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TonB-dependent outer-membrane proteins and siderophore utilization in *Pseudomonas fluorescens* Pf-5

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Abstract The soil bacterium *Pseudomonas fluorescens* Pf-5 produces two siderophores, a pyoverdine and enantio-pyochelin, and its proteome includes 45 TonB-dependent outer-membrane proteins, which commonly function in uptake of siderophores and other substrates from the environment. The 45 proteins share the conserved β-barrel and plug domains of TonB-dependent proteins but only 18 of them have an N-terminal signaling domain characteristic of TonB-dependent transducers (TBDTs), which participate in cell-surface signaling systems. Phylogenetic analyses of the 18 TBDTs and 27 TonB-dependent receptors (TBDRs), which lack the N-terminal signaling domain, suggest a complex evolutionary history including horizontal transfer among different microbial lineages. Putative functions were assigned to certain TBDRs and TBDTs in clades including well-characterized orthologs from other *Pseudomonas* spp. A mutant of Pf-5 with deletions in pyoverdine and enantio-pyochelin biosynthesis genes was constructed and characterized for iron-limited growth and utilization of a spectrum of siderophores. The mutant could utilize as iron sources a large number of pyoverdines with diverse structures as well as ferric citrate, heme, and the siderophores ferrichrome, ferrioxamine B, enterobactin, and aerobactin. The diversity and complexity of the TBDTs and TBDRs with roles in iron uptake clearly indicate the importance of iron in the fitness and survival of Pf-5 in the environment.

Keywords Pyoverdine · Iron-acquisition · TonB-dependent receptors · *Pseudomonas fluorescens*

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Introduction

TonB-dependent outer-membrane proteins are important components of the bacterial cellular machinery for the uptake of substrates from the environment. These

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proteins bind with high affinity to specific substrates external to the cell as the first step in the energy-dependent transport of the substrate into the periplasmic space. The energy for transport across the outer membrane is supplied by TonB proteins (Postle and Kadner 2003). TonB-dependent outer-membrane proteins are best known as receptors for siderophores, high-affinity iron-chelating compounds that are produced by microorganisms under iron-limiting conditions. Siderophores are exported from the cell, where they chelate ferric ions in the environment. Specific ferric-siderophore complexes are recognized by cognate TonB-dependent outer-membrane proteins, which initiate the process of iron transport into the cell where the iron becomes available for metabolic functions (Hider and Kong 2010). The roles of TonB-dependent outer-membrane proteins as receptors for siderophores, vitamin B12, and certain phages have been recognized for decades (Postle and Kadner 2003) but their broader functions in the uptake of sucrose (Blanvillain et al. 2007) maltodextrins (Lohmiller et al. 2008), nickel (Schauer et al. 2007), sulfate (Kahnert et al. 2002), and other substrates have been recognized only recently. Most bacteria have less than 14 TonB-dependent outer-membrane proteins in their proteomes but certain environmental bacteria, such as *Caulobacter crescentus* (Eisenbeis et al. 2008) and *Xanthomonas campestris* pv. *campestris* have very large numbers (Blanvillain et al. 2007). This is also the case for *Pseudomonas fluorescens* Pf-5, a well-characterized soil bacterium that colonizes seed and root surfaces and protects plants from infection by certain soil-borne plant pathogens (Loper and Gross 2007). The proteome of *P. fluorescens* Pf-5 includes 45 TonB-dependent outer-membrane proteins.

In environments in which iron is limited, fluorescent pseudomonads such as *P. fluorescens* produce pyoverdines. These siderophores are composed of a dihydroxyquinoline chromophore, an acyl side chain (either dicarboxylic acid or amide) bound to the amino group of the chromophore, and a peptide chain of variable length and composition. The structures of more than 60 pyoverdines from different strains and species of *Pseudomonas* have now been determined (Meyer et al. 2008). Strains of *P. aeruginosa* produce pyoverdines falling into three structural groups (Meyer et al. 1997), and two of the 34 TonB-dependent outer-membrane proteins in the proteome of PAO1 are responsible for the uptake of these ferric-pyoverdines (Lamont and Martin 2003). Other *Pseudomonas* spp. differ in the range of pyoverdine structures that they can utilize as iron sources. *P. entomophila* L48 utilizes a wide range of pyoverdines, whereas the related species *P. putida* KT2440 can utilize relatively few of these siderophores to acquire iron from the environment (Matthijs et al. 2009). In addition to pyoverdine, a second siderophore having a lower affinity for iron than pyoverdine is produced by many strains of *Pseudomonas* spp. (Cornelis 2010). For example, pyochelin is produced by *P. aeruginosa*, and its optical antipode enantio-pyochelin is produced by *P. fluorescens* Pf-5 (Youard et al. 2007). Furthermore, pseudomonads have a remarkable capacity to utilize heterologous siderophores produced by diverse taxa of bacteria and fungi (Cornelis and Matthijs 2002). Of the 34 TonB-dependent outer-membrane proteins in the proteome of *P. aeruginosa* PAO1, eight serve as receptors for the heterologous siderophores enterobactin, aerobactin, ferrichrome, ferrioxamine B, heme or ferric-citrate (Cornelis et al. 2008; Cornelis and Bodilis 2009). A more complex structure–function relationship likely exists in *P. fluorescens* Pf-5, with its 45 TonB-dependent outer-membrane proteins including six putative ferric-pyoverdine receptors (Paulsen et al. 2005).

In addition to their roles as outer membrane receptors, certain TonB-dependent outer-membrane proteins serve as components of cell-surface signaling (CSS) systems used by bacteria to sense signals from the extracellular medium and transmit them into the cytoplasm (Ferguson et al. 2007). Typically, CSS systems have three components: an alternative sigma factor of the extracytoplasmic function (ECF) family, a sigma factor regulator (anti-sigma factor) located in the cytoplasmic membrane, and a TonB-dependent outer-membrane protein having an N-terminal signaling domain. This signaling domain interacts with the C-terminus of the cognate anti-sigma factor, which releases the ECF sigma factor to function in transcription of specific target genes (Ferguson et al. 2007). Therefore, upon substrate binding, TonB-dependent outer-membrane proteins having the N-terminal signaling domain initiate a signaling pathway that controls the transcription of target genes. Genes encoding the three CSS components are typically clustered in the bacterial genome.

In this study, a combination of bioinformatic, phylogenetic and functional analyses were employed

to characterize the 45 TonB-dependent outer-membrane proteins of *P. fluorescens* Pf-5. Motifs defining constituent domains were identified and the presence or absence of the N-terminal signaling domain was used to distinguish the 27 TonB-dependent receptors (TBDRs) from the 18 TonB-dependent transducers (TBDTs) in the Pf-5 proteome. Phylogenetic analyses of the TonB-dependent outer-membrane proteins from Pf-5 and characterized orthologs from other *Pseudomonas* spp. allowed the assignment of putative functions to certain Pf-5 TBDRs and TBDTs. A mutant of Pf-5 with deletions in pyoverdine and enantio-pyochelin biosynthesis genes was constructed and characterized for iron-limited growth and utilization of a spectrum of siderophores. Pf-5 exhibited a remarkable capacity to utilize pyoverdines with diverse structures produced by different *Pseudomonas* spp., as well as ferric citrate, heme, and the siderophores ferrichrome, ferrioxamine B, enterobactin, and aerobactin.

Materials and methods

Bacterial strains and growth conditions

Pseudomonas strains were grown on King's medium B (KMB) (King et al. 1954) at 27°C. *Escherichia coli* and *Enterobacter cloacae* were grown on Luria–Bertani (LB) at 37°C. Antibiotics were used at the following concentrations (μg/ml): gentamicin (Gm) 40 (*P. fluorescens*) and 12.5 (*E. coli*), kanamycin (Km) 50, streptomycin (Sm) 100, tetracycline (Tet) 200 (*P. fluorescens*) and 20 (*E. coli*).

Pyoverdine peptide chain prediction

Pyoverdines produced by many strains of *Pseudomonas* spp. have unknown structures, but the amino acid composition of the peptide chain of these pyoverdines can be predicted bioinformatically from the nucleotide sequences of genes encoding the corresponding non-ribosomal peptide synthetases (NRPSs). Predicted amino acid sequences for the NRPSs for each strain were submitted to the NRPS/PKS predictor (Bachmann and Ravel 2009) and the NRPS predictor (http://www-ab.informatik.uni-tuebingen.de/software/NRPSpredictor) which uses the methods of Stachelhaus et al. (1999) and Rausch et al. (2005).

Sequence compilation and domain analysis

Alignments of amino acid sequences of the TonB-dependent outer-membrane proteins of Pf-5 were done using the multiple sequence alignment tool T-Coffee (Notredame et al. 2000). Characteristic domains of TonB-dependent outer-membrane proteins were identified according to Pfam (Finn et al. 2010), using default settings with an E-value cutoff of 1.0. Additional domain analysis was done using the EMBL_EBI InterProScan domain search tool.

Secondary structure prediction

PSIPRED GenTHREADER (McGuffin et al. 2000) and a beta barrel prediction model (Bigelow et al. 2004) were used to predict secondary structure of the 45 TonB-dependent outer-membrane proteins in the Pf-5 genome.

Phylogenetic analysis

Amino acid sequences of TonB-dependent outer-membrane proteins were submitted to the NCBI database of non-redundant protein sequences to identify the five to ten best hits for each using the PSI-BLAST algorithm (Altschul et al. 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0.2 (Tamura et al. 2007). The Clustal W (Thompson et al. 1994) based alignment option with a gap open penalty of 15 and a gap extension penalty of 0.3 was used to align the amino acid sequences. The aligned sequences were masked to remove gaps. The masked sequences were then subjected to bootstrapped maximum parsimony analysis. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The maximum parsimony tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). The %GC for each gene encoding a TonB-dependent outer membrane protein was compiled and those differing significantly from the Pf-5 genomic

average of 63.3% were identified by chi square analysis.

Construction of mutants of Pf-5

Mutants of Pf-5 were constructed using overlap extension PCR methods modified from Choi and Schweizer (2005). The *ofaA* (PFL_2145) mutant of Pf-5 is described in Hassan et al. (2010). The *pvdI* deletion mutant was made by the method described in Hassan et al. (2010) using the primers pyv UpF-Bam, pyv UpR-FRT, pyv DnF-FRT, and pyv DnR-Bam (Table 1). The *pchA* and *pchC* gene constructs were made by modified methods as described below. The *pchC* (PFL_3490) gene was amplified with primers PFL3490-Up and PFL3490-Low (Table 1) using iProof DNA polymerase (Bio-Rad, Hercules, CA, USA) and cloned into pCR-blunt (Invitrogen, Carlsbad, CA, USA). The GmR-*gfp* gene cassette was amplified from pPS858 (Hoang et al. 1998) with primers Gm-F and Gm-R using KOD DNA polymerase (Novagen (Merk), Darmstadt, Germany). The GmR-gfp cassette was used to interrupt the *pchC* gene by cloning into a unique *Psh*AI site. The interrupted *pchC* gene was re-amplified with PFL3490-Up and PFL3490-Low primers using KOD DNA polymerase and ligated into the *Sma*I site of pEX18Tc (Hoang et al. 1998). This construct was introduced into Pf-5 as described in Hassan et al. (2010). The *pchA* (PFL_3488) gene construct was made by PCR amplification of 5′ and 3′ regions of *pchA* with the primers 3488 UpFHind, 3488 UpR, 3488 DnF, and 3488 DnRHind (Table 1). The resulting PCR products were combined in a second round of PCR with the primers 3488 UpFHind and 3488 DnRHind added during the third-cycle extension, yielding a product consisting of the 5′ and 3′ regions of the *pchA* gene with the middle portion of the gene deleted. The final PCR product was digested with *Hin*dIII and cloned into pEX18Tc (Hoang et al. 1998). The *pchA* deletion construct was transformed into One Shot TOP10 Chemically Competent *E. coli* (Invitrogen) and then into the mobilizing strain *E. coli* S17-1 (Simon et al. 1983). Pf-5 transconjugants were selected on KMB (King et al. 1954) with streptomycin (100 µg/ml, innate resistance of Pf-5) and tetracycline (200 µg/ml). Resulting colonies were grown for 3 h without selection in LB broth and plated on LB with 5% sucrose to favor growth of resolved merodiploids. Colonies growing on sucrose were patched onto KMB containing tetracycline (200 µg/ml) to confirm resolution of merodiploids. Tetracyline-sensitive clones were screened for presence of the *pchA* deletion by PCR and the PCR product sequenced to confirm correct incorporation of the deleted allele.

Table 1 Primers used in the construction of mutants of *P. fluorescens* Pf-5

Primer	Sequence 5′–3′
pchA	
3488 UpFHind	GACGAAGACGAAGCTTTTCTACCTGCGCGAGCAACA
3488 UpR	TGCTCGCGGATAACAGGCAGGATTCACTCATC
3488 DnF	AATCCTGCCTGTTATCCGCGAGCATGAGCAA
3488 DnRHind	GTGGTTGTGGAAGCTTATTCCTTCGCCATAAACCGC
pvdI	
pyv UpF-Bam	CTCTGCTTCTGGATCCTCGGTTTCTTCGTCAACACC
pyv UpR-FRT	TCAGAGCGCTTTTGAAGCTAATTCGGAGGTGTAGATCGAATAGGC
pyv DnF-FRT	AGGAACTTCAAGATCCCCAATTCGTGCTGGATGCATCCTTGCAA
pyv DnR-Bam	CACACCATCAGGATCCATCTGCCAGAACAGCCATTG
pchC	
PFL3490-Up	CGGCCAGGCTGTACACCAC
PFL3490-Low	TACCTGAGCACCGAGCAGC
Gm-F	CGAATTAGCTTCAAAAGCGCTCTGA
Gm-R	CGAATTGGGGATCTTGAAGTTCCT

Arbitrary PCR

Tn*5* insertions in an extant set of pyoverdine-deficient mutants (Kraus and Loper 1992) were mapped by using arbitrary PCR. Genomic DNA flanking the Tn*5* insertion was amplified in two rounds of PCR reactions. In the first round, Primer 1 was complementary to sequences of Tn*5* and Primer 2 was a degenerate primer. The 5′ end of the degenerate primer was 5′-GGTCCG, a sequence that occurs 350 times, at an average of every 600 bp, in the pyoverdine regions of Pf-5. This primer also contained 10 random nucleotides and a previously-described 20-nucleotide sequence (Das et al. 2005). Round 2, Primer 1 was composed of the 3′ 20 nucleotides from the round one degenerate primer. Round 2, Primer 2 was complementary to Tn*5* at a location internal to the Round 1, Primer 1 sequence. The final product was sequenced to identify the DNA flanking the Tn*5* insertion.

Round 1, Primer 1: 5′-GGGCAGTACGGCGAGGAT-3′
Round 1, Primer 2: 5′-GGTCCGNNNNNNNNNNACTGATCAGCTGCGCACCGG-3′
Round 2, Primer 1: 5′-ACTGATCAGCTGCGCACCGG-3′
Round 2, Primer 2: 5′-CCTTTCTGATCGCCTCGG-3′

Iron limited growth

Pf-5 and derivative strains were tested for iron limited growth on KMB containing the iron chelator 2,2′-dipyridyl (Sigma–Aldrich, St Louis, MO, USA) at 0, 100, 200, 400, 600, and 800 μM. Bacterial cells from overnight cultures grown in KMB broth were collected by centrifugation and suspended in water to 0.1 OD_{600}. This suspension was diluted to 10^{-2}, 5 μl of the diluted cell suspension was placed on the agar surface, and bacterial growth was observed following 24 h incubation at 27°C. Each strain was tested in at least two experiments, each evaluating two replicate plates.

Enantio-pyochelin extraction and detection

Production of enantio-pyochelin in Pf-5 and derivative strains was analyzed using the following method: for each treatment, four tubes each containing 5 ml M9 minimal medium (Sambrook et al. 1989) broth were inoculated with 5 μl of overnight culture and incubated at 27°C for 48 h at 200 rpm. Two cultures were combined for each of two replicates and centrifuged at 7000 rpm for 10 min. Supernatants were decanted into 50 ml polypropylene conicle screw-cap centrifuge tubes and adjusted to pH 2.0 with 1 M HCl. The enantio-pyochelin was extracted by adding 0.5 volumes ethyl acetate and vortexing. The organic and aqueous phases were separated by centrifugation at 7000 rpm for 10 min. The organic top layer was transferred to 5 ml glass tubes and dried under vacuum. Dried samples were resuspended in 100 μl methanol and stored at −20°C. Enantio-pyochelin extracts were separated on thin layer chromatography plates (silica gel 60 F_{254} on aluminum, EM Science, Gibbstown, NJ, USA) using n-butyl alcohol/water/acetic acid 4:1:1 (v/v/v) as the mobile phase (Youard et al. 2007). Compounds were viewed by fluorescence at 365 nm and by spraying with 2 M $FeCl_3$ in 0.1 M HCl.

CAS agar assay

Pf-5 and mutants were tested for siderophore production by observing zones surrounding colonies grown on CAS (Chrome azurol S) agar for pseudomonads (Schwyn and Neilands 1987). 10 μl of a 0.1 OD_{600} cell suspension was spotted on the agar surface, plates were incubated at 27°C, and observed for zone formation. Each mutant was tested in at least two experiments, each evaluating two replicate plates. In some experiments, CAS agar was amended with $FeCl_3$ to a final concentration of 1 mM.

Crossfeeding assays

Pseudomonas spp. producing diverse pyoverdines (test strains presented in Table 2) were evaluated for their capacities to provide iron to the *pvdI–pchC* mutant of Pf-5 (indicator strain) in crossfeeding experiments. Cells from test strains and the indicator strain were collected from overnight cultures grown in KMB broth and suspended in water to 0.1 OD_{600}. Cell suspensions of the indicator strain were further diluted to 10^{-2} in sterile water. 10 μl of each test strain suspension was placed on the surface of KMB amended with 2,2′-dipyridyl at 400 μM or 600 μM. 5 μl of the diluted cell suspension of the indicator strain was spotted on the agar surface at a distance of

1 cm from each test strain. An alternative method was used for those test strains that did not grow on KMB amended with 2,2′-dipyridyl at 400 μM or 600 μM. For those strains, an agar plug (6 mm) obtained from a 48 h culture on KMB was substituted for the cell suspension on the surface of the test plate. Plates were incubated at 27°C, and growth of the indicator strain was observed at 24 and 36–48 h. Each test strain was evaluated in at least two experiments, each evaluating two replicate plates.

Siderophore utilization assays

The capacity of Pf-5 to utilize specific ferric-siderophore complexes as sources of iron was evaluated. Cells of a *pvdI–pchC* mutant of Pf-5 were collected from overnight cultures grown in KMB broth, suspended in water to 0.1 OD_{600}, diluted to 10^{-2} in sterile water, and 100 μl of the diluted sample was spread on the surface of KMB amended with 400 μM or 600 μM 2,2′-dipyridyl. Filter paper disks (5 mm diameter) were placed at the center of the agar surface, and 10 μl of a purified siderophore solution or water (negative control) was placed on the filter paper disk. Plates were incubated at 27°C for 24 h and then scored for the presence of bacterial growth in a halo surrounding the disk. The following compounds were tested: 20 mM ferric citrate in water, 7.7 mM hemin chloride in 10 mM NaOH, 5 mg hemoglobin in 1 ml PBS (phosphate-buffered saline), 20 mM desferrioxamine in 10 mM Tris–HCl pH 8.8, and 10 mM ferrichrome in 0.5 M Tris–HCl, pH 8.8. All of the compounds were obtained from Sigma–Aldrich. Each assay was done twice, with each experiment evaluating two replicate plates.

Results

Identification of conserved domains within the TonB-dependent outer-membrane proteins of *P. fluorescens* Pf-5

Analysis of the amino acid sequences of each of the 45 TonB-dependent outer-membrane proteins in the Pf-5 proteome revealed the conserved transmembrane pore and receptor domains of this protein family. Sequences characteristic of an outer membrane-spanning pore, formed by a β-barrel made up of repeated β-strands (Interpro: IPR000531) were identified in all 45 deduced peptide sequences (Online Resource 1). Two domains involved in substrate binding, a receptor domain (Pfam: PF00593) comprising a highly conserved region of the pore, and a plug domain (Pfam: PF07715) (Cobessi et al. 2005; Shultis et al. 2006; Pawelek et al. 2006), were also identified in 43 TonB-dependent outer-membrane proteins. The receptor domain was not identified in the proteins PFL_2919 and PFL_3612. A TonB box, defined as the five to seven amino acids required for interaction with TonB (Peacock et al. 2004), was not identified consistently in the 45 proteins following analysis of sequence alignments with known TonB boxes in other *Pseudomonas* spp. The lack of conservation of this motif across the TonB-dependent outer-membrane proteins of Pf-5 may be related to the presence of four putative TonB proteins in the Pf-5 genome (Paulsen et al. 2005). Multiple copies of TonB are also present in other species of *Pseudomonas* (Zhao and Poole 2002; Huang et al. 2004).

An N-terminal signaling domain (Pfam: PF07660), which is known to interact with regulatory proteins controlling the expression of ECF sigma factors (Ferguson et al. 2007), was identified in 18 of the 45 TonB-dependent outer-membrane proteins (Online Resource 1). Seventeen of the genes encoding these proteins are immediately adjacent to or clustered with genes encoding ECF sigma factors and associated regulatory proteins (anti-sigma factors) in the Pf-5 genome (Online Resource 2). One gene (PFL_4092) is located in a pyoverdine biosynthesis gene cluster also containing the corresponding ECF sigma factor gene FpvI (PFL_4080), but the corresponding anti-sigma factor encoding gene FpvR (PFL_2903) is distal in the genome.

The 27 TonB-dependent outer-membrane proteins lacking an N-terminal signaling domain range in length from 654 to 859 amino acids (72.9–93.8 kDa) whereas the 18 proteins having an N-terminal signaling domain are typically larger, ranging from 806 to 944 amino acids (88.05–104.48 kDa) (Online Resource 1). Alignment of all 45 proteins showed a lack of conservation over much of the sequence between the groups, which is due partially to differences in protein length. Therefore, our phylogenetic analyses considered the TBDRs and TBDTs separately, revealing differences that could, in some cases, be assigned to distinct substrates.

Table 2 Crossfeeding of *Pseudomonas* strains with Pf-5

Test Strains	Cross-feeding	Composition of peptide chain or siderotype	Reference or source
Six amino acids			
P. fluorescens B10	+	εLys-OHAsp-Ala-aThr-Ala-cOHOrn	Teintze et al. (1981)
P. lini DLE411J	+	*Lys-OHAsp-Ala-Thr-Ala-OHOrn*	Meyer (2007)
P. putida CS111 syn SB8.3	+	*Ala-Lys-Thr-Ser-OHOrn-OHOrn*	Meyer (2007)
P. putida CFML90-40	+	Asp–Ala–Asp–AcOHOrn–Ser–cOHOrn	Meyer et al. (2007)
P. putida biotype B ATCC 17470 syn 9BW	+	Ser-εLys-OHHis-aThr-Ser-cOHOrn	Budzikiewicz (1997)
Seven amino acids			
P. aeruginosa ATCC 27853	+	Ser-FOH-OrnOrn-Gly-aThr-Ser-cOHOrn (Type II pyoverdine)	Tappe et al. (1993)
P. aeruginosa Pa6	+	Ser-cDab-FOHOrn-Gln-Gln-FOHOrn-Gly (Type III pyoverdine)	Gipp et al. (1991)
P. fluorescens CLR711 syn PL7	+	Ser-AcOHOrn-Ala-Gly-aThr-Ala-cOHOrn	Barelmann et al. (2002)
P. chlororaphis ATCC 9446	+	Ser-Lys-Gly-FOHOrn-(Lys-FOHOrn-Ser)	Meyer (2000)
P. fluorescens biotype A type strain ATCC 13525	+	Ser-Lys-Gly-FOHOrn-(Lys-FOHOrn-Ser)	Meyer (2000)
P. fluorescens SBW25	+	Ser-Lys-Gly-FOHOrn-(Lys-FOHOrn-Ser)	Moon et al. (2008)
P. fluorescens WCS374	+	Ser-Lys-Gly-FOHOrn-(Lys-FOHOrn-Ser)	Djavaheri (2007)
P. fluorescens WCS374 Pvd-	–		Marugg et al. (1985)
P. fluorescens CTRp112 syn PL8	+	Lys-AcOHOrn-Ala-Gly-aThr-Ser-cOHOrn	Barelmann et al. (2002)
P. putida DSM3601 syn CFML90-33	–	Asp-Lys-Thr-OHAsp-Thr-aThr-cOHOrn	Sultana et al. (2001)
P. syringae ATCC 19310	–	εLys-OHAsp-Thr-(Thr-Ser-OHAsp-Ser)	Jülich et al. (2001)
P. syringae pv. *syringae* B728A	–	Lys-Asp-Thr-Thr-Ser-Asp-Ser	Bioinformatic prediction, this study
P. syringae pv. *tomato* DC3000	–	Lys-Asp-Thr-Thr-Ser-Asp-Ser	Bioinformatic prediction, this study
P. cichorii	–	εLys-OHAsp-Thr-(Thr-Gly-OHAsp-Ser)	Bultreys et al. (2004)
P. libanensis CFBP4841	–	Ala–Orn–OHAsp–Ser–Orn–Ser–cOHOrn	Meyer et al. (2008)
Eight amino acids			
P. aeruginosa PA14	+	Ser-Arg-Ser-OHOrn-Lys-OHOrn-Thr-Thr	Bioinformatic prediction, this study
P. aeruginosa PA14 Pvd-	–		Liberati et al. (2006)
P. aeruginosa PAO1	+	Ser-Arg-Ser-FOHOrn-(Lys-FOHOrn-Thr-Thr) (Type I pyoverdine)	Demange et al. (1990)
P. chlororaphis D-TR133	+	Asp-FOHOrn-Lys-(Thr-Ala-Ala-FOHOrn-Ala)	Meyer et al. (2008)
P. fluorescens CHA0	+	Asp-FOHOrn-Lys-(Thr-Ala-Ala-FOHOrn-Lys)	Wong-Lun-Sang et al. (1996)
P. fluorescens Pf-5	+	Asp-OHOrn-Lys-Thr-Ala/Gly-Ala/Gly-OHOrn-Lys	Bioinformatic prediction, this study
P. fluorescens Pf-5 Pvd-	–		
P. salomonii CFBP2022	+	*Ser–Orn–FOHOrn–Ser–Ser–Lys–FOHOrn–Ser*	Meyer et al. (2008)
Pseudomonas sp. 7SR1	–	Ser-AcOHOrn-Ala-Gly-(Ser-Ser-OHAsp-Thr)	Fernández (2003)
P. fluorescens CTR1015 syn PL9	–	Ser-AcOHOrn-Ala-Gly-(Ser-Ser-OHAsp-Thr)	Meyer (2007)

Table 2 continued

Test Strains	Cross-feeding	Composition of peptide chain or siderotype	Reference or source
Nine amino acids			
P. costantinii CFBP5705	+	Ser-AcOHOrn-Gly-aThr-Thr-Gln-Gly-Ser-cOHOrn	Fernández et al. (2001), Meyer (2007)
P. fluorescens A6	+	Lys-AcOHOrn-Gly-aThr-Thr-Gln-Gly-Ser-cOHOrn	Beiderbeck et al. (1999)
P. putida ATCC 12633	−	Asp-Lys-OHAsp-Ser-Thr-Ala-Glu-Ser-cOHOrn	Meyer et al. (2007)
P. putida WCS358	−	Asp-εLys-OHAsp-Ser-aThr-Ala-Thr-Lys-cOHOrn	Budzikiewicz (2004)
P. putida CFBP2461	−	Asp-εLys-OHAsp-Ser-aThr-Ala-Thr-Lys-cOHOrn	Fernández et al. (2003)
P. monteilii DSM14164	−	Asp–Lys–AcOHOrn–Ala–Ser–Ser–Gly–Ser–cOHOrn	Meyer et al. (2008)
P. fluorescens Pf0-1	+	Ala-AcOHOrn-Orn-Ser-Ser-Ser-Arg-OHAsp-Thr	Meyer et al. (2008)
P. fluorescens Pf0-1 Pvd-	−		M. Silby
Ten amino acids			
P. fluorescens DSM50106	+	Ser–Lys–Gly–FOHOrn–Ser–Ser–Gly–(Orn–FOHOrn–Ser)	Meyer et al. (2008)
P. rhodesiae DSM14020	+	*Ser–Lys–FOHOrn–Ser–Ser–Gly– (Lys–FOHOrn–Ser–Ser)*	Meyer et al. (2007, 2008)
P. fluorescens Pfl 17400	−	Ala-Lys-Gly-Gly-OHAsp-Gln-Dab-Ser-Ala-cOHOrn	Meyer (2007)
P. tolaasii NCPPB 2192	−	Ser-Lys-Ser-Ser-Thr-Ser-AcOHOrn-Thr-Ser-cOHOrn	Meyer et al. (2008)
Unknown structures			
P. flectens CFBP3281	+	Unknown	http://www.straininfo.net/strains/621587
P. fluorescens biotype F type strain ATCC17513	+	Unknown	Stanier et al. (1966)
P. fluorescens biotype G type strain ATCC17518	+	Unknown	Stanier et al. (1966)
P. fluorescens CFBP2130	+	Unknown	http://www.straininfo.net/strains/757032
P. marginalis pv. *alfalfae* CFBP2039	+	Unknown	http://www.straininfo.net/strains/544626
P. marginalis pv. *marginalis* CFBP2037	+	Unknown	http://www.straininfo.net/strains/17707
P. marginalis pv. *pastinacae* CFBP2038	+	Unknown	http://www.straininfo.net/strains/544628
P. reactans NCPPB387	+	Unknown	http://www.straininfo.net/strains/53319
P. blatfordae CFBP3280	−	Unknown	http://www.straininfo.net/strains/757233
P. fluorescens biotype B type strain ATCC17467	−	Unknown	Stanier et al. (1966)
P. fluorescens biotype C type strain ATCC17559	−	Unknown	Stanier et al. (1966)
P. mosselii MFY161	−	Unknown	Isolated from a blood culture in Evreux, France
P. putida GB-1	−	Unknown	Wu et al. (2010)
P. viridiflava CFBP2107	−	Unknown	http://www.straininfo.net/strains/270228
P. corrugata CFBP2431	−	Corr	Meyer et al. (2002), Meyer (2007)
P. fluorescens C7R12	−	PL1	J.-M. Meyer, personal communication
P. frederiksbergensis DSM13022	−	Fred	Meyer et al. (2002), Meyer (2007)
P. fuscovaginae CFBP2065	−	G17	Meyer et al. (2002), Meyer (2007)

Table 2 continued

Test Strains	Cross-feeding	Composition of peptide chain or siderotype	Reference or source
P. gessardii CIP105469	–	Gess-bren	Meyer and Geoffroy (2004)
P. graminis DSM11363	–	Gram	Meyer et al. (2002), Meyer (2007)
P. kilonensis CFBP5372	–	Kilo	Meyer and Geoffroy (2004)
P. plecoglossicida DSM15088	–	Plec	Meyer et al. (2002), Meyer (2007)
P. thivervalensis CFBP5754	–	Thiv/ML45	Meyer et al. (2007)

Underline denotes D-amino acids. Parentheses define cyclic residues. cOHOrn is cyclo-hydroxy-ornithine. FOHOrn is δN-formyl-δN-hydroxy-ornithine. εLys is Lys linked by its ε-NH2. OHAsp is threo-β-hydroxy-aspartic acid. Dab is diamino-butanoic acid. OHH is threo-β-hydroxy-histidine. aThr is allo-Thr. AcOHOrn is δN-acetyl-δN-hydroxy-ornithine. Italicized peptide chains are inferred from siderotyping analysis (Meyer et al. 2008). These pyoverdines are in the same siderotype as a pyoverdine having the structure provided. Stereochemistry is not shown for pyoverdines with no amino acids underlined. Pvd- indicates a pyoverdine-deficient mutant

Phylogenetic analysis of TonB-dependent receptors (TBDRs)

The compiled best hits from PSI_BLAST of the 27 TBDRs were aligned and subjected to maximum parsimony analysis, using two TBDRs from *Helicobacter* spp. as an outgroup. A tree with 22 distinct clades was generated (Fig. 1). The majority of the clades are composed exclusively of TBDRs from *Pseudomonas* spp., but nine of the 22 clades include TDBRs present in proteomes of diverse genera representing the alpha-, beta- and gamma-proteobacteria. For eight of the nine TBDR genes corresponding to the proteins in clades having a member from genera other than *Pseudomonas* spp., the %GC differs significantly from the Pf-5 genomic mean of 63.3% (Online Resource 2). The diversity of genera with orthologous TBDRs implies that horizontal gene transfer of TBDR genes is a possible mode for acquisition.

Of the 27 TBDRs, only PFL_3498 (*fetA*) has a demonstrated function in *P. fluorescens*, serving as the receptor for enantio-pyochelin (Hoegy et al. 2009). Putative functions were assigned to four other TBDRs (PFL_2663, PFL_0648, PFL_5511, PFL_0213) (Fig. 1) based on clustering with and similarity to sequences of functionally characterized TBDRs in other bacteria, as well as the identity of adjacent genes in the Pf-5 genome. PFL_2663 is 82% identical at the amino acid level to PfeA of PAO1 (PA2688), which functions as a receptor for the ferric complex of enterobactin, a catecholate siderophore produced by *E. coli* and other species of the Enterobacteriaceae (Dean and Poole 1993). In the Pf-5 genome, PFL_2663 is clustered with orthologs of *pfeS* and *pfeR* (Fig. 2c), involved in the regulation of *pfeA* (Dean et al. 1996), and *pfeE*, which functions in esterification of enterobactin prior to transport across the cytoplasmic membrane (Zhu et al. 2005). Amino acid sequences of each pair of orthologs in the syntenic *pfe* clusters of Pf-5 and PAO1 have 66–82% identity. Therefore, evidence for the role of PFL_2663 as a ferric-enterobactin receptor is provided both by sequence similarity to *pfeA* and conservation of the *pfe* gene cluster.

PFL_0648 is a putative copper receptor, having 73% identity at the amino acid level to PA3790 (*oprC*), which encodes a TBDR that binds copper and is thought to function in copper utilization in PAO1

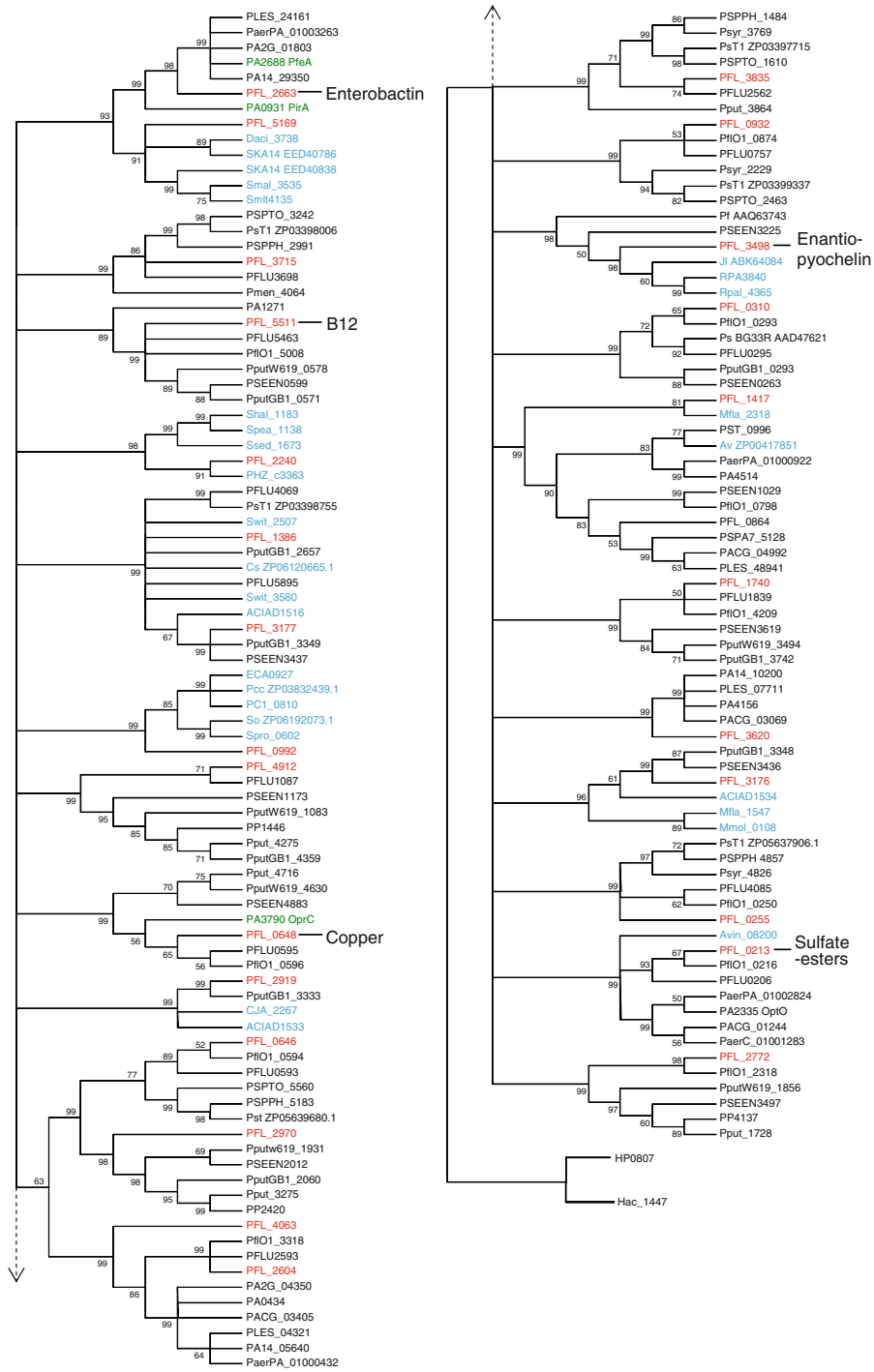
(Yoneyama and Nakae 1996). PFL_0648 is in a three-gene cluster that is conserved in *Pseudomonas* spp. but located in different genomic regions in *P. fluorescens* and *P. aeruginosa* (Fig. 2a). PFL_5511 is a putative receptor for vitamin B12 (cobalamin), exhibiting 29% identity at the amino acid level to BtuB, the characterized B12 receptor of *E. coli*. Protein structure analysis using PSIPRED GenTHREADER matched the TBDR encoded by PFL_5511 ($2e^{-17}$) to BtuB from *E. coli*. In Pf-5, this TBDR is adjacent to a gene encoding a putative periplasmic binding protein for cobalamin (PFL_5512) (Fig. 2e), whereas the ortholog in PAO1 (PA1271) is adjacent to a cobalamin biosynthesis gene cluster. PFL_0213 is a putative receptor for sulfate esters, exhibiting 73% identity at the amino acid level to SftP, a TBDR required for growth of *P. putida* strain S-313 on aryl- or alkylsulfate esters (Kahnert et al. 2002). Contiguous to PFL_0213 are homologs for the sulfate ester/sulfonate transporter (*atsRBC*), a LysR-type regulator (*sftR*), an oxygenolytic alkylsulfatase (*atsK*), and an arylsulfotransferase (*astA*) clustered with *sftP* in *P. putida* S-313 (Fig. 2b), providing further evidence for the putative function of PFL_0213 as a sulfate ester receptor. Analysis of the sequenced *Pseudomonas* genomes indicates conservation of the gene cluster across the genus, with duplications of genes having metabolic functions evident in the genomes of *P. fluorescens* and those with metabolic and regulatory functions evident in *P. aeruginosa*.

Phylogenetic analysis of TonB-dependent transducers (TBDTs)

The compiled best PSI-BLAST hits for the 18 TBDTs were aligned and subjected to maximum parsimony analysis generating a tree with ten distinct, well-supported clades (Fig. 3). Close orthologs having known functions in *P. aeruginosa* PAO1 were also included. Two sequences from *Caulobacter* spp. were used as an outgroup to root the tree. Of the ten clades, two include TBDTs from bacteria other than *Pseudomonas* spp. PFL_3612 clusters with TBDTs from *Yersinia* spp., *Stenotrophomonas* spp., and *Pectobacterium wasabiae*, gamma-proteobacteria found in terrestrial or aquatic environments. PFL_2527, which falls in the pyoverdine clade, clusters with TBDTs from the beta-proteobacteria *Achromobacter piechaudii* ATCC 43553, a human pathogen, and

Fig. 1 Phylogenetic analysis of TonB-dependent receptors. ▶ Phylogenetic analysis of the 27 TBDRs of *P. fluorescens* Pf-5 (PFL) and orthologs was done using the Maximum Parsimony method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the proteins analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The tree is rooted with two *Helicobacter* spp. TBDRs. Pf-5 proteins are shown in *red* font; proteins with known functions are shown in *green* font, and proteins from genera other than *Pseudomonas* are shown in *blue* font. Putative functions assigned to Pf-5 TBDRs are labeled. The tree has been divided into two portions to improve visualization, and positions where the tree is joined are indicated with *dotted lines*. Abbreviations for species represented in the tree are as follows: *Acinetobacter* sp. ADP1 (ACIAD), *Azotobacter vinelandii* DJ (Avin), *Azotobacter vinelandii* AvOP (Av), *Caulobacter segnis* ATCC 21756 (Cs), *Cellvibrio japonicus* Ueda107 (CJA), *Delftia acidovorans* SPH-1 (Daci), *Helicobacter acinonychis* str. Sheeba (Hac), *Helicobacter pylori* 26695 (HP), *Janthinobacterium lividum* (Jl), *Methylobacillus flagellatus* KT (Mfla), *Methylotenera mobilis* JLW8 (Mmol), *P. aeruginosa* 2192 (PA2G), *P. aeruginosa* C3719 (PACG), *P. aeruginosa* LESB58 (PLES), *P. aeruginosa* PA14 (PA14), *P. aeruginosa* PACS2 (PaerPA), *P. aeruginosa* PAO1 (PA), *P. entomophila* (PSEEN), *P. fluorescens* Pf0-1 (Pfl01), *P. fluorescens* SBW25 (PFLU), *P. mendocina* ymp (Pmen), *P. putida* F1 (Pput), *P. putida* GB1 (PputGB1), *P. putida* KT2440 (PP), *P. putida* W619 (PputW619), *P. stutzeri* A1501 (PST), *P. syringae* pv. *phaseolicola* 1448A (PSPPH), *P. syringae* pv. *syringae* B728a (Psyr), *P. syringae* pv. *tomato* T1 (PsT1), *Pectobacterium atrosepticum* SCRI1043 (ECA), *Pectobacterium carotovorum* subsp. *carotovorum* PC1 (PC1), *Pectobacterium carotovorum* subsp. *carotovorum* WPP14 (Pcc), *Phenylobacterium zucineum* HLK1 (PHZ), *Pseudomonas filiscindens* (Pf), *Pseudomonas* sp. BG33R (Ps BG33R), *Pseudomonas syringae* pv. *tomato* DC3000 (PSPTO), *Rhodopseudomonas palustris* CGA009 (RPA), *Rhodopseudomonas palustris TIE-1* (Rpa1), *Serratia odorifera* 4Rx13 (So), *Serratia proteamaculans* 568 (Spro), *Shewanella halifaxensis* HAW-EB4 (Shal), *Shewanella pealeana* ATCC 700345 (Spea), *Shewanella sediminis* HAW-EB3 (Ssed), *Sphingomonas wittichii* RW1 (Swit), *Stenotrophomonas maltophilia* K279a (Smlt), *Stenotrophomonas maltophilia* R551-3 (Smal), *Stenotrophomonas* sp. SKA14 (SKA14)

Janthinobacterium sp. and *Methylovorus* sp. SIP3-4, which are found in soil and aquatic environments, respectively. The capacity to utilize pyoverdines as iron sources has not been observed outside of *Pseudomonas* spp. and *Azotobacter vinelandii* to date (Cornelis et al. 2008), but these results highlight the possibility that such capacity exists in other bacteria. For six of the 18 TBDT genes, the %GC differs statistically from the Pf-5 genomic mean of 63.3% (Online Resource 2), but none of the six corresponding proteins are in clades with genera other than *Pseudomonas* spp. Therefore, while horizontal gene transfer of the TBDTs provides the most plausible



explanation for the presence of diverse genera of proteobacteria in certain clades, we did not uncover convincing evidence for recent horizontal acquisition as a mechanism of inheritance of these genes by Pf-5.

Five of the 10 TBDT clades include characterized proteins known to function in iron uptake in other *Pseudomonas* spp. (Fig. 3). Four TBDTs (PFL_1371, PFL_2365, PFL_4627, and PFL_5378) are in a large

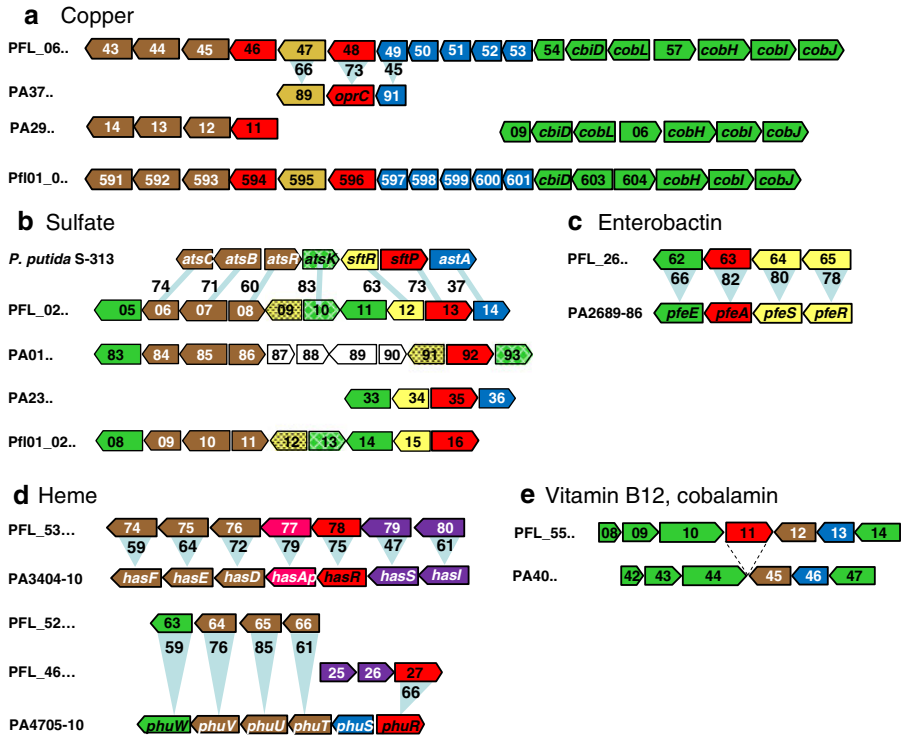


Fig. 2 Gene clusters with TBDRs and TBDTs of known function. Gene clusters in *P. fluorescens* Pf-5 (PFL_), *P. aeruginosa* PAO1 (PA), *P. putida* S-313, and *P. fluorescens* Pf0-1 (Pfl01_) with characterized or putative functions in the uptake of **a** Copper, **b** Sulfate, **c** Enterobactin, **d** Heme, or **e** Cobalamin (B12). Predicted gene functions are denoted by color: *red*, TBDR or TBDT; *brown*, ABC transport; *gold*, membrane protein (other than ABC transport); *green*, biosynthesis; *purple*, ECF sigma factor and anti-sigma factor; *yellow*, regulatory (other than ECF sigma factor); *pink*, hemophore; *blue*, hypothetical. Genes whose functions appear unrelated to that of the TBDR/TBDT are shown in *white*. Orthologs not readily identifiable by their position in the gene cluster are indicated by identical patterns. *Light blue lines* and *triangles* connect orthologs, and accompanying numbers indicate the percent identity of amino acid sequences

clade also containing HasR and HxuC, which function in heme uptake in *P. aeruginosa* PAO1 (Cornelis and Bodilis 2009; Ochsner et al. 2000). PFL_5378 is 75% identical to PA3408 (HasR), the hemophore receptor in *P. aeruginosa* PAO1, and is clustered with orthologs of genes functioning in hemophore production and uptake (Fig. 2d). PFL_1371 is 61% identical to PA1302 (HxuC) with no conservation of contiguous genes beyond the sigma factors and anti-sigma factors adjacent to the transducers. The deduced amino acid sequence of PFL_4627 is 66% identical to PA4710 (PhuR), a heme receptor (Cornelis et al. 2009), but PFL_4627 is clustered with an ECF sigma factor/anti-sigma factor gene pair whereas PA4710 is clustered with other genes having a demonstrated role in heme uptake in *P. aeruginosa* (Fig. 2d). PA4710 does not have an N-terminal signaling domain so it was not included in the phylogenetic analysis of TBDTs in the Pf-5 genome. This large clade also includes PFL_2365, which is 69% identical to PA4897 (OptI), a TBDT that is iron regulated in *P. aeruginosa* (Cornelis et al. 2009).

Two of the 10 TBDT clades include proteins with known or putative roles in the uptake of ferric-complexes of citrate or aerobactin. PFL_0982 and PFL_4039 fall in a clade with PA3901 (FecA) (Fig. 3), which functions in ferric citrate uptake in *P. aeruginosa* PAO1 (Marshall et al. 2009). PFL_0982 is clustered with an ECF sigma factor and anti-sigma factor pair orthologous to PA3900 (*fecR*) and PA3899 (*fecI*), as determined by reciprocal best-hit analysis, suggesting that the PFL_0982-PFL_0984 cluster is likely to function in ferric-citrate uptake. Another clade includes PFL_3154, which is similar (49% identity) to the TBDR PA4675 (ChtA) involved in aerobactin, rhizobactin 1021 and

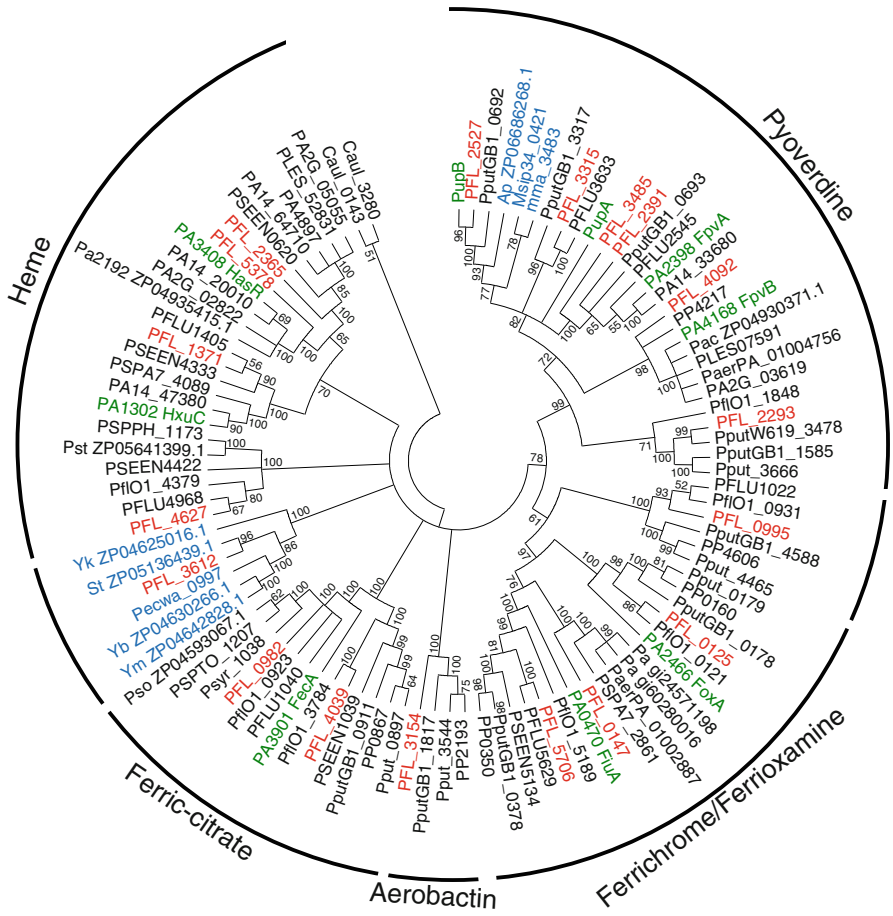


Fig. 3 Maximum parsimony analysis of TonB-dependent transducers. A phylogenetic analysis of the 18 TBDTs of *P. fluorescens* Pf-5 (PFL) and orthologous transducers was done using the Maximum Parsimony method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The tree is rooted with two transducers from *Caulobacter* sp. K31 as an outgroup. Pf-5 proteins are shown in *red* font; proteins with known functions are shown in *green* font; and proteins from genera other than *Pseudomonas* are shown in *blue*. Putative substrates assigned to Pf-5 TBDTs are labeled on the periphery of the circle. Abbreviations for species represented in the tree are as follows: *Achromobacter piechaudii* ATCC 43553 (Ap), *Caulobacter* sp. K31 (Caul), *Janthinobacterium* sp. *Marseille* (mma), *Methylovorus* sp. SIP3-4 (Msip34), *P. aeruginosa* (Pa), *P. aeruginosa* 2192 (PA2G), *P. aeruginosa* PA14 (PA14), *P. aeruginosa* PA7 (PSPA7), *P. aeruginosa* PACS2 (PaerPA), *P. aeruginosa* PAO1 (PA), *P. entomophila* (PSEEN), *P. fluorescens* Pf0-1 (Pfl01), *P. fluorescens* SBW25 (PFLU), *P. putida* F1 (Pput), *P. putida* GB1 (PputGB1), *P. putida* KT2440 (PP), *P. putida* W619 (PputW619), *P. syringae* pv. *oryzae* str. 1_6 (Pso), *P. syringae* pv. *phaseolicola* 1448A (PSPPH), *P. syringae* pv. *tabaci* ATCC 11528 (Pst), *Pectobacterium wasabiae* WPP163 (Pecwa), *Stenotrophomonas* sp. SKA14 (St), *Yersinia bercovieri* ATCC 43970 (Yb), *Yersinia kristensenii* ATCC 33638 (Yk), *Yersinia mollaretii* ATCC 43969 (Ym)

schizokinen uptake by *P. aeruginosa* (Cuiv et al. 2006). ChtA lacks a signaling domain so was not included in the phylogenetic analysis.

Three TBDTs (PFL_0125, PFL_0147, and PFL_5706) are in a large clade that also contains TBDTs functioning in the uptake of the hydroxamate siderophores ferrioxamine and ferrichrome in *P. aeruginosa*. PFL_0125 is 66% identical to FoxA (PA2466), which is a ferrioxamine uptake receptor in PAO1 (Hannauer et al. 2010). PFL_0125 and *foxA* are components of syntenous clusters with orthologous genes encoding an ECF sigma factor, anti-sigma factor, and putative transmembrane protein. PFL_5706 is 66% identical to PA0470 (FiuA), the

ferrichrome receptor of *P. aeruginosa* PAO1 (Hannauer et al. 2010). PFL_5706 is also clustered with an ECF sigma factor and anti-sigma factor pair orthologous to PA0471 and PA0472, as determined by reciprocal best-hit analysis, suggesting that the PFL_5704-PFL_5706 cluster is likely to function in ferrichrome uptake. Recently, Hannauer et al. (2010) reported that, in *P. aeruginosa*, both FiuA and FoxA transport ferrichrome, which suggests that the Pf-5 TBDTs in this clade may also exhibit relaxed specificities in the transport of these hydroxamate siderophores (Hannauer et al. 2010). The three Pf-5 TBDTs, PFL_0125, PFL_5706 and PFL_0147, are all contained within a well-supported clade, suggesting that PFL_0147 may also function in uptake of ferrichrome, ferrioxamine, or both siderophores. PFL_0995 and orthologs from other *Pseudomonas* spp. form a clade related to the ferrichrome/ferrioxamine clade with a bootstrap of 61, indicating a possible role for these proteins in the uptake of hydroxamate siderophores.

Another large clade includes characterized pyoverdine receptors FpvA and FpvB from *P. aeruginosa* PAO1 (Cobessi et al. 2005; Ghysels et al. 2004) and PupA and PupB from *P. putida* WCS358 (Bitter et al. 1991; Koster et al. 2006). Pf-5 has six TBDTs falling within this clade (Fig. 3), whose sequences are 35% to 68% identical to FpvA or FpvB of *P. aeruginosa* PAO1 at the amino acid level. PFL_4092 is present within one of the four pyoverdine gene clusters in the Pf-5 genome (Fig. 4) whereas the other five TBDTs in this clade are clustered with ECF sigma factor and anti-sigma factor gene pairs at dispersed locations in the Pf-5 genome. In this clade, PFL_2293 appears to be ancestral, and PFL_4092 forms its own subclade with FpvB from *P. aeruginosa* PAO1. The other four TBDTs in this clade (PFL_2391, PFL_3315, PFL_2527, and PFL_3485) are more closely related to each other and to FpvA, PupA and PupB.

Characterization of siderophore-biosynthesis mutants of *P. fluorescens* Pf-5

Arbitrary polymerase chain reaction (PCR) was used to map Tn*5* insertions in nine Pf-5 mutants deficient in pyoverdine production (Pvd^-) (Kraus and Loper 1992). Three insertions were mapped to *pvdL*, a non-ribosomal peptide synthetase involved in the biosynthesis of the pyoverdine chromophore (Fig. 4). Three Tn*5* insertions mapped to the non-ribosomal peptide

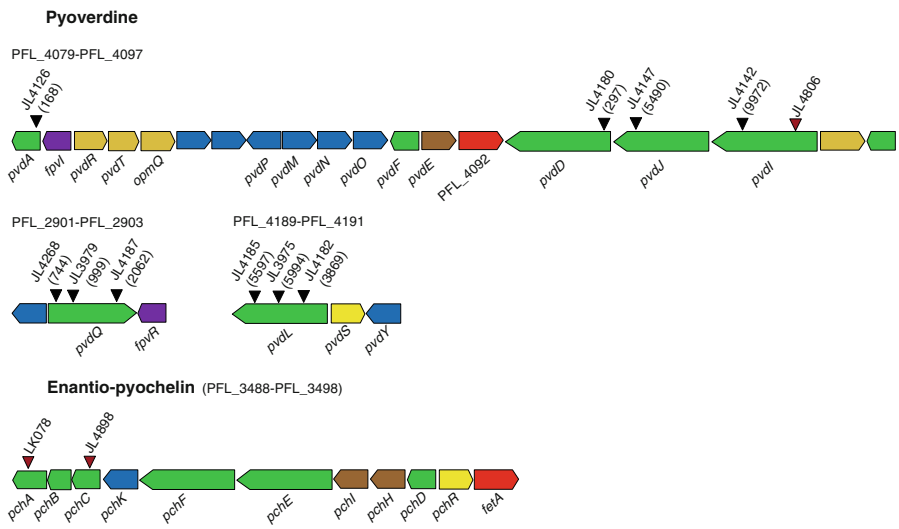


Fig. 4 Locations of mutations in the pyoverdine or enantio-pyochelin gene clusters of Pf-5. *Arrows* denote genes functioning in siderophore biosynthesis (*green*), ABC transport (*brown*), ECF sigma factor and anti-sigma factor (*purple*), membrane proteins (other than ABC transport) (*gold*), regulatory (other than ECF sigma factor) (*yellow*), unknown function and hypothetical (*blue*). The TonB-dependent outer-membrane proteins are in *red*. *Black triangles* denote sites of Tn*5* insertions eliminating pyoverdine production, and *red triangles* denote sites of deletions eliminating pyoverdine or enantio-pyochelin production by Pf-5. Strain numbers of mutants having the designated mutations are shown above the triangles. In parentheses below strain number is the nucleic acid position of the Tn*5* insertion

synthetases involved in biosynthesis of the pyoverdine peptide chain: one each in *pvdD, pvdI,* and *pvdJ*. Three insertions mapped to *pvdQ*, an acylase functioning in maturation of the pyoverdine (Koch et al. 2010). Therefore, the Tn*5* insertions were mapped to three of the four pyoverdine gene clusters predicted from bioinformatic analysis of the Pf-5 genome, providing functional support for these predictions (Fig. 4).

To further characterize siderophore biosynthesis and uptake in Pf-5, we made unmarked deletions in the pyoverdine and enantio-pyochelin gene clusters of Pf-5. A pyoverdine deficient mutant, constructed by deletion of a sequence internal to *pvdI* (PFL_4095), lacked the characteristic fluorescence of the pyoverdine siderophore when cultures grown on KMB were viewed under UV light. Mutants in enantio-pyochelin biosynthesis were constructed by deletion of a sequence internal to *pchA* (PFL_3488), or *pchC* (PFL_3490) (Fig. 4). PchA catalyses the first step in the synthesis of salicylate from chorismate (Gaille et al. 2003) whereas PchC is a thioesterase involved in subsequent conversion of salicylate to pyochelin (Reimmann et al. 2004). Enantio-pyochelin was detected by TLC in culture extracts of Pf-5 but not the *pchA* mutant. Less than wildtype levels were detected in extracts of the *pchC* mutant (data not shown). A *pchC* mutant of *P. aeruginosa* also produces low levels of pyochelin compared to wild type (Reimmann et al. 2004).

Double mutants were created by stacking deletions in *pvdI* with *pchA* or *pchC*. These mutants were evaluated for growth under iron-limited conditions imposed by amending KMB with varying concentrations of the iron chelator 2,2′-dipyridyl. The wildtype Pf-5 grew on KMB amended with up to 800 μM 2,2′-dipyridyl whereas the Pvd^- Tn*5* mutants and the *pvdI* deletion mutant grew only on KMB containing 600 μM or less of the chelator, as expected due to the known role of pyoverdine production in iron-limited growth of *Pseudomonas* spp. The *pchC* and *pchA* mutants grew on KMB containing up to 800 μM 2,2′-dipyridyl, indicating that enantio-pyochelin is not required for iron-limited growth of pyoverdine-producing strains. In contrast, the double *pvdI–pchC* and *pvdI–pchA* mutants did not grow on KMB containing 400–800 μM 2,2′-dipyridyl, demonstrating the role of both siderophores in iron-limited growth of Pf-5.

The mutants were also characterized by observing their phenotypes on CAS agar, the universal siderophore detection medium (Schwyn and Neilands 1987). This medium contains a blue dye (CAS) that turns orange when iron is removed. Typically, siderophore production results in an orange zone surrounding a colony. In preliminary experiments, we found that Pf-5 also caused a cleared halo with a deep blue margin (Fig. 5), whereas this type of halo was not generated by an *ofa*A mutant of Pf-5 deficient in the production of orfamide A, an anionic biosurfactant (Gross et al. 2007). The clearing zone was also observed surrounding colonies of Pf-5, but not the *ofaA* mutant, on CAS agar amended with 1 mM $FeCl_3$ (data not shown). This clearing could be related to the formation of micelles around the CAS dye, which has been reported for anionic surfactants (Callahan and Cook 1984). Clear zones with blue margins were seen on CAS agar plates spotted with 10 μl of 1 mg/ml orfamide A or 1% sodium dodecyl sulfate (SDS) (data not shown), an anionic surfactant known to form micelles with CAS (Callahan and Cook 1984). Therefore, the *pchA*, *pchC*, and *pvdI* mutations were introduced into an *ofaA* mutant of Pf-5, which lacks orfamide A production, so that siderophore production could be assessed on CAS agar without interference from the biosurfactant. By visualizing halos surrounding mutant colonies on CAS agar, we confirmed that both siderophores chelate iron and observed no additional siderophore produced by Pf-5 on this medium (Fig. 5).

Utilization of diverse siderophores by *P. fluorescens* Pf-5

The ability of Pf-5 to utilize a diverse set of pyoverdines as iron sources was assessed in cross-feeding experiments. Sixty-one strains of *Pseudomonas* spp. were tested, 34 of which produce pyoverdines of known amino acid composition (Table 2). Nine strains produce pyoverdines representing distinct siderotypes, although their structures are not known. The length and amino acid composition of the pyoverdine peptide chain was predicted bioinformatically from genomic sequence data for four strains (Pf-5, *P. syringae* B728A, *P. syringae* DC3000, and *P. aeruginosa* PA14) (Online Resource 3). As stated above, the *pvdI–pchC* mutant of Pf-5 did not grow on KMB amended with 400 μM 2,2′-dipyridyl under the conditions of this assay. When grown in proximity to 32 of the 61 test strains of *Pseudomonas* spp.,

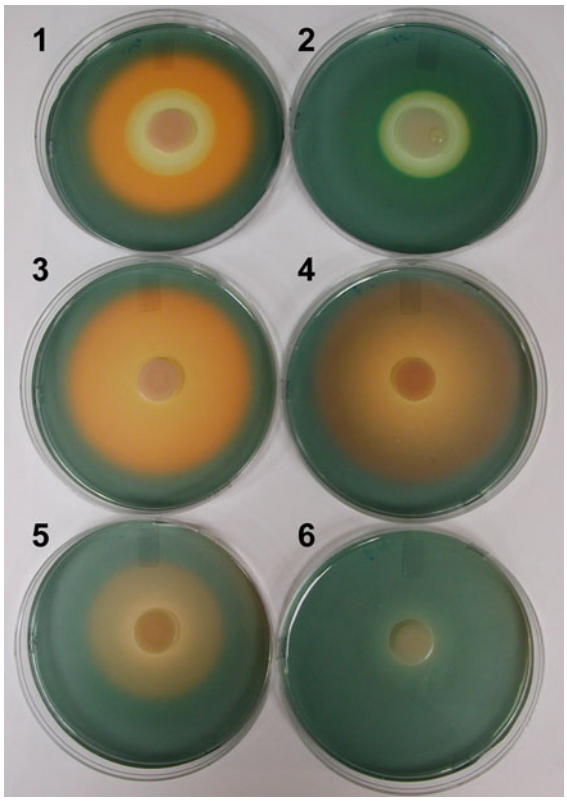


Fig. 5 Siderophore and orfamide A production detected as halos surrounding colonies of Pf-5 or derivative strains on the universal siderophore detection medium, CAS. On CAS agar, an *orange halo* surrounding a colony of strain Pf-5 (1) indicates siderophore production, and a smaller clear halo is due to orfamide production. The clear zone is also evident surrounding a colony of a *pvdI–pchA* mutant of Pf-5 (2). Pyoverdine production is visualized as an orange halo surrounding the *pchA–ofaA* mutant (3) whereas enantio-pyochelin production is visualized as the halo surrounding colonies of the Δ*pvdI–ofaA* mutant (4). A smaller halo surrounds colonies of the Δ*pvdI–pchC–ofaA* mutant (5), which is attributed to residual levels of enantio-pyochelin or salicylate biosynthesis. No halo surrounds colonies of Δ*pvdI–pchA–ofaA* mutant (6), indicating the lack of detectable siderophore production by this mutant. Plates were incubated at room temperature for 12 days

however, the *pvdI–pchC* mutant grew on this iron-limited medium, indicating its capacity to utilize siderophores produced by the test strains as iron sources. Pvd^{-} mutants were available for four of the crossfeeding strains, and these mutants did not cross-feed the *pvdI–pchC* mutant of Pf-5 (Table 2), indicating that the pyoverdine was responsible for crossfeeding. The 32 strains of *Pseudomonas* spp. that crossfed the *pvdI–pchC* mutant represent 17 pyoverdine structures. Therefore, Pf-5 can utilize a diverse set of pyoverdines as iron sources.

Pf-5 was also tested for utilization of non-pyoverdine siderophores as iron sources. In the presence of ferric-citrate, ferrichrome, desferrioxamine, hemoglobin, and hemin chloride, the *pvdI–pchC* mutant grew on the iron-limited medium, indicating that Pf-5 can utilize these compounds as iron sources. The capacity of Pf-5 to utilize aerobactin and enterobactin was assessed in cross-feeding experiments. The *pvdI–pchC* mutant grew on KMB amended with 400 μM 2,2′-dipyridyl when placed in proximity to a colony of *E. cloacae* EcCT-501, which produces enterobactin and aerobactin (Table 3). Similarly, the *pvdI–pchC* mutant grew on the medium when placed near colonies of an enterobactin-deficient mutant of *E. cloacae*, which produces aerobactin, or an aerobactin-deficient mutant of *E. cloacae*, which produces enterobactin. In contrast, the *pvdI–pchC* mutant did not grow on the medium when placed near a colony of a mutant of *E. cloacae* deficient in the production of both siderophores, indicating that Pf-5 can utilize both aerobactin and enterobactin as iron sources. Taken together, the results of these experiments confirm the siderophore utilization patterns that were predicted from the phylogenetic analyses.

Discussion

The 45 TonB-dependent outer-membrane proteins in the proteome of *P. fluorescens* Pf-5 (Paulsen et al. 2005) comprise 27 TBDRs and 18 TBDTs that share conserved β-barrel and plug domains but differ in the presence of an N-terminal signaling domain. Phylogenetic and bioinformatic analyses suggest a complex evolutionary history for the TonB-dependent outer-membrane proteins in Pf-5 including horizontal transfer among different microbial lineages. In a recent phylogenetic analysis of 4,600 TonB-dependent outer-membrane proteins, Mirus et al. (2009) reported that, with few exceptions, the proteins cluster according to their substrate rather than taxonomy (Mirus et al. 2009). The results of our study also provide convincing evidence of lateral transmission of these proteins among diverse groups of bacteria.

Iron is a limiting factor for many soil microorganisms including Pf-5, which uses pyoverdine and enantio-pyochelin to retrieve iron from its

Table 3 Crossfeeding of the *pvdI–pchC* mutant of Pf-5 by *Enterobacter cloacae*

Genotype abbreviations: Aerobactin (*iuc*). Enterobactin (*ent*) (Costa and Loper 1994)

E. cloacae strain	Genotype	Siderophores produced	Iron limited growth of JL4900
EcCT-501	Field isolate	Enterobactin & aerobactin	+
LA122	Δ*iuc*	Enterobactin	+
LA266	Δ*ent*	Aerobactin	+
LA235	Δ*iuc* Δ*ent*	None	−

surroundings (Youard et al. 2007; Hoegy et al. 2009). Here, we showed that Pf-5 can utilize a broad spectrum of exogenous siderophores as sources of iron. Phylogenetic analysis of the TBDTs in the Pf-5 genome indicated a high level of redundancy for the uptake of certain compounds, notably ferrioxamine, ferric-citrate, heme, and pyoverdines. The number of TBDTs in certain phylogenetic clades, such as those with putative functions in heme and pyoverdine acquisition, exceeds the number found in other bacteria such as *P. aeruginosa* PAO1, which also has multiple TonB-dependent outer-membrane proteins functioning in the uptake of ferrioxamine, enterobactin, heme and pyoverdines (Cornelis and Matthijs 2002; Cornelis et al. 2008). The diversity and complexity of the TBDTs with roles in iron uptake clearly indicate the importance of iron in the biology of Pf-5.

Pseudomonas fluorescens Pf-5 was isolated from soil (Howell and Stipanovic 1979) and establishes populations in the rhizosphere when inoculated onto seed or root surfaces (Brodhagen et al. 2004; Kraus and Loper 1992; Sarniguet et al. 1995). The roles of TonB-dependent outer-membrane proteins in enhancing the access of bacteria to limited resources in the rhizosphere or bulk soil has been demonstrated only for iron and sulfur to date. Siderophore-mediated competition for iron is a major determinant in interactions between certain strains of *Pseudomonas* spp., and the capacity to utilize a pyoverdine produced by a competing strain was shown to enhance the fitness of *P. fluorescens* living on root surfaces (Raaijmakers et al. 1995). Furthermore, levels of iron available to *Pseudomonas* spp. in the rhizosphere are known to be enhanced by siderophores produced by other rhizosphere bacteria. For example, a pyoverdine-producing strain of *Pseudomonas* spp. and enterobactin- and aerobactin-producing strains of *E. cloacae* enhanced the levels of iron available to *P. putida* in the rhizosphere, assessed using an iron biosensor (Loper and Henkels 1999). The results from these studies indicate that TonB-dependent outer-membrane proteins confer an advantage to *Pseudomonas* spp. in the rhizosphere due to enhanced iron uptake. Similarly, the capacity to utilize sulfur esters is necessary for optimal survival of *P. putida* in agricultural and grassland soils (Kahnert et al. 2002), and the sulfur-inducible TonB-dependent receptor SftP appears to function in sulfate ester metabolism. In addition to the SftP ortholog PFL_0213, several other genes encoding TonB-dependent receptors are linked to transport proteins with putative functions in sulfur transport in the Pf-5 genome (data not shown), and their role in sulfur metabolism is an intriguing area for future study. In addition to their roles in iron and sulfur uptake, TonB-dependent outer-membrane proteins are likely to function more broadly in the acquisition of resources by environmental prokaryotes like *P. fluorescens*, and future investigations should reveal novel roles of these transport systems in the ecology of soil and rhizosphere bacteria.

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