Ferripyochelin uptake genes are involved in pyochelin-mediated signalling in *Pseudomonas aeruginosa*

Laurent Michel,[†] Aude Bachelard and Cornelia Reimmann

Département de Microbiologie Fondamentale, Université de Lausanne, CH-1015 Lausanne, Switzerland

Correspondence

Cornelia Reimmann
Cornelia.Reimmann@unil.ch

In response to iron starvation, *Pseudomonas aeruginosa* produces the siderophore pyochelin. When secreted to the extracellular environment, pyochelin chelates iron and transports it to the bacterial cytoplasm via its specific outer-membrane receptor FptA and the inner-membrane permease FptX. Exogenously added pyochelin also acts as a signal which induces the expression of the pyochelin biosynthesis and uptake genes by activating PchR, a cytoplasmic regulatory protein of the AraC/XylS family. The importance of ferripyochelin uptake genes in this regulation was evaluated. The *fptA* and *fptX* genes were shown to be part of the *fptABCX* ferripyochelin transport operon, which is conserved in *Burkholderia* sp. and *Rhodospirillum rubrum*. The *fptB* and *fptC* genes were found to be dispensable for utilization of pyochelin as an iron source, for signalling and for pyochelin production. By contrast, mutations in *fptA* and *fptX* not only interfered with pyochelin utilization, but also affected signalling and diminished siderophore production. It is concluded from this that pyochelin-mediated signalling operates to a large extent via the ferripyochelin transport system.

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INTRODUCTION

Iron is essential for most living organisms, including bacteria. However, despite its abundance on earth, iron is not freely available to micro-organisms under aerobic conditions, as it forms poorly soluble ferric hydroxides in the environment or is tightly bound to transport and storage proteins in mammalian hosts (Andrews *et al.*, 2003). To acquire ferric ions, bacteria have evolved a number of strategies; the most common involves high-affinity iron-chelating molecules, termed siderophores (Guerinot, 1994; Wandersman & Delepelaire, 2004).

In Gram-negative bacteria, iron uptake is mediated by specific transport systems consisting of an outer-membrane receptor, which is energized by the TonB-ExbB-ExbD system (Moeck & Coulton, 1998), and an inner-membrane permease, which often belongs to the family of periplasmic binding protein-dependent ABC transporters (Köster, 2001). The entire ferrisiderophore complex may be transported to the cytoplasm, where iron is released from the chelator, e.g. in the case of iron uptake by ferrichrome (Köster, 1997). Alternatively, iron release can occur in the periplasm and the siderophore does not cross the inner

membrane, e.g. during ferric citrate transport (Hussein *et al.*, 1981).

Biosynthesis of siderophores and their cognate uptake systems is tightly regulated to ensure that they are produced only when needed and to avoid accumulation of iron, which can be deleterious to the cell (because free ferrous iron can catalyse the generation of hydroxyl radicals through the Fenton reaction: Andrews *et al.*, 2003). Under iron-rich conditions, the Fur protein represses, directly or indirectly, the expression of siderophore biosynthesis and uptake genes (Escobar *et al.*, 1999; Hantke, 2001; Prince *et al.*, 1993; Vasil & Ochsner, 1999). Fur-mediated repression is alleviated when iron becomes limiting, allowing a basal level of gene expression to occur. Full expression often requires the presence of the siderophore. By a variety of different mechanisms involving extracellular cytoplasmic function (ECF) sigma/anti-sigma factors, two-component regulatory systems or AraC-type regulators, the siderophore induces the expression of genes necessary for its uptake and, in certain cases, also for its biosynthesis (Poole & McKay, 2003; Visca *et al.*, 2002). In this type of regulation, also known as siderophore-mediated signalling (Lamont *et al.*, 2002), perception of the siderophore can occur either at the cell surface, in the periplasm, or in the cytoplasm; examples are provided by pyoverdine-, enterobactin- and pyochelin-mediated signalling in the Gram-negative bacterium *Pseudomonas aeruginosa*. This opportunistic human pathogen produces two siderophores, pyoverdine and pyochelin

[†]Present address: BIOMERIT Research Centre, Department of Microbiology, National University of Ireland, Cork, Ireland.

Abbreviation: Dha, dihydroaeruginosate.

(Cox, 1980; Cox & Adams, 1985; Meyer & Abdallah, 1978; Rinehart *et al.*, 1995), but can promote iron uptake also with a variety of heterologous siderophores of fungal and bacterial origin (Poole & McKay, 2003). Pyoverdine is perceived at the cell surface, where interaction of the ferripyoverdine complex with the outer-membrane receptor FpvA transmits a signal to the anti-sigma factor FpvR. This inner-membrane-spanning protein then activates two ECF-sigma factors, PvdS and FpvI, which are required for the transcription of pyoverdine biosynthesis and uptake genes, respectively (Beare *et al.*, 2003; Lamont *et al.*, 2002; Redly & Poole, 2003). Perception of the heterologous *Escherichia coli* siderophore enterobactin occurs in the periplasm by the sensor kinase PfeS, which activates its cognate response regulator PfeR by phosphorylation such that PfeR becomes able to upregulate the transcription of the outer-membrane receptor gene *pfeA* (Dean & Poole, 1993; Dean *et al.*, 1996). Pyochelin sensing occurs in the cytoplasm of *P. aeruginosa*. We have shown recently that pyochelin, possibly in its iron-loaded form (Michel *et al.*, 2005), is the intracellular effector required by the AraC-type regulator PchR (Heinrichs & Poole, 1993, 1996) to activate the expression of the two pyochelin biosynthesis operons *pchDCBA* (Serino *et al.*, 1997) and *pchEFGHI* (Reimann *et al.*, 1998, 2001), and of the *fptA* gene, encoding the outer-membrane ferripyochelin receptor (Ankenbauer & Quan, 1994). (Note that until the iron status of the PchR effector has been confirmed by additional experiments, the more general term 'pyochelin' will be used here.) As addition of pyochelin to the growth medium triggers expression of these target genes in pyochelin-negative mutants, it can be concluded that the siderophore needs to be translocated to the cytoplasm in order to act as a PchR effector. Genes involved in pyochelin-mediated iron uptake could thus be involved in pyochelin-mediated signalling and hence might also affect pyochelin production.

The *fptA* gene, located immediately downstream of the pyochelin biosynthesis genes (Fig. 1), is followed by three contiguous ORFs, PA4220 (= *fptB*), PA4219 (= *fptC*) and *fptX*, which was reported recently to encode an inner-membrane permease required for growth with pyochelin as an iron source (Ó Cuív *et al.*, 2004). Here we demonstrate that these genes form a ferripyochelin transport operon and we evaluate their importance in pyochelin utilization, signalling, and pyochelin production.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains, plasmids and cosmids are listed in Table 1. Bacteria were routinely grown on nutrient agar and in nutrient yeast broth (Stanisich & Holloway, 1972) at 37 °C. For β -galactosidase assays, *P. aeruginosa* strains were cultivated in GGP medium (Carmi *et al.*, 1994), in which limited iron availability allows the expression of pyochelin biosynthesis and uptake genes. Pyochelin-utilization assays were performed with M9 minimal medium (Sambrook & Russell, 2001) using 0.5% glycerol as a carbon source. Antibiotics were added to the growth media at the following concentrations:

ampicillin (Ap) 100 $\mu\text{g ml}^{-1}$, kanamycin (Km) 25 $\mu\text{g ml}^{-1}$, spectinomycin (Sp) 50 $\mu\text{g ml}^{-1}$ and tetracycline (Tc) 25 $\mu\text{g ml}^{-1}$ for *E. coli*; carbenicillin (Cb) 250 $\mu\text{g ml}^{-1}$, Sp 1000 $\mu\text{g ml}^{-1}$ and Tc 125 $\mu\text{g ml}^{-1}$ for *P. aeruginosa*. To monitor β -galactosidase expression, X-Gal was incorporated into solid media at a final concentration of 0.02%. To counterselect *E. coli* donor cells in matings with *P. aeruginosa*, chloramphenicol (Cm) was used at a concentration of 10 $\mu\text{g ml}^{-1}$; mutant enrichment was performed with Tc at a final concentration of 20 $\mu\text{g ml}^{-1}$ and Cb at a final concentration of 2000 $\mu\text{g ml}^{-1}$, as described previously (Ye *et al.*, 1995).

DNA manipulations and cloning procedures. Small- and large-scale preparations of plasmid DNA were made with the cetyltrimethylammonium bromide method (Del Sal *et al.*, 1988) and the Jetstar kit (Genomed), respectively. DNA fragments were purified from agarose gels with the GeneClean II kit (Bio 101) or the MinElute Gel Extraction Kit (Qiagen). DNA manipulations were performed according to standard procedures (Sambrook & Russell, 2001). Transformation of *E. coli* and *P. aeruginosa* was carried out by electroporation (Farinha & Kropinski, 1990). All constructs involving PCR techniques were verified by sequence analysis. Sequencing was performed with the BigDye Terminator Cycle Sequencing Kit and an ABI-PRISM 373 automatic sequencer (Applied Biosystems) or was carried out at Microsynth (<http://www.microsynth.ch>). Sequences were compared with the DNA sequence available from the *P. aeruginosa* sequencing project (<http://www.pseudomonas.com>). Database searches were conducted at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) with BLAST algorithms, and protein sequences were analysed with the Expert Protein Analysis System (<http://www.expasy.org>).

Construction of plasmids used for complementation (Fig. 1). Plasmid pME7034, which carries *fptA*, was generated as follows. A 1.2 kb *EcoRI*–*EcoRV* fragment containing the promoter and the 5' region of *fptA* was excised from pMO012405 and cloned into pUCPSK, giving pME7033. The resulting plasmid was digested with *EcoRI* and *SmaI* and the remaining part of *fptA* was added on a pMO012405-derived 1.7 kb *EcoRI*–*XhoI* fragment, made blunt at its *XhoI* end by T4 DNA polymerase. To construct pME7036, which carries the entire *fptABCX* operon, a 4.8 kb *EcoRI*–*NruI* fragment from pMO012405 was cloned into pUK21 between *EcoRI* and *StuI*, giving pME7032. The 4.8 kb insert was then excised with *EcoRI* and *SpeI* and cloned into pME7033, cleaved with the same enzymes. Expression of *fptA* and *fptABCX* in pME7034 and pME7036, respectively, occurs from the *fptA* promoter as the vector-encoded *lac* promoter is located at the end of the cloned genes. Plasmid pME7204, expressing the *fptX* gene under *P_{lac}* control, was generated by inserting the 1.6 kb *NotI*–*SpeI* fragment from pME7032 into pUCPSK, linearized with the same enzymes.

Generation of translational *lacZ* fusions to *fptB*, *fptC* and *fptX* (Fig. 1). The *fptB'*–*lacZ* fusion carried by pME7518 was constructed as follows. First, a 0.2 kb fragment containing the 3' part of *fptA* and the first 2 codons of *fptB* was PCR-amplified from chromosomal DNA of PAO1 with primers yfpB-1 (GGCGTGA-GCATGCGCCAGG; *SphI*-tagged) and fptB-1 (ACGTCTGCAG-CGGCATCAGAACGCGCCCCG; *PstI*-tagged), cleaved with *SphI* and *PstI* and cloned into pME7215. From the resulting plasmid, a 2.8 kb fragment was excised with *BglII* and *PstI* and ligated to *BamHI* and *PstI*-linearized pME6015. An *fptC'*–*lacZ* fusion was obtained in a similar way. A 0.5 kb PCR fragment, amplified from PAO1 chromosomal DNA with primers yfpB-1 and yfpB-2 (ACGTCTGCAGCGCCACTTCAACCGCGCCCC; *PstI*-tagged), was trimmed with *SphI* and *PstI* and cloned into pME7215. A 3.1 kb *BglII*–*PstI* fragment, carrying *fptA*, *fptB* and the first 2 codons of *fptC*, was excised from the resulting plasmid and cloned into pME6015 to give pME7517. To generate a translational *lacZ* fusion

Fig. 1. Physical map of the pyochelin gene cluster on the chromosome of *P. aeruginosa* PAO1 and of plasmids constructed in this study. The Ω-Sp/Sm cassette used to generate the *fptX* mutants PAO6368, PAO6396 and PAO6397 (see Table 1) is designated by Ω. Deletions are indicated by Δ and filled triangles represent the location of the vectors *lacZ* promoter. Arrows indicate the direction of transcription or the transcriptional units. Restriction sites used for subcloning experiments are indicated (note that only the locations relevant to this study are shown); artificially introduced sites are marked by an asterisk.

Table 1. Strains, plasmids and cosmids

Name	Relevant characteristics	Reference or source
<i>E. coli</i> strains		
DH5 α	<i>recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1 Δ(lacZYA-argF) U169</i> (Φ 80d <i>lacZ</i> Δ M15)	Sambrook & Russell (2001)
S17-1	<i>pro thi hsdR recA</i> ; chromosomal RP4 (Tra ⁺ Tc ^s Km ^s Ap ^s); Tp ^r Sm ^r	Simon <i>et al.</i> (1983)
<i>P. aeruginosa</i> strains		
PAO1	Wild-type	ATCC 15692
PAO6297	Δ <i>pchBA</i>	Reimmann <i>et al.</i> (1998)
PAO6368	<i>fptX</i> :: Ω -Sp/Sm	This study
PAO6383	Δ <i>pvdF</i> , Δ <i>pchBA</i>	This study
PAO6387	Δ <i>fptC</i>	This study
PAO6388	Δ <i>pchBA</i> Δ <i>fptC</i>	This study
PAO6390	Δ <i>pvdF</i> Δ <i>pchBA</i> Δ <i>fptC</i>	This study
PAO6396	Δ <i>pchBA</i> <i>fptX</i> :: Ω -Sp/Sm	This study
PAO6397	Δ <i>pvdF</i> Δ <i>pchBA</i> <i>fptX</i> :: Ω -Sp/Sm	This study
PAO6423	Δ <i>fptB</i>	This study
PAO6424	Δ <i>pchBA</i> Δ <i>fptB</i>	This study
PAO6428	Δ <i>fptA</i>	This study
PAO6429	Δ <i>pchBA</i> Δ <i>fptA</i>	This study
PAO6540	Δ <i>pvdF</i> Δ <i>pchBA</i> Δ <i>fptB</i>	This study
PAO6541	Δ <i>pvdF</i> Δ <i>pchBA</i> Δ <i>fptA</i>	This study
Plasmids		
pBLS II KS, SK	Cloning vector, ColE1 replicon; Ap ^r	Stratagene
pHP45 Ω	ColE1 replicon carrying a Ω -Sp/Sm cassette; Sp ^r Sm ^r Ap ^r	Prentki & Krisch (1984)
pME3087/3088	Suicide vectors; ColE1 replicon; Tc ^r	Voisard <i>et al.</i> (1994)
pME6015	Cloning vector for construction of translational <i>lacZ</i> fusions; Tc ^r	Schnider-Keel <i>et al.</i> (2000)
pME7032	pUK21 carrying a 4.8 kb <i>EcoRI</i> – <i>NruI</i> fragment containing <i>fptB</i> , <i>fptC</i> and <i>fptX</i> ; Km ^r	This study
pME7033	pUCPSK carrying a 1.2 kb <i>EcoRV</i> – <i>EcoRI</i> fragment containing the 5' part of <i>fptA</i> ; Ap ^r	This study
pME7034	pUCPSK derivative carrying <i>fptA</i> ; Ap ^r	This study
pME7036	pUCPSK derivative carrying <i>fptABCX</i> ; Ap ^r	This study
pME7038	pME3087 derivative carrying <i>fptX</i> :: Ω -Sp/Sm; Tc ^r	This study
pME7041	pUCPSK carrying a 3.46 kb <i>EcoRV</i> – <i>SmaI</i> fragment containing the 3' part of <i>pchI</i> , the entire <i>fptA</i> and <i>fptB</i> genes and the 5' part of <i>fptC</i> ; Ap ^r	This study
pME7043	pME3087 derivative carrying Δ <i>fptC</i> ; Tc ^r	This study
pME7152	pME3087 carrying the <i>pvdF</i> gene with an in-frame deletion on a 0.8 kb <i>BglII</i> – <i>PstI</i> fragment cloned between <i>BamHI</i> and <i>PstI</i> ; Tc ^r	This study
pME7153	Translational <i>fptA</i> '–' <i>lacZ</i> fusion; Tc ^r	Michel <i>et al.</i> (2005)
pME7158	pME3087 derivative carrying Δ <i>fptA</i> ; Tc ^r	This study
pME7160	translational <i>pchD</i> '–' <i>lacZ</i> fusion; Tc ^r	Michel <i>et al.</i> (2005)
pME7204	pUCPSK derivative expressing <i>fptX</i> from <i>plac</i> ; Ap ^r	This study
pME7208	pME3087 derivative carrying Δ <i>fptB</i> ; Tc ^r	This study
pME7215	pUK21 derivative carrying a 3.47 kb <i>ClaI</i> – <i>SmaI</i> fragment from pME7041 with <i>fptA</i> , <i>fptB</i> and the 5' part of <i>fptC</i> ; Km ^r	This study
pME7517	Translational <i>fptC</i> '–' <i>lacZ</i> fusion on pME6015; Tc ^r	This study
pME7518	Translational <i>fptB</i> '–' <i>lacZ</i> fusion on pME6015; Tc ^r	This study
pME7520	Translational <i>fptX</i> '–' <i>lacZ</i> fusion on pME6015; Tc ^r	This study
pME7521	Translational <i>fptC</i> '–' <i>lacZ</i> fusion lacking the <i>fptA</i> promoter; Tc ^r	This study
pME7522	Translational <i>fptB</i> '–' <i>lacZ</i> fusion lacking the <i>fptA</i> promoter; Tc ^r	This study
pME7523	Translational <i>fptX</i> '–' <i>lacZ</i> fusion lacking the <i>fptA</i> promoter; Tc ^r	This study
pUCPKS, SK	ColE1-pRO1600 shuttle vectors; Ap ^r	Watson <i>et al.</i> (1996)
pUK21	Cloning vector; ColE1 replicon; Km ^r	Vieira & Messing (1991)
Cosmids		
pMO012405	pLA2917 derivative with 23 kb chromosomal DNA from PAO1 encoding genes downstream of <i>pchF</i> ; Tc ^r	J. Mattick, University of Queensland, Australia

to *fptX*, a 2 kb PCR fragment was amplified with *yfpB*-1 and 4218-1 (ACGTCTGCAGAAGCATGGTGGTCTCCGGTG; *Pst*I-tagged), cleaved with *Sph*I and *Pst*I and cloned into pME7215 as described above. Digestion with *Bgl*II and *Pst*I yielded a 4.6 kb fragment containing *fptA*, *fptB*, *fptC* and the first two codons of *fptX*, which was cloned into pME6015 to generate pME7520. Plasmids pME7522, pME7521 and pME7523 lacking the *fptA* promoter region were generated by removing the 1.2 kb *Eco*RI–*Eco*RI fragment from pME7518, pME7517 and pME7520, respectively.

***P. aeruginosa* mutant constructions.** Gene replacement mutants were generated as described previously (Ye *et al.*, 1995) using suicide plasmids constructed as follows. To create in-frame *fptA* deletions, plasmid pME7041 (Fig. 1) was cleaved with *Sph*I and *Bcl*II, treated with T4 DNA polymerase and religated, thus removing codons 54–659 (note that the second *Bcl*II site present on pME7041 had not been cleaved during this experiment). The remaining 1.65 kb insert was excised with *Hind*III and *Bam*HI and cloned into the suicide vector pME3087 to give pME7158. This plasmid was then used to delete the *fptA* gene in *P. aeruginosa* PAO1 and PAO6297, generating the corresponding mutants PAO6428 and PAO6429, respectively.

Chromosomal in-frame deletions in *fptB* were constructed as follows. First, a 0.97 kb *Xma*III–*Xma*III fragment containing *fptB* was excised from pME7041 and cloned into pBLS II KS linearized with *Not*I. The resulting plasmid served as template in an inverse PCR reaction with the *Bgl*II-tagged primers *fptB*-2 (ACGTAGATCTGGCGGGGCGCGGTTGAAG) and *fptB*-3 (GATCAGATCTAAGCCCGACTGGCGCGGCATC). The amplified fragment was cleaved with *Bgl*II and religated, giving a plasmid containing *fptB* with a deletion of codons 7–88 (Δ *fptB*). This Δ *fptB* gene, together with flanking DNA, was inserted on a 0.73 kb *Sac*I–*Hind*III fragment into pME3087 to give the suicide construct pME7208, which was used to generate the Δ *fptB* mutants PAO6423 and PAO6424.

The construction of the in-frame *fptC* deletion mutants PAO6387 and PAO6388 required several steps as well. A 0.54 kb *Xho*I–*Xma*I fragment and a 0.4 kb *Not*I–*Bam*HI fragment, both originating from pME7032 and treated with T4 DNA polymerase at their *Xma*I and *Not*I ends, respectively, were ligated together and inserted into pBLS II KS between the *Xho*I and *Bam*HI sites. This generated an *fptC* deletion derivative (Δ *fptC*) lacking codons 128–445. The 0.94 kb *Xho*I–*Bam*HI fragment containing Δ *fptC* was then excised with *Kpn*I and *Bam*HI and cloned into pME3087 to yield the suicide construct pME7043, which was used to mutate *fptC* in strains PAO1 and PAO6297.

In strains PAO6368 and PAO6396, the *fptX* gene was mutated by insertion. A 1 kb *Apa*I–*Pst*I fragment originating from pME7032 and carrying the 5' part of *fptX* was first cloned into pBLS II KS, excised as a *Kpn*I–*Pst*I fragment and inserted into pME3087. The 2 kb Ω -Sp/Sm cassette from pHP45 Ω was then cloned into the *Bam*HI site located 80 codons downstream of the *fptX* start codon. This generated the suicide plasmid pME7038 used to mutate *fptX* in strains PAO1 and PAO6297.

To generate pyoverdine-negative mutants, in-frame deletions were constructed in the *pvdF* gene as follows. Primers *pvdF*-1 (ACGTA-GATCTTGCCCGGTATTAGCGGC; *Bgl*II-tagged) and *pvdF*-2 (TCGAAAGCTTCAGAGCTTCTCGGCGAC; *Hind*III-tagged) were used to PCR-amplify the *pvdF* gene with its promoter region from chromosomal DNA of PAO1. The 1 kb PCR product was digested with *Bgl*II and *Hind*III and cloned into pUK21. A 0.22 kb *Xma*I fragment was removed from this plasmid to create an in-frame deletion in the *pvdF* ORF and the remaining insert was excised with *Bgl*II and *Pst*I, and cloned into the suicide vector pME3087, cleaved with the same enzymes. The resulting plasmid pME7152 was then mobilized from *E.*

coli S17-1 to *P. aeruginosa* PAO6297, PAO6429, PAO6424, PAO6388 and PAO6396, and chromosomally integrated, with selection for tetracycline resistance. Excision of the vector via a second crossing-over was obtained by enrichment for tetracycline-sensitive cells (Ye *et al.*, 1995), generating the mutants PAO6383, PAO6541, PAO6540, PAO6390 and PAO6397, respectively. Deletion mutants were generally identified either by Southern blotting or by PCR analysis, while Ω -insertion mutants could also be screened for by their Sp/Sm-resistant phenotype.

Identification of salicylate, dihydroaeruginosate (Dha) and pyochelin in culture supernatants of *P. aeruginosa*. *P. aeruginosa* strains were grown in GGP medium for the time indicated. For HPLC analysis, ethyl acetate extracts of acidified culture supernatants were dried by evaporation, dissolved in 60% (v/v) methanol/10 mM H₃PO₄ and injected into an HPLC system as described previously (Reimann *et al.*, 1998). Compounds were identified by their retention times and UV spectra. Dha and salicylate were quantified at 256 and 237 nm, respectively. Pyochelin, which exists as a mixture of two interconvertible isomers, pyochelin I and pyochelin II (Rinehart *et al.*, 1995), was quantified at 258 nm and 254 nm, respectively.

Pyochelin-utilization assays. Utilization of pyochelin as an iron source was measured with liquid growth assays as follows. Erlenmeyer flasks (50 ml) with 15 ml M9-glycerol minimal medium containing, or not, the iron chelator 2,2'-dipyridyl at 500 μ M were inoculated to OD₆₀₀ 0.02 with precultures grown in M9-glycerol medium. HPLC-purified pyochelin was added at 20 μ M final concentration and growth at 37 °C and 220 r.p.m. was recorded for 150 h.

β -Galactosidase assays. Fifty millilitre Erlenmeyer flasks containing 15 ml GGP medium were inoculated with 0.15 ml portions of precultures grown in the same medium. Incubation was at 37 °C and 220 r.p.m. for 12 h. When required, pyochelin, which was isolated from *P. aeruginosa* PAO1 and purified by HPLC (Reimann *et al.*, 1998), was added at a final concentration of 20 μ M. β -Galactosidase activities were determined by the method of Miller (Sambrook & Russell, 2001) using cells permeabilized with 5% (v/v) toluene.

RT-PCR. Total RNA was extracted from a stationary-phase *P. aeruginosa* PAO1 culture in GGP medium by using the hot-phenol extraction method (Leoni *et al.*, 1996). Residual DNA was digested with 40 U RNase-free DNase I (Roche). Four micrograms of total RNA was added to a 38 μ l reaction mixture (Omniscrypt RT kit, Qiagen) containing primer *fptB*-bw (GACCACGCGCCAGCA-ACCCG), *fptC*-bw (GGATGTGACGCGCTTCCTCG) or *fptX*-bw (CGACCCAGGGTGCCAGAGG), respectively, but lacking reverse transcriptase. Reaction mixtures were divided in two equal parts to which either 1 μ l (=4 U) of reverse transcriptase was added (positive reaction) or RNase-free water (negative control). Incubation was for 2 h at 37 °C for reactions using *fptB*-bw (RT-1) or *fptC*-bw (RT-2), and at 42 °C when *fptX*-bw was used (RT-3). Subsequent PCR reactions were performed on 2 μ l of each RT mixture, using primer pairs *fptA*-fw (GACTACAGCGTCGACTACCG) and *fptB*-bw (for RT-1), *fptB*-fw (CGACCCAGCGCGGCTATCTG) and *fptC*-bw (for RT-2), and *fptC*-fw (GCGGATTGCTCGGCGTCGCC) and *fptX*-bw (for RT-3).

RESULTS

fptB, *fptC* and *fptX* are transcribed from the *fptA* promoter

PA4220 (Fig. 1), originally described as ORF2 (Ankenbauer

& Quan, 1994), seems to be translationally coupled to *fptA* as its predicted start codon overlaps with the *fptA* stop codon. The nucleotide sequence of PA4220 predicts a 93 amino acid cytoplasmic membrane protein of 9569 Da of unknown function. The following ORF, PA4219 [ORF 3 (Ankenbauer & Quan, 1994)], may be larger than originally proposed if translation initiates at a GTG codon located immediately downstream of PA4220. In this case, PA4219 would encode a protein of 501 amino acids with a molecular mass of 53 460 Da, predicted to be located in the cytoplasmic membrane as well. The *fptX* gene overlaps with PA4219 (Fig. 1) by a few bases and encodes a 414 amino acid protein of 43 151 Da, which belongs to a new family of inner-membrane permeases involved in siderophore transport (Ó Cuív *et al.*, 2004). To test the possibility that PA4220, PA4219 and *fptX* could form an operon together with *fptA*, translational *lacZ* fusions to the second codon of each of these three genes were constructed, with or without the *fptA* promoter. β -Galactosidase activities were measured under iron-limiting growth conditions in *P. aeruginosa* PAO1. As shown in Table 2, all three fusions containing the *fptA* promoter (pME7518, pME7517, pME7520) were well expressed whereas expression dropped 1000–10 000-fold in the three constructs pME7522, pME7521 and pME7523 lacking the *fptA* promoter. Note that the expression of *fptX* was much stronger than the expression of PA4220 and PA4219. This is likely due to the fact that *fptX* is preceded by a plausible ribosome-binding site (GGAGA) whereas this is not the case for PA4220 and PA4219. The GTG initiation codon of PA4219 may also contribute to the low expression of this gene. From these results we conclude that (i) the two ORFs of unknown function, PA4220 (which we name now *fptB*) and PA4219 (now named *fptC*), are transcribed and translated; (ii) *FptC* is indeed larger than reported earlier and that translation is initiated at the proposed GTG codon located immediately downstream of *fptB*; and (iii) *fptB*, *fptC* and *fptX* form an operon together with *fptA*. The operon structure was confirmed in addition by an RT-PCR analysis,

Fig. 2. Transcriptional analysis of the *fptABCX* genes. RNA extracted from strain PAO1 grown under iron limitation was used in reverse transcription reactions containing (+), or not (–) reverse transcriptase. Reaction products generated with primers *fptB*-bw, *fptC*-bw, or *fptX*-bw, respectively, were used in subsequent PCR reactions to amplify specific DNA fragments of 420 bp, 421 bp and 400 bp, respectively, corresponding to transcripts between *fptAB* (primer pairs *fptA*-fw and *fptB*-bw), *fptBC* (primer pairs *fptB*-fw and *fptC*-bw) and *fptCX* (primer pairs *fptC*-fw and *fptX*-bw). No amplification occurred in control experiments performed without reverse transcriptase. M, 1 kb ladder (Promega).

which revealed co-transcription of *fptA* with *fptB*, *fptB* with *fptC*, and *fptC* with *fptX* (Fig. 2).

Utilization of pyochelin as an iron source requires *fptA* and *fptX* but not *fptB* and *fptC*

We evaluated the role of the *fptABCX* genes in pyochelin utilization in strains defective for the production of pyoverdine and pyochelin. As shown in Fig. 3(a), the Δ *pvdF*, Δ *pchBA* mutant PAO6383 was unable to grow in iron-limited M9 medium but growth could be restored to a

Table 2. Expression of *fptB*, *fptC* and *fptX* requires the *fptA* promoter

Cultures of *P. aeruginosa* PAO1 carrying the plasmids indicated were grown at 37 °C and 220 r.p.m. for 12 h in 50 ml Erlenmeyer flasks containing 15 ml GGP medium inoculated with 0.15 ml portions of precultures grown in the same medium. β -Galactosidase activities represent the means $\pm$ SD of three parallel experiments.

Plasmid	Translational fusion	Presence of <i>fptA</i> promoter	β -Galactosidase activity (Miller units)
pME7518	PA4220 (<i>fptB</i>)'–'lacZ	+	6846 $\pm$ 616
pME7522	PA4220 (<i>fptB</i>)'–'lacZ	–	0.4
pME7517	PA4219 (<i>fptC</i>)'–'lacZ	+	1519 $\pm$ 49
pME7521	PA4219 (<i>fptC</i>)'–'lacZ	–	0.7
pME7520	PA4218 (<i>fptX</i>)'–'lacZ	+	29822 $\pm$ 383
pME7523	PA4218 (<i>fptX</i>)'–'lacZ	–	26 $\pm$ 2

Fig. 3. Role of the *fptABCX* genes in pyochelin-mediated growth promotion of iron-starved *P. aeruginosa* strains. M9 minimal medium containing (◆), or not (■) the iron chelator 2,2'-dipyridyl (500 μ M) was inoculated with PAO6383 ($\Delta pvdF$, $\Delta pchBA$; a), PAO6540 ($\Delta pvdF$, $\Delta pchBA$, $\Delta fptB$; b), PAO6390 ($\Delta pvdF$, $\Delta pchBA$, $\Delta fptC$; c), PAO6541 ($\Delta pvdF$, $\Delta pchBA$, $\Delta fptA$; d), PAO6541 carrying pME7036 (*fptABCX*; e), PAO6397 ($\Delta pvdF$, $\Delta pchBA$, *fptX*:: Ω Sp/Sm; f), and PAO6397 carrying pME7204 (*fptX*; g). Pyochelin was added at 20 μ M to M9 medium containing 2,2'-dipyridyl (●). OD₆₀₀ values represent the means and standard deviations of three parallel experiments.

large extent when the medium was supplemented with 20 μ M pyochelin as an iron source. Similar results were obtained with the PAO6383 derivatives affected in *fptB* (PAO6540, Fig. 3b) or *fptC* (PAO6390, Fig. 3c), indicating that these two genes were not required for pyochelin utilization. In contrast, mutations in *fptA* (PAO6541, Fig. 3d) or *fptX* (PAO6397, Fig. 3f) strongly interfered with pyochelin-mediated growth promotion, thus confirming the proposed functions for FptA and FptX in pyochelin-mediated iron uptake (Ankenbauer & Quan, 1994; Ó Cuív *et al.*, 2004). Complementation of PAO6541 and PAO6397 with pME7036 (*fptABCX*) and pME7204 (*fptX*), respectively, fully restored pyochelin-mediated growth promotion (Fig. 3e, g).

fptA and *fptX* are important for pyochelin-mediated signalling

We have previously shown that expression of the ferripyochelin receptor gene and of all pyochelin biosynthesis genes is strongly reduced in pyochelin-negative mutants but that expression is restored to wild-type levels when the growth medium is supplemented with pyochelin (Michel *et al.*, 2005; Reimann *et al.*, 1998). To test whether pyochelin-mediated signalling involves genes of the *fptABCX* operon, we measured the expression of translational *lacZ* fusions to

fptA and *pchD* in PAO1, PAO6297 ($\Delta pchBA$), and its derivatives PAO6429 ($\Delta pchBA$, $\Delta fptA$), PAO6424 ($\Delta pchBA$, $\Delta fptB$), PAO6388 ($\Delta pchBA$, $\Delta fptC$) and PAO6396 ($\Delta pchBA$, *fptX*:: Ω Sp/Sm), in both the presence and the absence of exogenously added pyochelin. As shown in Table 3, both fusions were poorly expressed in all pyochelin-negative mutants but addition of 20 μ M pyochelin restored expression of *fptA'*-*lacZ* and *pchD'*-*lacZ* to wild-type levels in PAO6297, PAO6424 and PAO6388, suggesting that *fptB* and *fptC* are not required for pyochelin-mediated signalling. In contrast, addition of pyochelin to strains PAO6429 and PAO6396 stimulated *fptA* and *pchD* expression to a certain extent, but expression remained below wild-type level. *fptA* and *fptX* thus play an important role in pyochelin-mediated signalling without being absolutely essential.

fptA and *fptX* were also required for maximal expression of *fptA'*-*lacZ* and *pchD'*-*lacZ* in pyochelin-producing strains (Table 4), indicating that pyochelin needs to be released to the extracellular medium and then taken up again before it can interact with PchR.

Plasmid pME7204, which expresses *fptX* from the vector's *lac* promoter, fully restored the expression of *fptA'*-*lacZ* in the *fptX* mutant PAO6396. By contrast, the *fptA* mutation in strain PAO6429 was complemented only partially by

Table 3. Effect of *fptA*, *fptB*, *fptC* and *fptX* mutations on the expression of pyochelin biosynthesis and uptake genes

P. aeruginosa strains carrying pME7153 (*fptA'*-*'lacZ*) or pME7160 (*pchD'*-*'lacZ*) were grown at 37 °C and 220 r.p.m. for 12 h in 50 ml Erlenmeyer flasks containing 15 ml GGP medium inoculated with 0.15 ml portions of precultures grown in the same medium. Pyochelin (Pch) was added to the culture medium at a final concentration of 20 µM. β-Galactosidase activities represent the means ± SD of three parallel experiments.

Strain	Genotype	β-Galactosidase activity (Miller units)			
		<i>fptA'</i> - <i>'lacZ</i>		<i>pchD'</i> - <i>'lacZ</i>	
		-Pch	+ Pch	-Pch	+ Pch
PAO1	Wild-type	4851 ± 263	5183 ± 290	2516 ± 267	2825 ± 58
PAO6297	Δ <i>pchBA</i>	19 ± 1	5128 ± 850	21 ± 3	2589 ± 133
PAO6429	Δ <i>pchBA</i> , Δ <i>fptA</i>	18 ± 1	667 ± 70	19 ± 2	383 ± 49
PAO6424	Δ <i>pchBA</i> , Δ <i>fptB</i>	17 ± 2	5790 ± 464	24 ± 1	2572 ± 173
PAO6388	Δ <i>pchBA</i> , Δ <i>fptC</i>	12 ± 4	5026 ± 238	25 ± 2	2607 ± 41
PAO6396	Δ <i>pchBA</i> , <i>fptX</i> ::Ω	18 ± 2	3742 ± 267	24 ± 1	1410 ± 121

plasmid pME7034, which expresses *fptA* from its natural, iron-regulated promoter (Table 5). Full complementation was achieved only when the entire *fptABCX* operon was provided *in trans* on plasmid pME7036. It thus seems that the deletion in *fptA*, although in-frame, exerts a polar effect on the expression of the downstream genes. Indeed, plasmid pME7204 was able to partially complement the mutation in PAO6429 as well (Table 5).

Impact of the *fptABCX* operon on the formation of salicylate, Dha and pyochelin

During pyochelin biosynthesis, chorismate is converted to salicylate, which is then coupled to two molecules of cysteine by a thiotemplate mechanism (Serino *et al.*, 1995; Gaille *et al.*, 2002; Reimann *et al.*, 1998; Quadri *et al.*, 1999). Dha, a byproduct of the pathway, is formed by *P. aeruginosa* in only small amounts and consists of a single cysteine moiety coupled to salicylate (Serino *et al.*, 1997). We evaluated the importance of the *fptABCX* genes on the production of these metabolites (Table 6). Compared to strain PAO1, the

production of Dha and pyochelin was reduced about threefold in the *fptA* mutant PAO6428 and by about 20 % in the *fptX* mutant PAO6368. Mutations in *fptB* and *fptC* did not affect the production of Dha and pyochelin and, as in the wild-type, salicylate was not detected as this compound is converted quantitatively to Dha and pyochelin under these experimental conditions. Small amounts of salicylate accumulated only in PAO6428, where a reduced pyochelin formation slowed down the entire biosynthetic pathway.

Taken together, these data show that the *fptA* and *fptX* genes are not only involved in pyochelin-mediated iron uptake as shown previously (Ankenbauer & Quan, 1994; Ó Cuív *et al.*, 2004), but are also important for pyochelin-mediated signalling, which ultimately affects the production of the siderophore itself.

DISCUSSION

We have shown in this work that the genes *fptB*, *fptC* and *fptX* are transcribed from the PchR-dependent (Heinrichs & Poole, 1996) and Fur-regulated (Ankenbauer & Quan, 1994) *fptA* promoter, thus forming an *fptABCX* operon (Table 2 and Fig. 2). Given the results of Table 2, additional, operon-internal promoters are unlikely. It is not clear, however, why the in-frame deletion in *fptA* affected *fptX* expression (Table 5) and it is thus possible that the regulation of the *fptABCX* operon is more complex. BLAST searches with microbial genomes reveal similar *fptABCX* operons in *Burkholderia pseudomallei* K96243, *Burkholderia* sp. 383 and *Rhodospirillum rubrum*. Whereas pyochelin production is documented in *Burkholderia* sp. (Darling *et al.*, 1998; Visser *et al.*, 2004) and pyochelin biosynthetic genes are highly conserved in the genomes of *B. pseudomallei* K96243 and *Burkholderia* sp. 383, there is no evidence for pyochelin formation by *R. rubrum*. The presence of an operon similar to *fptABCX* suggests, however, that *R. rubrum* might be able to utilize pyochelin as a heterologous siderophore for iron uptake.

Table 4. Impact of *fptA* and *fptX* mutations on signalling in pyochelin-producing strains

P. aeruginosa strains carrying pME7153 (*fptA'*-*'lacZ*) or pME7160 (*pchD'*-*'lacZ*) were grown at 37 °C and 220 r.p.m. for 12 h in 50 ml Erlenmeyer flasks containing 15 ml GGP medium inoculated with 0.15 ml portions of precultures grown in the same medium. β-Galactosidase activities represent the means ± SD of three parallel experiments.

Strain	Genotype	β-Galactosidase activity (Miller units)	
		<i>fptA'</i> - <i>'lacZ</i>	<i>pchD'</i> - <i>'lacZ</i>
PAO1	Wild-type	5436 ± 882	2442 ± 222
PAO6428	Δ <i>fptA</i>	192 ± 19	248 ± 73
PAO6368	<i>fptX</i> ::Ω	1298 ± 480	948 ± 504

Table 5. Complementation of *fptA* and *fptX* mutants

P. aeruginosa strains carrying pME7153 (*fptA*'-'*lacZ*) and, where indicated, pME7204, pME7034, or pME7036, respectively, were grown at 37 °C and 220 r.p.m. for 12 h in 50 ml Erlenmeyer flasks containing 15 ml GGP medium inoculated with 0.15 ml portions of precultures grown in the same medium. Pyochelin (Pch) was added to the culture medium at a final concentration of 20 µM. β-Galactosidase activities represent the means±SD of three parallel experiments. The β-galactosidase activities of the uncomplemented mutants are included in Table 3.

Strain	Genotype	Plasmid	Gene(s) carried	β-Galactosidase activity (Miller units)	
				–Pch	+Pch
PAO6297	Δ <i>pchBA</i>	–	–	16±2	4715±981
PAO6396	Δ <i>pchBA</i> , <i>fptX</i> ::Ω	pME7204	<i>fptX</i>	22±2	5265±841
PAO6429	Δ <i>pchBA</i> , Δ <i>fptA</i>	pME7034	<i>fptA</i>	32±12	1168±92
PAO6429	Δ <i>pchBA</i> , Δ <i>fptA</i>	pME7036	<i>fptABCX</i>	18±1	4448±158
PAO6429	Δ <i>pchBA</i> , Δ <i>fptA</i>	pME7204	<i>fptX</i>	26±4	2342±264

Previous work has established that *fptA* and *fptX* are both involved in pyochelin-mediated iron uptake (Ankenbauer & Quan, 1994; Ó Cuív *et al.*, 2004). We have shown here that these genes are also important in pyochelin-mediated signalling in pyochelin-deficient and in pyochelin-producing strains (Tables 3 and 4) and, as a consequence, affect the production of pyochelin (Table 6). This is consistent with the fact that pyochelin, possibly in its iron-loaded state, acts as an effector for the cytoplasmic PchR regulator (Michel *et al.*, 2005). After FptA-promoted translocation of ferripyochelin across the outer membrane, the inner-membrane permease FptX is believed to be necessary for subsequent ferripyochelin uptake into the cytoplasm. FptX belongs to a new family of single-subunit siderophore transporters which differs from classical, binding-protein-dependent ABC proteins, such as FhuBCD, which is required for ferric hydroxamate uptake in *E. coli* (Braun & Killmann, 1999). Members of this new family seem to lack associated proteins that function in energy coupling or as periplasmic binding proteins (Ó Cuív *et al.*, 2004). The proteins encoded by the *fptB* and *fptC* genes are not expected to have such a role and we have shown here that they are

not essential for pyochelin utilization (Fig. 3), signalling (Tables 3 and 4) or pyochelin production (Table 6). Given their co-regulation with *fptA* and *fptX* and their conservation in *Burkholderia* sp. and *R. rubrum*, it is difficult to believe that *fptB* and *fptC* should not be involved in these processes at all. We cannot exclude that their functions are redundant in *P. aeruginosa* such that *fptB* and *fptC* mutants do not show a distinct phenotype.

FptX seemed less important for signalling than FptA (Tables 3 and 4) and the *fptX* mutation did not entirely abolish, but rather delayed, pyochelin utilization (Fig. 3). While this can be explained at least in part by the polar nature of the *fptA* mutation (Table 5) it also indicates that, in the absence of *fptX*, ferripyochelin may enter the cytoplasm by an alternative permease, as transport across the inner membrane exhibits less specificity than at the outer membrane. The FhuBCD permease, for instance, facilitates the uptake of several hydroxamate siderophores, each of which requires its own receptor at the outer membrane (Köster, 2001). Some hydroxamate siderophores can also be transported via a heterologous permease related to FptX (Ó

Table 6. Effects of *fptA*, *fptB*, *fptC* and *fptX* mutations on salicylate, Dha and pyochelin formation

GGP medium (30 ml) was inoculated with 0.3 ml portions of precultures grown in the same medium. After incubation at 37 °C and 220 r.p.m. for 35 h, supernatants were extracted and analysed for their amount of salicylate, Dha and pyochelin by HPLC. The values given represent the means±SD obtained for three parallel experiments.

Strain	Genotype	Salicylate (nmol ml ⁻¹)*	Dha (nmol ml ⁻¹)*	Pyochelin (nmol ml ⁻¹)*
PAO1	Wild-type	<8.0	88±14	897±22
PAO6428	Δ <i>fptA</i>	46±2	23±3	316±32
PAO6423	Δ <i>fptB</i>	<8.0	75±9	835±94
PAO6387	Δ <i>fptC</i>	<8.0	103±13	881±140
PAO6368	<i>fptX</i> ::Ω	<8.0	62±8	717±55

*Concentration in culture supernatant.

Cuív *et al.*, 2004), illustrating that classical ABC-type permeases and single-subunit siderophore transporters may replace each other in some cases.

Mutations in ferripyochelin uptake genes also affected metabolite production (Table 6) although the effect was less pronounced than on gene expression (Tables 3 and 4). This is likely due to the fact that under the experimental conditions used, gene expression is not the only parameter which determines the amount of product formed. Indeed, we have shown previously that pyochelin formation could be significantly increased when the growth medium was supplemented with cysteine (Gaille *et al.*, 2003), indicating that the intracellular cysteine pool is another important factor.

In conclusion, we have shown here that pyochelin-mediated signalling (and hence pyochelin production) involves the ferripyochelin uptake functions FptA and FptX. These results thus confirm and extend our previous work demonstrating that pyochelin, possibly in its iron-loaded form, acts as an intracellular effector of the AraC-type regulator PchR (Michel *et al.*, 2005). In the absence of ferripyochelin uptake functions, pyochelin-mediated signalling is, however, not entirely abolished, indicating that target gene induction may occur by an alternative signalling pathway as well.

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