). On the basis of our docking experiments, a number of the amino-acid differences between the three ferredoxins occur at the FusA–ferredoxin interface. Sequence divergence at the protein–substrate interface has been observed for the neisserial transferrin substrate interface has been observed for the neisserial transferrin and the family Pasteurellaceae6,17. Studies on FusA homologues in these species are limited, however upregulation in response to iron limitation and the presence of host factors has been demonstrated24. In a recent study, the E. coli homologues of both FusA and the predicted periplasmic protease FusC were shown to be important for the fitness of the UPEC strain CFT073 in systemic infection of mice. This study also showed that the genes encoding these proteins, designated yddB and pqqL, belong to a single operon, which also contains yddA, a FudD homologue25. Interestingly, the order of the genes in this operon differs from that of the Fus operon, with the gene order varying further in homologues from Proteus and Yersinia (Fig. 1c). The maintenance of these genes in the same gene cluster, despite rearrangement, strongly suggests that they have a highly integrated function. While further investigation is required, together these data strongly suggest that the Fus proteins are members of a family that play an important role in the acquisition of iron from small iron-containing host proteins.

Our structural analysis of FusA shows that it is the member of a unique family of TBDRs. The extracellular and plug domain loops are highly extended, which is characteristic of a TBDR with a bulky protein substrate. However, the structure formed by these loops and the pattern of loop extension is distinct from other protein-binding TBDRs, showing that FusA has evolved to specifically bind its ferredoxin substrate26. Interestingly, sequence analysis of FusA homologues reveals the same pattern of loop extension, with the difference in length of homologues largely accounted for by the size of these extensions. Our modelling of the FusA–ferredoxin complex shows that the structures formed by these loops forms a large binding surface with the ferredoxin molecule and grasp it in a glove like fashion. It is tempting to speculate that this grasping motion of FusA, predicted by NMA simulations, represents the initial stages of substrate import in vivo. Whether the intact ferredoxin is then imported by FusA or somehow the iron sulphur cluster is liberated at the bacterial cell surface remains to be proven. However, the fact that the ferredoxin-containing pectocins are able to cross the outer membrane and enter the periplasm, through interaction with FusA suggests that importation of the intact ferredoxin is plausible26. Importation of the ferredoxin by FusA is also reasonable given that its periplasmic protease FusC, which could cleave the ferredoxin on import, liberating the iron–sulphur cluster for import across the inner membrane by the ABC transporter FusD.

In summary this study reports and the structural characterization of a TBDR receptor that binds to iron–sulphur cluster containing ferredoxin domains.

**Discussion**

Despite the universal importance of iron as a limiting nutrient for microbial growth and in pathogenesis, our understanding of the systems that bacteria use for obtaining it from their host is far from comprehensive4. This situation is even more pronounced for bacterial phytopathogens, where very little is known about the role that specific iron import systems play in infection1. The discovery of FusA demonstrates that, like their mammalian pathogenic counterparts, phytopathogenic bacteria can use a TBDR to specifically target iron-containing host proteins23. The presence of the FusA in Dickeya and Pantoaea, which are closely related to Pectobacterium, suggests the ferredoxin uptake system represents an important iron acquisition tool for soft rot pathogens16. FusA homologues however are not limited in their distribution to phytopathogens, with our analysis of microbial genomes revealing that more distantly related clades are present in proteobacterial species that adopt a commensal or pathogenic lifestyle with mammalian hosts. These species include E. coli and members of the genera *Shigella* and *Yersinia* and the family Pasteurellaceae6,17. Studies on FusA homologues in these species are limited, however upregulation in response to iron limitation and the presence of host factors has been demonstrated24. In a recent study, the E. coli homologues of both FusA and the predicted periplasmic protease FusC were shown to be important for the fitness of the UPEC strain CFT073 in systemic infection of mice. This study also showed that the genes encoding these proteins, designated yddB and pqqL, belong to a single operon, which also contains yddA, a FudD homologue25. Interestingly, the order of the genes in this operon differs from that of the Fus operon, with the gene order varying further in homologues from *Proteus* and *Yersinia* (Fig. 1c). The maintenance of these genes in the same gene cluster, despite rearrangement, strongly suggests that they have a highly integrated function. While further investigation is required, together these data strongly suggest that the Fus proteins are members of a family that play an important role in the acquisition of iron from small iron-containing host proteins.

**Methods**

**Expression and purification of ferredoxins and pectocins.** The open reading frames (ORFs) for pectocin M1 and M2 and the plant ferredoxins (minus the stop codon) were cloned into pET21a (Invitrogen) and expressed in *E. coli* BL21 (DE3).
Cells were grown at 37 °C and protein expression was induced by the addition of 0.3 mM isopropyl-β-D-thiogalactoside (IPTG) at an OD600 of ~0.6. Cultures were grown for a further 6 h at 28 °C. Cells were collected and resuspended in 50 mM Tris-HCl, pH 7.9, 500 mM NaCl, 20 mM imidazole, 5% glycerol, 100 μg lysozyme, and Complete EDTA-free protease inhibitor cocktail tablets (Roche) were added. After disruption by sonication the supernatant was clarified by centrifugation and applied to a HisTrap-nickel agarose column (GE Healthcare) equilibrated in a buffer containing 50 mM Tris-HCl, pH 7.9, 500 mM NaCl, 20 mM imidazole and 5% glycerol. Bound protein was eluted with a linear gradient of 20–250 mM imidazole in lysis buffer. Pectocin-containing fractions were identified based on colour and analysis by SDS-PAGE. Pectocin-containing fractions were pooled and dialysed into 50 mM Tris-HCl, pH 7.5, 50 mM NaCl and purified using a Superdex S75 26/60 column (GE Healthcare) equilibrated in the same buffer.

**Cytotoxicity and growth enhancement assays.** The cytotoxicity of purified pectocins was tested using the soft agar overlay method27. A volume of 200 μl of mid-log phase culture of the test strain was added to 6 ml of 0.6% agar melted and cooled to 42 °C. The molten agar was then overlaid onto Lysogeny broth (LB) medium with or without 100–400 μM 2,2'-bipyridine. Purified proteins (0.02–10 mg ml−1) were spotted directly onto the surface of the overlay, once solidified. Zones of growth inhibition or enhancement.

**Identification of FusA.** A 2 l suspension culture of *P. atrosepticum* (Pba) LMG2386 was grown in LB with 200 μM bipyridine until stationary phase was reached. Cells were collected by centrifugation, resuspended in 20 ml of 50 mM Tris, 10 mM EDTA, pH 7.2. Protease inhibitors and 2 mg ml−1 lysozyme were added and cells were lysed by sonication. Cellular debris were removed by centrifugation at 8,000 g for 10 min and membranes were then pelleted by ultracentrifugation at 100,000 g for 4 °C, for 1 h. Pelleted membranes were resuspended with a tight-fitting homogenizer in 20 ml of 50 mM Tris, 0.5% Sarkosyl (pH 7.2), left to solubilize for 20 min at room temperature, and the outer membrane fraction pelleted by centrifugation at 100,000 g at 4 °C, for 1 h. The pellet was resuspended as before in 20 ml of 20 mM Tris, 1% n-octyl-β-D-glucoside (pH 7.2) and solubilized for 12 h at 4 °C. Purified pectocin M1 in 50 mM Tris, 50 mM NaCl, pH 7.5 was immobilized on a 1 ml His-trap column. Bound pectocin M1 was washed with 20 column volumes of Tris buffer (50 mM Tris, 500 mM NaCl and 10 mM imidazole, pH 8.0). The solubilized outer membrane (OM) fraction from *Pba* LMG2386 was passed through the column, which was subsequently washed with 20 column volumes of Tris buffer as above. Proteins were eluted from the column with Tris buffer containing an increasing concentration of imidazole (20, 50, 100 and 150 mM with pectocin M1 eluting at 100 mM imidazole). Control experiments were performed in which the *Pba* LMG2386-solubilized OMs were passed down a column with no pectocin bound and imidazole elutions were undertaken for a column to which no OM fraction had been added. Proteins were visualized by Coomassie and silver staining of SDS-PAGE gels, and bands unique to the pectocin M1 plus OM fraction experiment were excised and identified by peptide mass fingerprinting.

**Cloning and expression of FusA.** To study FusA in vitro, homologues from a number of *Pectobacterium* strains (including *Pba* LMG2386 and LMG2410) were screened for expression in *E. coli*. Despite extensive optimization, only the FusA homologue from the *P. atrosepticum* strain SCRI1043 (FusA1043) yielded significant quantities of pure folded monodisperse protein (Supplementary Fig. 4) and was utilized for further studies. The method for achieving this expression was as follows: the FusA ORF from *Pba* SCRI1043 (FusA1043) was amplified by PCR in its entirety or lacking the region coding for the 20-aa C-terminal signal sequence (Δ20) (forward full length: GCATCCATATGACAAATGATACCT GTAAGATGTC; forward Δ20: GCATCCATATGACAAATGATACCT GTAAGATGTC; reverse: GCATCCATATGACAAATGATACCT GTAAGATGTC; reverse GCATCCATATGACAAATGATACCT GTAAGATGTC; reverse GCATCCATATGACAAATGATACCT GTAAGATGTC) and cloned into pET28a at the NdeI and Xhol restriction sites. The full-length ORF was ligated into pET28a at the NdeI and Xhol restriction sites to encode a protein with a C-terminal His-tag and this construct was used to produce protein for structural and NMR studies. Plasmids were transformed into *E. coli* BL21 (DE3) for expression. Cells expressing full-length FusA1043 were induced at OD600 = 0.6 with 0.1 mM IPTG and grown at 28 °C for 12 h. The OM fraction from these cells was isolated, and co-elution experiments performed as for *Pba* LMG2386 membranes. Cells expressing FusA1043Δ20 as inclusion bodies were induced at OD600 = 0.6 with 0.1 mM IPTG and grown at 30 °C for 36 h in auto-inducing super broth28. Inclusion bodies were washed, refolded and purified based on the method described by Saleem et al.29. Cells were collected and lysed as for pectocin purification, the insoluble fraction (containing the inclusion bodies) was isolated by centrifugation at 18,000g for 25 min and homogenized using a tight-fitting homogenizer in with 50 mM Tris, 1.5% N,N-dimethyldodecylamine N-oxide (LDAO), pH 7.5 and incubated at room temperature with shaking for 30 min. Inclusion bodies were pelleted by centrifugation at 18,000g for 25 min and homogenized once more in 50 mM Tris, 1.5% LDAO (v/v), pH 7.5, pelleted and washed once in 50 mM Tris (pH 7.5) before pelleting a final time. Inclusion bodies were then denatured in denaturing buffer (10 mM Tris, 1 mM EDTA, 8 M urea and 1 mM dithiothreitol (pH 7.5)) at a ratio of 0.5 g of inclusion body to 40 ml of buffer using a tight-fitting homogenizer, followed by incubation with shaking at 56 °C for 30 min. Insoluble material was then removed by centrifugation at 8,000g for 10 min.

**Figure 6 | Plant ferredoxins with high levels of sequence homology enhance the growth of *Pectobacterium* species to greatly differing extents.** (a) Growth enhancement of a soft agar overlay of *P. carotovorum* LMG2442 and LMG2410, on iron-limiting LB agar due to the application of threefold serially diluted purified plant ferredoxin at a starting concentration of 1 mg ml−1. Ferredoxins from Arabidopsis and potato both enhance growth in these strains, whereas for maize ferredoxin no growth enhancement is observed. Refer to Supplementary Table 3 for a list of observed phenotypes from different *Pectobacterium* strains (experiment repeated >10 times). (b) Sequence alignment of Arabidopsis, potato, maize ferredoxins and the pectacin M1 and M2 ferredoxin domains showing the overall high level of amino-acid identity and the position of sequence divergence between the homologues.
FusA in denaturing buffer was then added drop wise to an equal volume of rapidly stirring refolding buffer (20 mM Tris, 1 M NaCl and 5% (v/v) LDAO (pH 7.9)), followed by stirring for 1.5 h. Refolded FusA was then dialysed (10–15,000 molecular weight cutoff membrane) into 2 x 5 of dialysis buffer (20 mM Tris, 0.5 M NaCl and 0.1% (v/v) LDAO (pH 7.9)) overnight at 4°C. Refolded FusA was then purified with Ni-affinity chromatography, with dialysis buffer used for binding. FusA to the nickel column and dialysis buffer with 0.5 M imidazole used to elute the protein. The protein was further purified using a superdex 2000 26/60 column equilibrated in 50 mM Tris, 200 mM NaCl and 0.1% (v/v) LDAO (pH 7.9). For crystallization experiments in β-OG, FusA was exchanged into buffer containing 0.8–1% β-OG, by immobilization on a nickel column. Normal β-OG was recovered by washing 10 column volumes of 50 mM NaCl and 0.8–1% (v/v) β-OG (pH 7.9), and eluted with the same buffer with 0.5 M imidazole. The protein was then concentrated to 10–15 mg ml⁻¹ before dialysis (10–15,000 molecular weight cutoff membrane) for 20 h against 50 mM Tris, 200 mM NaCl and 0.8–1% (v/v) β-OG (pH 7.9), to remove imidazole and normalize β-OG concentration to refold. Successive cycles of refolding of FusA was confirmed by analytical gel filtration and circular dichroism. Refolded FusA was then concentrated and dialysed against a final buffer of 50 mM Tris, 50 mM NaCl and 0.1% LDAO for storage or 50 mM sodium phosphate and 0.1% LDAO for NMR experiments.

Circular dichroism measurements. Circular dichroism data were obtained using a Jasco J-810 spectropolarimeter (Jasco UK Ltd).

Creation of ΔfusA strains. The suicide pMRS101 plasmid was utilized in the creation of deletion mutants in Pectobacterium spp.21,23. The initial and final 1,000 bp of fusA gene were amplified by PCR and fused with a stop codon inserted at the end of the PCR products. This cassette was inserted into pMRS101 at the EcoRI and the ColE1 origin of replication excised by NotI digestion and re-ligation. After this step the vector (designated pKNFRKO2386) was propagated in E. coli SM10 λpir with 50 μg ml⁻¹ streptomycin as the selection agent. The vector was then transformed into the Pectobacterium knockout target (LMG2386) by electroporation and cells were selected on 50 μg ml⁻¹ streptomycin. It was found to be important to use 50 μg ml⁻¹ streptomycin rather than the published 25 μg ml⁻¹ to prevent the isolation of spontaneously resistant colonies. Isolated streptomycin resistant colonies had the entire pKNFRKO plasmid recombined into the Pectobacterium genome via recombination in one 1,000 bp fragment. pMRS101 possesses sacB gene, which imparts sucrose sensitivity and hence strain classification. The sucrose-susceptible phenotype was restored to ΔfusA by complementation with a plasmid containing a copy of fusA driven by a T5 promoter, spots tests were performed as above with the addition of 1 mM IPTG.

Crystallization and structural solution of FusA. Crystallization trials for FusA were conducted on refolded FusA1043 in a buffer containing 50 mM Tris, 200 mM NaCl and 1% β-OG (pH 7.9), ~800 conditions from commercial screens (Memplus, Memgold I/II, Morpheus, JCSG 800 conditions from commercial screens) were tested32. Very small needle-like crystals (5–20 μm) grew in a number of conditions after ~18 months. For Ferara crystals from JCSG condition D6 (0.2 M MgCl₂, 0.1 M Tris and 20% PEG 8000, pH 8.5) were looped and cryoprotected by increasing the PEG 8000 concentration to 35% before cryocooling to 100 K in liquid nitrogen. For Ferara crystals from PACT condition D1 (0.1 M Malic acid/ME/Tis and 25% PEG 1500, pH 8.0) were looped and cryocooled to 100 K in liquid nitrogen directly in mother liquid. In these conditions no crystals were obtained. The crystals were collected for both ferredoxin crystals at the Fe-K edge wavelength (1.74 Å) at DLS at 100 K. The best data sets diffracted to 2.34 and 2.46 Å for Ferara and Ferpet, respectively. Data sets could be solved both using Fe anomalous signal and by molecular replacement using the structure of spinach ferredoxin (Protein Data Bank ID: 1A70), using AutoSols and Phaser, respectively36,37. The ferredoxins were built in COOT and refined using REFMAC5 (refs 38,42).

Nuclear magnetic resonance. Fast-HSQC spectra43 were recorded for 15N-labelled Arabidopsis and potato ferredoxins (FusA and Ferara) using a JCSG plus PACT screen. Spectra were recorded in a Bruker AVANCE 600 MHz spectrometer. The interaction of these proteins with FusA was explored by using spectra with 5% D2O (pH 6.9). 3D 1H-15N-13C HNCA, HNCO and 15N-1H-13C HNCO spectra were acquired on the 15N-labelled sample, and 3D HNCA, HNCO and 15N-1H-13C HNCO spectra were acquired on the 15N-labelled sample. 3D HNCO, HNCA, HNCACB and HN(C)CA spectroscopy were acquired on the triple labelled 15N-13C-1H PM1fer sample. Spectra were processed with ARAZAR (W. Boucher, www.bio.cam.ac.uk/azara) using the MaxEnt method, and backbone assignment was carried out with the CCPNmr software package.

Analytical ultracentrifugation. Sedimentation velocity was carried out in a Beckman Coulter Optima XL-I analytical ultracentrifuge using an An-50 Ti 4-hole rotor. FusA (90 μl) at concentrations ranging from 0.2 to 10 mg ml⁻¹ was loaded into a 3 mm path-length centriplate and spun at 49,000 rpm for ~12 h at 15°C. The samples were inspected every 7 min, and absorbances were recorded in the range of 5.8–7.2 cm, and radial step-size of 0.005 cm). The buffer used was 50 mM Tris, 200 mM NaCl and 2 mM ζ-sulpho myristic acid (C14SF), pH 7.5, and this buffer lacking C14SF as a reference. Data were analysed with SEFDIT25 using the continuous (c) distribution model. SDINTERP was used to calculate the partial specific volume, the buffer density and viscosity at 15 and 20°C.

HADDOCK docking. Docking was performed using the HADDOCK server http://haddock.science.uu.nl/services/HADDOCK2.2/ (ref. 22). For docking of the PM1fer domain an atomic model was generated using modele24; this model along with the Arabidopsis and potato ferredoxin crystal structures were refined using water refinement in HADDOCK, and an ensemble of 10 conformations generated, one from each of the 10 experimental HADDOCK docking runs. All protein loops were removed from the PM1fer model and used to build a model of the PM1fer crystal structure. The model was constructed utilizing these experimental maps and used to phase the 22-stranded β-barrels that make up the domains of the PM1fer crystal structure. The β-barrel-gene prediction programme BOCTOPUS proved highly accurate in predicting the position of transmembrane strands in the FusA sequence, which was invaluable in the initial stages of model construction35. Model quality was assessed using the Molprobity webserver31. Subsequent to the solving the structure of FusA, crystallization of FusA in complex with Ferara, and in complex with PM1fer was attempted. FusA at 15 mg ml⁻¹ in a buffer containing 50 mM Tris, 200 mM NaCl and 1% β-OG (pH 7.9) was mixed with an equal volume of the ferredoxin at 5 mg ml⁻¹ in 50 mM Tris and 200 mM NaCl (pH 7.9). Despite screening ~800 conditions, no crystals were obtained.
extracellular pocket of FusA and the other from spurious interactions with the outside of the FusA barrel. As such in subsequent rounds the inside of the extracellular loops of FusA were defined as passively interacting with the ferredoxins. There residues on FusA were 100, 101, 102, 107, 101, 144, 145, 146, 147, 148, 149, 150, 151, 153, 156, 158, 159, 162, 163, 234, 236, 239, 243, 327, 376, 378, 380, 382, 383, 384, 385, 396, 397, 398, 399, 400, 401, 403, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 473, 474, 492, 497, 585, 587, 589, 590, 592, 594, 595, 596, 597, 598, 599, 601, 602, 603, 604, 605, 606, 648, 650, 652, 653, 654, 655, 656, 658, 705, 706, 710, 712, 713, 715, 726, 727, 732, 770, 771, 772, 773, 774, 775, 776, 778, 779, 790, 791, 793, 795, 796, 837, 839, 841, 843, 844, 845, 846, 847, 848, 849, 850, 851 and 852. For PM1 and PM2 the following residues were defined as active (based on average NMR CSP values cutoff of 90, network-based cluster analysis was then performed to identity FusA homologues were performed using the HMMER (phmmer) algorithm against the Uniprot representative sequences (rp75) and NCBI references (RefSeq) databases, respectively. Sequences identified in these searches were performed in five modes, with DQ and DP step cutoffs of 30 and 100, respectively. The docked structure of FusA and PM1en was deposited in the GenBank Database with the accession codes FusA from Erwinia chrysanthemi 3937 genes that are regulated during plant infection. Acta Crystallogr. D 67, 356–357 (2011).

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Acknowledgements
We thank the Diamond Light Source for access to beamlines I02, I03, I04, I04-1 and I24 (proposal numbers MX6638 and MX6659). The work was funded by the BBSRC (BB/L02022X/1), a Kelvin-Smith Scholarship from the University of Glasgow and a Sir Henry Wellcome Fellowship awarded to R.G.; the Wellcome Trust; award number 093592/Z/10/Z.

Author contributions
Conceived and designed the experiments: D.W., R.G., I.J. and A.M.J.J.B.; performed the experiments: R.G., I.J., K.M. and A.M.J.J.B.; analysed the data: D.W., R.G., I.J., A.M.J.J.B., S.K., B.S., A.W.R. and O.B.; contributed reagents/materials/analysis tools: D.W., S.K., B.S., A.W.R. and O.B.; wrote the paper: D.W., R.G., I.J., A.M.J.J.B., A.W.R., B.J.C., J.M., O.B. and B.S.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Grinter, R. et al. Structure of the bacterial plant-ferredoxin receptor FusA. Nat. Commun. 7, 13308 doi: 10.1038/ncomms13308 (2016).

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