Microbiology (2004), **150**, 1671–1680 DOI 10.1099/mic.0.27035-0

FpvB, an alternative type I ferripyoverdine receptor of *Pseudomonas aeruginosa*

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Received 16 January 2004
Revised 19 March 2004
Accepted 25 March 2004

Under conditions of iron limitation, *Pseudomonas aeruginosa* secretes a high-affinity siderophore pyoverdine to scavenge Fe(III) in the extracellular environment and shuttle it into the cell. Uptake of the pyoverdine–Fe(III) complex is mediated by a specific outer-membrane receptor protein, FpvA (ferripyoverdine receptor). Three *P. aeruginosa* siderovars can be distinguished, each producing a different pyoverdine (type I–III) and a cognate FpvA receptor. Growth of an *fpvA* mutant of *P. aeruginosa* PAO1 (type I) under iron-limiting conditions can still be stimulated by its cognate pyoverdine, suggesting the presence of an alternative uptake route for type I ferripyoverdine. *In silico* analysis of the PAO1 genome revealed that the product of gene PA4168 has a high similarity with FpvA. Inactivation of PA4168 (termed *fpvB*) in an *fpvA* mutant totally abolished the capacity to utilize type I pyoverdine. The expression of *fpvB* is induced by iron limitation in Casamino acids (CAA) and in M9-glucose medium, but, unlike *fpvA*, not in a complex deferrated medium containing glycerol as carbon source. The *fpvB* gene was also detected in other *P. aeruginosa* isolates, including strains producing type II and type III pyoverdines. Inactivation of the *fpvB* homologues in these strains impaired their capacity to utilize type I ferripyoverdine as a source of iron. Accordingly, introduction of *fpvB in trans* restored the capacity to utilize type I ferripyoverdine.

INTRODUCTION

Iron is an essential element for almost all organisms, involved as an indispensable enzymic co-factor in many cellular processes. But despite being one of the most abundant elements on earth, the amount of free iron in typical bacterial niches like soil or for mammalian host pathogens, is usually below the minimal concentration to support bacterial growth (Ratledge & Dover, 2000).

Gram-negative bacteria have therefore developed numerous strategies for acquiring iron. A common mechanism is the production of low-molecular-mass iron-chelating compounds named siderophores (Guerinot, 1994), which are secreted to scavenge iron outside the cell. Besides siderophores, cell surface receptors are produced, which function as gated porin channels that recognize and internalize the ferri-siderophore complexes in concert with the TonB protein that energizes the receptor protein (Ratledge & Dover, 2000).

These proteins are characterized by a large C-terminal domain of 22 antiparallel β-strands, which form a so-called β-barrel that spans the outer membrane (Koebnik *et al.*, 2000). Unlike outer-membrane porins, TonB-dependent outer-membrane proteins also contain an additional domain known as 'cork' or 'plug' that transiently blocks the β-barrel domain and by using energy transduced by TonB, allowing selective uptake of cognate siderophore/ion complexes (Ferguson *et al.*, 1998).

Fluorescent pseudomonads form a group of Gram-negative bacteria which respond to iron-deficiency by secreting yellow-green siderophores termed pyoverdines or pseudobactins. All characterized pyoverdines and pseudobactins comprise a conserved dihydroxyquinoline chromophore linked to an acyl group and a short (6–12 amino acids)

Abbreviations: EDDHA, ethylenediaminedihydroxyphenylacetic acid; Gm, gentamicin; Tc, tetracycline.

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type-specific peptide chain (Ravel & Cornelis, 2003). Pyoverdines of a single strain have the same peptide but may differ in the nature of the acyl group. Typing methods exist to classify fluorescent pseudomonads according to the pyoverdine they produce, so-called siderotyping (Meyer *et al.*, 2002a).

The type species of the group, *Pseudomonas aeruginosa*, is an opportunistic human pathogen associated with infections of compromised individuals and a notorious hospital pathogen. Experiments in animal models have shown the importance of pyoverdine for *P. aeruginosa* virulence (Meyer *et al.*, 1996; Takase *et al.*, 2000). The contribution of pyoverdine to *P. aeruginosa* virulence is not restricted to its siderophore activity. Recent work has shown that pyoverdine can be considered a signal molecule which orchestrates a synergic action between itself and other virulence determinants [e.g. exotoxin A (ToxA); endoprotease (PrpL)] to retrieve iron from host cells and proteins (Lamont *et al.*, 2002; Shen *et al.*, 2002). Different branches of this pyoverdine signalling pathway thereby regulate expression of virulence determinants and pyoverdine synthesis on the one hand and pyoverdine uptake via its receptor on the other hand (Beare *et al.*, 2003).

Three siderotypes of *P. aeruginosa* can be distinguished, producing three structurally different types of pyoverdine (types I, II, III) (Cornelis *et al.*, 1989; Meyer *et al.*, 1997; De Vos *et al.*, 2001; Spencer *et al.*, 2003; Ernst *et al.*, 2003), each being recognized at the level of the outer membrane by a specific receptor (Cornelis *et al.*, 1989; De Chial *et al.*, 2003).

Even before its complete genome sequence became available, *P. aeruginosa* PAO1 (Stover *et al.*, 2000), which produces type I pyoverdine, was already the most intensively studied *P. aeruginosa* isolate. Genetics and physiology of pyoverdine biosynthesis and uptake are therefore also the best characterized for the *P. aeruginosa* type I pyoverdine of *P. aeruginosa* PAO1 (Ochsner *et al.*, 2002; Ravel & Cornelis, 2003; Lamont & Martin, 2003; Palma *et al.*, 2003; Heim *et al.*, 2003). The FpvA receptor for *P. aeruginosa* PAO1 pyoverdine has been intensively characterized using physiological, immunological, and molecular approaches (Poole *et al.*, 1993; Schalk *et al.*, 2001, 2002).

Recently, we have cloned the receptors for *P. aeruginosa* type II and type III pyoverdines and developed a multiplex PCR method for fast siderotyping of *P. aeruginosa* strains based on their specific *fpvA* sequences (De Chial *et al.*, 2003). In this work we present evidence for an additional type I pyoverdine receptor in *P. aeruginosa* PAO1 and in other strains producing type II and type III pyoverdines.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are shown in Table 1. Strains were grown with aeration at 37 °C either in Casamino acid medium (CAA, low iron medium) or in CAA containing 0·5 mg ml^{-1} of the iron(III) chelator ethylenediaminedihydroxyphenylacetic acid (EDDHA) to induce conditions of extreme iron limitation (Cornelis *et al.*, 1992). Sometimes the *P. aeruginosa* cells were grown in the iron-limiting succinate minimal medium (Meyer & Abdallah, 1978).

Primers and oligonucleotides. Primer and oligonucleotides used in this study are listed in Table 2.

Purification of pyoverdines. A 0·5–1 l culture in succinate minimal medium of the *P. aeruginosa* (type I, II or III) pyoverdine-producing strain was grown for 48 h at 37 °C. The culture supernatants were acidified to pH 6 and filtrated on a preparative scale on a XAD-4 amberlite column (2·5 × 15 cm) and eluted with a 1/1 (v/v) methanol/water mixture (Budzikiewicz, 1993). After evaporation of the methanol, the pyoverdine eluate was freeze-dried. The semi-pure pyoverdine powder was then dissolved in a small amount of water, and finally brought to the desired concentration (10 mM) after measurement of OD_{400}.

Physiological characterization of the mutants. Growth stimulation by the different pyoverdines on CAA plus EDDHA was done on agar plates by spreading 200 µl of a saturated *P. aeruginosa* CAA culture of the mutant on the agar surface on top of which filter discs impregnated with a 10 µM solution of the pyoverdines were deposited. Growth stimulation was recorded after 1 day, and the plates were photographed using a Fuji Digital Camera (Finepix S1 Pro). For more accurate analysis, growth was assessed in microtitre plates (300 µl CAA medium with EDDHA as described in the text), which were incubated for 48 h at 37 °C in a Bio-Screen incubator (Life Technologies), using the following parameters: shaking for 30 s per 3 min and readings recorded every 10 min (De Vos *et al.*, 2001).

Analysis of outer-membrane proteins. Outer-membrane proteins from bacteria grown under iron-limiting conditions (CAA) were prepared as described by Mizuno & Kageyama (1978). The protein content of the outer-membrane preparations was determined by the Lowry method, and analysed by SDS-PAGE (10 % polyacrylamide).

Generation of Δ*fpvA* Δ*fpvB* double mutants in *P. aeruginosa* PAO1 by allelic exchange. The *P. aeruginosa* PAO1 2429 bp genome fragment from position 4 663 835 to 4 666 264, which includes the PA4168 ORF with its putative ribosome-binding sites, was PCR-amplified with primers PA4168 F and PA4168 R, and cloned into pCR2.1 using the TA cloning kit (Invitrogen). The cloned insert was restricted with *Eco*RV and *Bam*HI and ligated to *Eco*RV/*Bam*HI-restricted pBR325, thereby replacing part of the tetracycline (Tc) resistance gene. Clones were selected by resistance for chloramphenicol and lost resistance to Tc. Restriction analysis confirmed insertion of the PA4168 ORF in pBR325. A *Sac*I–*Sac*I Tc cassette from the pTnModoTc plasposon (Dennis & Zylstra, 1998) was then inserted in the *Sac*I site, 1·5 kb from the start of the ORF. Tc-resistant clones were picked up and additional screening was done by restriction analysis. The selected clones were used to transform *Escherichia coli* GJ23 cell before mobilization of the disrupted PA4168 ORF by conjugation into *P. aeruginosa fpvA pvdDpchEF*. Recombinants were selected by their resistance to both Tc (100 µg ml^{-1}) and gentamicin (Gm) (100 µg ml^{-1}). The *fpvA* II and *fpvA* III mutants were constructed as described by De Chial *et al.* (2003). The *fpvA* mutant in *P. aeruginosa* PAO1 was obtained as described by Lamont *et al.* (2002) using the pEX18Gm vector (Hoang *et al.*, 1998). All mutations were confirmed by PCR amplification using the appropriate primers (Table 2: PA4168 GR Fw and PA4168 GR Rv, FpvAII-2F and FpvAII-2R, FpvAIII-3F and FpvAIII-3R), except for the *fpvA* mutation in *P. aeruginosa* PAO1, which was confirmed by Southern blotting.

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Table 1. Strains and vectors used in this study

Strain/plasmid	Characteristics	Source/reference
Pseudomonas aeruginosa		
PAO1	Wild-type, type I pyoverdine typed *P. aeruginosa* isolate	Holloway (1955); Stover *et al.* (2000)
PAO1-*pvdD pchEF*	Allelic mutant of *pvdD* and *pchEF*; unmarked	This study
PAO1-*pvdD pchEF fpvA*	Allelic mutant of *fpvA* in *pvdD* and *pchEF* background; Gm^R	This study
PAO1-*pvdD pchEF fpvB*	Allelic mutant of PA4168 (*fpvB*) in *pvdD* and *pchEF* background; Gm^R	This study
PAO1-*pvdD pchEF fpvA fpvB*	Allelic mutant of PA4168 (*fpvB*) in *pvdD*, *pchEF* and *fpvA* background	This study
PAO1Δ*pvdS*	Insertion-deletion of *pvdS*; Gm^R	Ochsner *et al.* (1996)
PAO1Δ*pvdD*	Insertion-deletion of *pvdD*; Gm^R	This study
PAO1 *fur*$^-$ (C6)	Mutant with a point mutation in *fur*	Barton *et al.* (1996)
A15	Wild-type, type III pyoverdine typed *P. aeruginosa* isolate	Meyer *et al.* (1997)
59.20	Wild-type, type III pyoverdine typed *P. aeruginosa* isolate	Meyer *et al.* (1997); De Chial *et al.* (2003)
59.20-3B2	Tn*5* pyoverdine-negative mutant in *pvdI* homologue; Gm^R	This study
59.20-*fpvA*	Allelic *fpvAIII* mutant; Gm^R	De Chial *et al.* (2003)
59.20-3B2 *fpvB*	Allelic exchange mutant of PA4168 (*fpvB*) homologue in *pvdI* background; Gm^R Tc^R	This study
59.20-*fpvA fpvB*	Allelic exchange mutant of PA4168 (*fpvB*) homologue in *fpvA* background; Tc^R	This study
7NSK2	Wild-type, type II pyoverdine typed *P. aeruginosa* isolate	Höfte *et al.* (1990)
7NSK2-*fpvA*	Allelic *fpvAII* mutant; Gm^R	De Chial *et al.* (2003)
7NSK2-MPFM1	Pyoverdine-negative mutant	Höfte *et al.* (1993)
7NSK2-*fpvA fpvB*	Allelic exchange mutant of PA4168 (*fpvB*) homologue in *fpvA* background; Gm^R Tc^R	This study
7NSK2-MPFM1 *fpvB*	Allelic exchange mutant of PA4168 (*fpvB*) homologue in pyoverdine-negative background; Tc^R	This study
E. coli		
DH5α	*supE44* Δ*lacU169* (*ϕ*80 *lacZ*ΔM15 *recA hsdR17 recA1 endA1 gyrA96 thi-1 relA1*)	Hanahan (1983)
GJ23	JC2692 (pGJ28) (R64*drd11*); Km^R Sm^R Tc^R	Van Haute *et al.* (1983)
SM10 (*λ* pir)	*Thi-1 thr leu tonA lacY supE recA*::RP4-2 tc::Mu; *λ* pir; Km^R	Herrero *et al.* (1990)
Top 10F	F′ [*lacI*^{q} Tn*10* (Tet^R) *mcrA*Δ(*mrr-hsdRMS-mcrBC*)*ϕ*80 *lacZ*ΔM15Δ *lacX74 recA1 araD139* Δ(*ara-leu*) 7697 *galU galK rpsL* (Str^R) *endA1 nupG*]	Invitrogen
Plasmid		
pCRII-2.1	TA cloning vector for PCR fragments; Ap^R, Km^R	Invitrogen
pBR325	ColE1 vector; Ap^R, Cm^R, Tc^R	Bolivar (1978)
pBBR1-MCS	Wide-host-range cloning vector; Cm^R	Kovach *et al.* (1994)

Generation of a Tn*5*-pyoverdine biosynthetic mutant in 59.20, a type III pyoverdine-producing *P. aeruginosa* strain. Mutagenesis was done by biparental mating of 59.20 with the donor strain *E. coli* SM10(*λ* pir) containing the suicide delivery system pUT (de Lorenzo *et al.*, 1990) and the transposon miniTn*phoA3*, as described by Pattery *et al.* (1999) and De Chial *et al.* (2003). Transconjugants were selected on CAA medium plates supplemented with appropriate antibiotics (100 mg Gm l^{-1}; 10 mg Tc l^{-1}). Candidate pyoverdine biosynthetic mutants were first selected on CAA for their lack of fluorescence, then for absence of growth in the presence of 0·5 mg EDDHA ml^{-1} and finally on CAA-EDDHA-pyoverdine to assure that the mutant was not pyoverdine-uptake deficient. The localization of the mutation was done by inverse PCR (IPCR) using primers PhoA, GM1, PhoA4 and GM2 as described previously (De Chial *et al.*, 2003). The sequence of the DNA flanking the transposon revealed that the insertion occurred in a gene with high similarity to the pyoverdine synthetase gene *pvdI* from *P. aeruginosa* PAO1 (Lehoux *et al.*, 2000; results not shown).

Generation of mutants in *fpvB* homologues of type II and type III pyoverdine-producing *P. aeruginosa* strains by allelic exchange. The *P. aeruginosa* PAO1 disrupted *fpvB* gene was also mobilized into a type II (7NSK2) and type III (59.20) pyoverdine-producing *P. aeruginosa* by conjugation to inactivate *fpvB* homologues in these strains.

RT-PCR detection of *fpvA* and *fpvB* transcripts. Total RNA was isolated from *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1*pvdD* grown overnight in CAA medium (iron-limited growth condititon) and CAA medium with 100 μM $FeCl_3$ (high-iron growth condition) with the high pure RNA purification kit (Roche). To avoid DNA

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Table 2. List of primers used in this study

Name	Sequence (5′–3′)
PA 4168 F	CCATCCAGGAACTGCAGAT
PA 4168 R	GGATCAGAGCGATACTTCA
PA4168 GR Fw	ACGACAGCTATGACGCCTAT
PA4168 GR Rv	TGTTCCTGGTCGAGCTTCAT
PA4168 SC Fw	GCATGAAGCTCGACCAGGA
PA4168 SC Rv	TTGCCCTCGTTGGCCTTGT
FpvA SS Fw	GAACAGGGCACCTACCTGTA
FpvA SS Rw	GATGCCGTTGCTGAACTCGTA
FpvAI-1F	CGAAGGCCAGAACTACGAGA
FpvAI-1R	TGTAGCTGGTGTAGAGGCTCAA
FpvA RT F	CTACTGGAACCTGCGCAACTA
FpvAII-2F	TACCTCGACGGCCTGCACAT
FpvAII-2R	GAAGGTGAATGGCTTGCCGTA
FpvAIII-3F	ACTGGGACAAGATCCAAGAGAC
FpvAIII-3R	CTGGTAGGACGAAATGCGAG
PAL 1	ATGGAAATGCTGAAATTCGGC
PAL 2	CTTCTTCAGCTCGACGCGACG

contamination, the kit protocol was slightly adapted. The purified RNA sample was mixed with binding buffer and again applied on the column so that DNase treatment could be repeated and the DNase easily removed in the following steps according to the manufacturer's instructions.

Complementary DNA, using as template these total RNAs, was synthesized with the first strand cDNA synthesis kit (Amersham Biosciences) following the manufacturer's instructions. The cDNAs served as template for a PCR with primers FpvAI RT F and FpvA I-1R (*fpvA* expression detection), PA4168 GR Fw and PA4168 GR Rv (*fpvB* expression detection) and PAL 1 and PAL 2 (*oprL* expression detection as housekeeping gene control; Lim *et al.*, 1997).

Microarray analysis. The expression of *fpvA* and *fpvB* were examined from microarray experiments as previously described (Ochsner *et al.*, 2002). The PAO1 *fur*$^-$ mutant contains a point mutation in Fur (A10G), which abrogates its ability to bind Fur boxes (Barton *et al.*, 1996). Strains were grown in dialysed trypticase soy broth (DTSB) or M9 minimal media as described by Ochsner *et al.* (2002).

RESULTS

A *P. aeruginosa* PAO1 *fpvA* mutant is still capable of using its cognate ferripyoverdine as source of iron

A siderophore-free background was created in *P. aeruginosa* PAO1 by making unmarked deletions in the *pvdD* (pyoverdine biosynthesis locus) and *pchEF* (pyochelin biosynthesis operon). This mutant cannot grow in the presence of the strong iron chelator EDDHA unless pyoverdine is present in the medium. An additional mutation was created by allelic exchange with a Gm cassette in *fpvA*, the gene that encodes the pyoverdine receptor (Poole *et al.*, 1993). In contrast to what was expected, the mutation in *fpvA* delayed, but did not abolish growth in the presence of type I pyoverdine (Fig. 1). This result clearly suggests that *P. aeruginosa* PAO1 is able to take up its cognate pyoverdine via an alternative receptor.

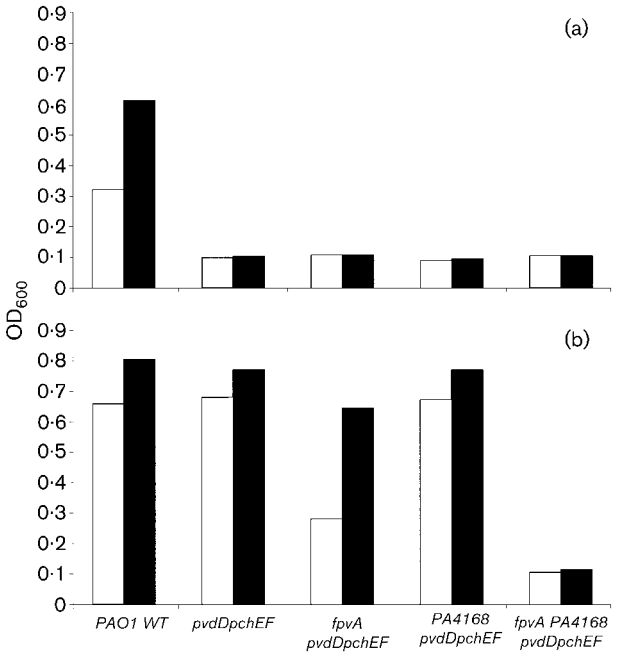

Fig. 1. Growth of wild-type *P. aeruginosa* PAO1 and corresponding mutants. All data are the means of three replica cultures in the Bio-Screen. (a) CAA medium with 0·5 mg EDDHA ml^{-1}. (b) CAA-medium with 0·5 mg EDDHA ml^{-1} and 50 μM purified type I pyoverdine. Values of OD_{600} are given for cultures grown for 12 (white bars) and 24 h (black bars).

Identification by *in silico* analysis of ORF PA4168 (*fpvB*), encoding a TonB-dependent receptor similar to FpvA

Using the FpvA sequence, the *P. aeruginosa* Genome Project Database (http://www.pseudomonas.com) was screened for a candidate alternative pyoverdine receptor gene, using the BLASTX algorithm. The product of ORF PA4168, annotated as a probable TonB-dependent receptor, showed the highest similarity to FpvA (54 %). Fig. 2(a) shows an alignment of the sequences of FpvA and FpvB (PA4168). Further analysis of the genomic context revealed that PA4169 encodes a transcriptional regulator while PA4167 encodes a putative oxidoreductase (Fig. 2b).

Inactivation of *fpvB* in *P. aeruginosa* PAO1 and PAO1 *fpvA*

Inactivation of the ORF PA4168 (*fpvB*) in the *pvdDpchEF* background did not affect growth in the presence of type I pyoverdine (Fig. 1). By allelic exchange with a Tc cassette, the *fpvB* gene was inactivated in the PAO1 *pvdD pchEF fpvA* mutant. With *fpvA* and *fpvB* simultaneously inactivated, the resulting mutant was now completely unable to grow in the presence of EDDHA when the cognate type I pyoverdine was present in the medium (Fig. 1). Moreover, the double *fpvA*

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fpvB mutant was unable to grow in the presence of 50 μM pyoverdine in the absence of EDDHA (results not shown). This result indicates that PA4168 contributes as a receptor for the utilization of type I pyoverdine in *P. aeruginosa* PAO1, justifying the renaming of the gene as *fpvB*.

Conservation of *fpvB* in different *P. aeruginosa* strains

Primers PA4168 SC Fw and PA4168 SC Rv were developed in order to amplify a 562 bp fragment of PA4168. An amplification product was obtained in 20 out of 27 independent clinical and environmental *P. aeruginosa* isolates, including seven type II strains, eight type III strains and three type I strains as typed by amplified fragment-length polymorphism (AFLP) (Pirnay *et al.*, 2002; results not shown), multiplex PCR for the presence of the *fpvA* gene (type I, II or III; De Chial *et al.*, 2003) and/or IEF (Pirnay *et al.*, 2002).

Ability of type II and type III producing *P. aeruginosa* strains to use heterologous type I ferripyoverdine

The *fpvA* genes of type III pyoverdine-producing *P. aeruginosa* clinical isolates 59.20 (positive for the

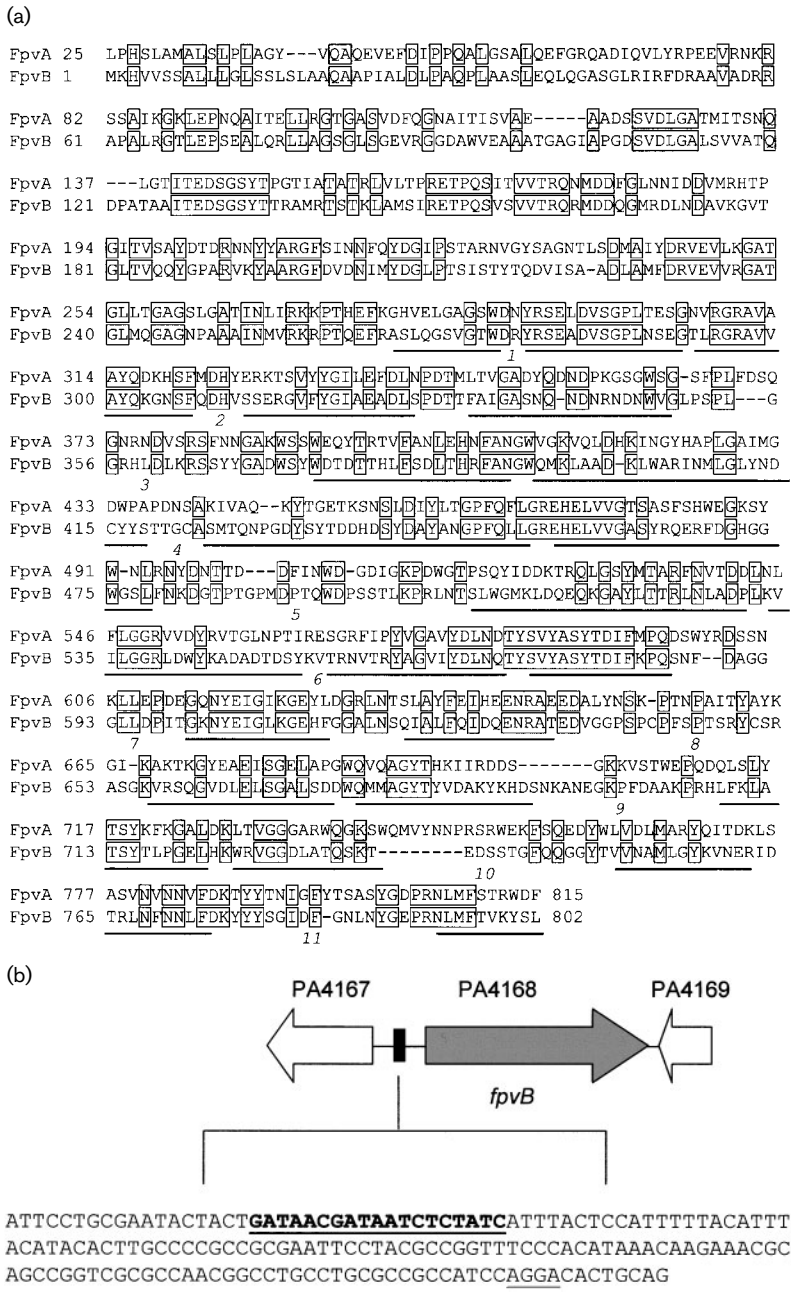

Fig. 2. (a) Alignment of the amino acid sequences of FpvA and FpvB of *P. aeruginosa* PAO1. Identical residues are boxed. The alignment is based on a larger non-redundant CLUSTALW alignment comprising ~100 sequences from other known or putative TonB-dependent receptor proteins, including *E. coli* FhuA, for which the crystal structure has been experimentally determined (Ferguson *et al.*, 1998). The relative position of the β-strands that comprise the *E. coli* FhuA β-barrel are shown as bold lines and the extracellular loops are numbered 1–11 according to the FhuA schema (Ferguson *et al.*, 1998). (b) Genomic organization of the PA4168–PA4169 region showing the intergenic region and the putative *fur* binding site (in bold). PA4167 encodes a putative oxidoreductase while PA4169 encodes a LysR regulator.

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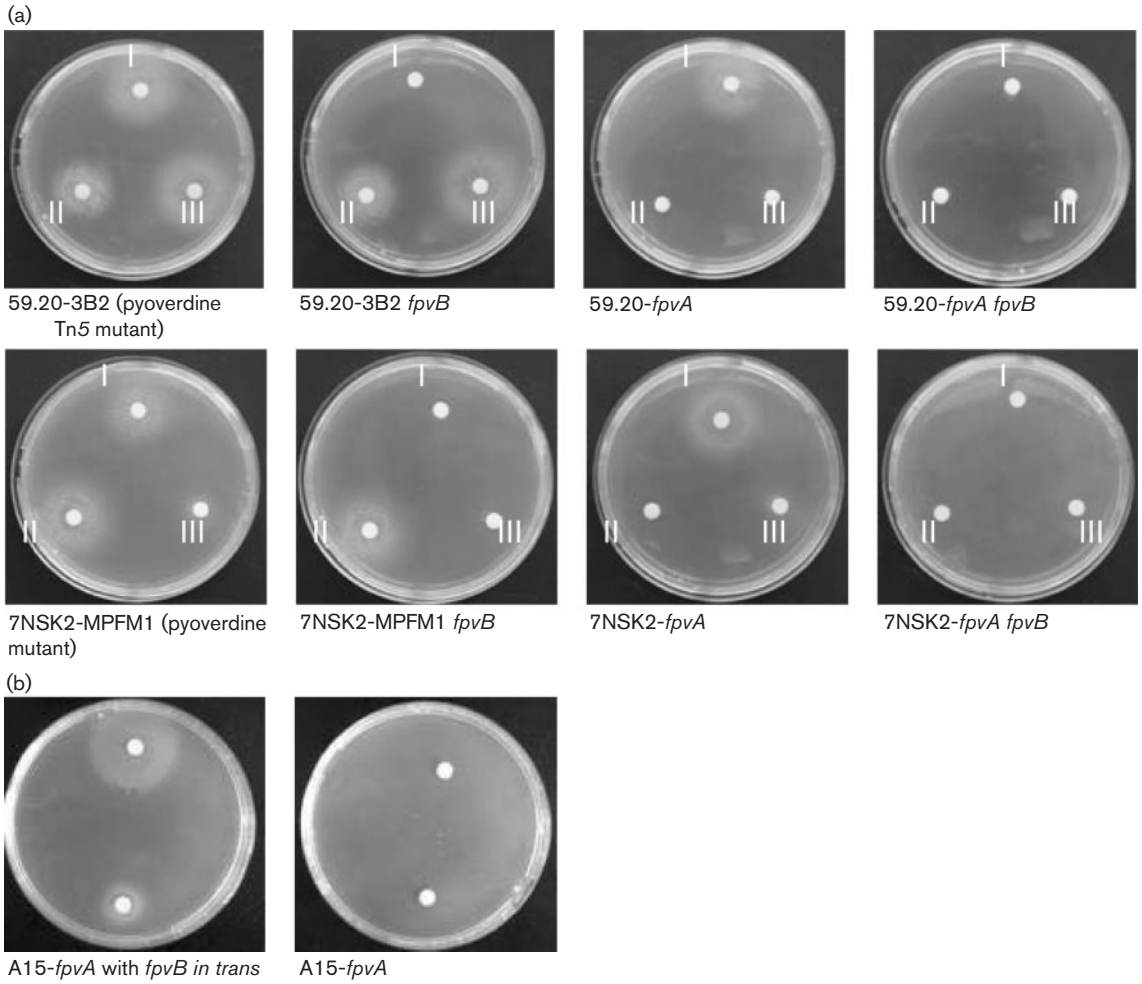

Fig. 3. (a) *P. aeruginosa* 59.20 (type III) and 7NSK2 (type II) and the corresponding pyoverdine receptor mutants tested for their capacity to utilize the three *P. aeruginosa* type pyoverdines (I, II, III). Growth stimulation is detected as a halo of growth surrounding the filter disc, impregnated with a particular pyoverdine type when the mutant is spread on EDDHA (0·5 mg ml^{-1}) containing CAA medium. For the generation of the *fpvA* and *fpvA fpvB* mutants the wild-type strain was used rather than a pyoverdine-negative mutant because the *fpvA* mutations already caused a pyoverdine-negative phenotype as shown by the absence of pigmentation on the plates (De Chial *et al.*, 2003). (b) Growth of the *fpvA* mutant of strain A15 (type III pyoverdine producer) with *fpvB* from *P. aeruginosa* PAO1 *in trans* in CAA medium containing EDDHA and type I pyoverdine (left plate, top) compared to the same mutant without the *fpvB* gene (right plate, top). Notice the slight stimulation by type III pyoverdine (bottom disc).

amplification of *fpvB*), A15 (negative for the amplification of *fpvB*) and the type II pyoverdine-producing plant rhizosphere isolate 7NSK2 (also positive for the *fpvB* gene; Höfte *et al.*, 1990) were inactivated by allelic exchange using a Gm cassette. With their *fpvA* genes inactivated, these strains were unable to use their cognate pyoverdines as a source of iron (Fig. 3a, b). However, on EDDHA-containing CAA medium, growth of 59.20 *fpvA* and 7NSK2 *fpvA* was stimulated by the heterologous type I pyoverdine (Fig. 3a) while the growth of strain A15 *fpvA* was not (Fig. 3b). This result suggests that the homologues of FpvB in other *P. aeruginosa* strains could serve as functional receptors for type I pyoverdine, allowing these strains to utilize this heterologous siderophore.

fpvB in trans allows *P. aeruginosa* A15 to use type I pyoverdine as a source of iron

When the receptor for the cognate type III pyoverdine, *fpvA*, is inactivated, *P. aeruginosa* A15 can no longer grow in the presence of EDDHA and its cognate pyoverdine. The same mutant is also unable to grow in the presence of the heterologous type I or type II pyoverdines. This is in agreement with the fact that in this strain no homologue of *fpvB* could be detected by PCR.

The *fpvB* gene of *P. aeruginosa* PAO1 was cloned in the plasmid vector pBBR 1 MCS. Conjugational transfer of the construct into A15Δ*fpvA* conferred the capacity for this mutant to grow in the presence of EDDHA when type I

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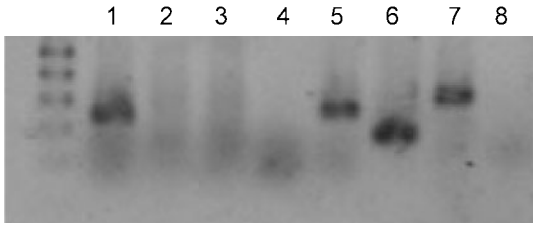

Fig. 4. RT-PCR detection of *fpvA* and *fpvB* transcripts in *P. aeruginosa* PAO1. Lanes: 1–4, cDNA prepared from RNA extracted from cells grown overnight in CAA plus 100 μM $FeCl_3$; 5–8, cDNA prepared from RNA extracted from cells grown overnight in CAA; 1 and 5, amplification of *oprL* RNA with PAL primers; 2 and 6, amplification of *fpvA* RNA; 3 and 7, amplification of *fpvB*; 4 and 8, control amplification with PAL primers on the crude RNA preparation.

pyoverdine was provided (Fig. 3b). Therefore, we can conclude that the provision of *fpvB in trans* confers the capacity to utilize type I pyoverdine. Interestingly, expression of *fpvB* also conferred some capacity to utilize type III pyoverdine (Fig. 3b).

Demonstration of the functionality of *fpvB* homologues in type II and type III pyoverdine-producing *P. aeruginosa* strains 59.20 and 7NSK2

7NSK2 and 59.20 *fpvA* mutants are both capable of using the heterologous type I pyoverdine as source of iron. Homologues of *fpvB* were detected in these strains by PCR and were inactivated using the same construct that was used to inactivate *fpvB* in PAO1. Double *fpvA fpvB* mutants of both 7NSK2 and 59.20 completely lost the ability of utilizing type I pyoverdine (Fig. 3a). A mutant of 59.20 was obtained that carries a transposon insertion in a homologue of the PAO1 *pvdI* gene (Lehoux *et al.*, 2000) and is therefore unable to produce pyoverdine and to grow in the presence of EDDHA. However, growth can be restored by each of the three types of *P. aeruginosa* pyoverdines. Inactivation of the *fpvB* homologue in this mutant abolished growth stimulation by type I pyoverdine, but not by type II or type III (Fig. 3). When *fpvA* is inactivated in 59.20, utilization of type II and III pyoverdines, but not of type I, is abolished. Likewise, inactivation of *fpvB* in 7NSK2 totally compromises the utilization of type I pyoverdine in this mutant, but does not affect the use of the cognate pyoverdine.

Regulation of *fpvB* expression

RT-PCR experiments show that iron limitation induces the transcription of *fpvA* and *fpvB* in CAA medium (Fig. 4).

In parallel, Affymetrix GeneChip microarray data (Ochsner *et al.*, 2002) were examined to determine if *fpvA* and *fpvB* were always observed to be coordinately expressed. As shown in Table 3, it is clear that *fpvA* and *fpvB* can be coordinately expressed, but they can also be independently expressed. In this case, we grew iron-deficient and iron-replete cells to stationary phase in either M9 minimal media with glucose as the carbon source or in deferrated DTSB, which is frequently used in studies on the iron-regulated expression of genes encoding virulence determinants (Barton *et al.*, 1996). The major carbon source in DTSB is glycerol. In this particular medium, no expression of *fpvB* could be detected whatever the iron status in the cell. Collectively these data clearly indicate that the expression *fpvA* and *fpvB* is differentially influenced by carbon source or another unknown factor present or absent in the media used in these experiments.

DISCUSSION

As for most Gram-negative bacteria with an aerobic lifestyle, acquiring iron is an important issue for *P. aeruginosa*. Therefore, it is not surprising that iron-uptake systems play an important role in both rhizosphere competence and pathogenesis of this opportunist pathogen with a ubiquitous nature (Cornelis & Matthijs, 2002). Illustrative for both flexibility needed for living in different environments and the importance of iron competitiveness in that matter are the variety of siderophore-mediated iron-uptake systems on which *P. aeruginosa* can rely (Cornelis & Matthijs, 2002; Poole & McKay, 2003). Not only does it synthesize two siderophores, pyochelin (low affinity) and pyoverdine (high affinity), together with their specific receptor proteins, but it is also able to take up heterologous siderophores of bacterial or fungal origin. This is reflected by the high number of

Table 3. Expression of *fpvA* and *fpvB* from microarray experiments as described by Ochsner *et al.* (2002)

Details are explained in the Methods section.

Microarray experimental conditions	Media used	-Fold increase in expression of *fpvB*	-Fold increase in expression of *fpvA*
PAO1 WT low Fe vs PAO1 WT high Fe	DTSB	No expression	25·5
PAO1 WT low Fe vs PAO1 WT high Fe	M9	26·6	156·0
PAO1 Δ*pvdS* vs PAO1 WT	DTSB	No expression	16·7
PAO1 Δ*pvdD* vs PAO1 WT	DTSB	No expression	25·6
PAO1 *fur*$^-$ (C6) high Fe vs PAO1 WT high Fe	DTSB	No expression	256

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candidate siderophore receptor genes present in the genome of *P. aeruginosa* PAO1 and other fluorescent pseudomonads (Cornelis & Matthijs, 2002). Few older reports mention the expression of an additional receptor for uptake of a heterologous pyoverdine (Koster *et al.*, 1993; Morris *et al.*, 1994). Closer *in silico* analysis suggested a certain degree of redundancy among these putative receptor genes in a way that different receptors would assure the uptake of a single siderophore (Cornelis & Matthijs, 2002). In this work, we present evidence for the existence of an alternative pyoverdine receptor in *P. aeruginosa* PAO1, supporting this concept of siderophore receptor redundancy (Cornelis & Matthijs, 2002). It started with the observation that a mutant in the *fpvA* pyoverdine receptor gene created in a siderophore-negative background [thanks to mutations in *pvdD* (pyoverdine biosynthesis) and *pchEF* operon (pyochelin biosynthesis)], still can overcome growth restriction imposed by the presence of the strong iron chelator EDDHA when provided with its cognate pyoverdine. However, in comparison with a pyoverdine biosynthesis mutant, an *fpvA* mutant shows a delayed growth response to the addition of pyoverdine. The PA4168 gene of the *P. aeruginosa* PAO1 genome represents the best candidate as second pyoverdine receptor gene with 54 % sequence identity at the amino acid level with FpvA. Simultaneous inactivation of *fpvA* and PA4168 in PAO1 resulted in the complete loss of the capacity to stimulate growth of the strain with its cognate pyoverdine. According to these data, PA4168 can be considered an alternative receptor of *P. aeruginosa* PAO1 for its cognate pyoverdine (type I) and may therefore be given the name *fpvB*. While it is possible that pyoverdine is usurping the receptor encoded by PA4168 (i.e. *fpvB*) that may actually preferentially bind an unknown siderophore ligand, it is also possible that these receptors are differentially expressed (as evidenced by our microarray data) so that pyoverdine would be accessible for iron gathering under distinct environmental conditions or nutrient availability. Unlike *fpvA*, *fpvB* is not part of the so-called pyoverdine locus where genes involved in biosynthesis and uptake of pyoverdine are clustered together with genes that control their expression (Ochsner *et al.*, 2002; Ravel & Cornelis, 2003).

In recent articles on the response of *P. aeruginosa* to iron limitation, Heim *et al.* (2003) demonstrated the production of the protein encoded by PA4168 using a proteomic analysis while Palma *et al.* (2003), using a microarray approach, described the early induction of PA4168 by iron limitation. In this study, we detected close homologues of *fpvB* in several clinical and environmental isolates of *P. aeruginosa*, including type II and type III pyoverdine-producing strains. It was already previously reported that certain type II and type III pyoverdine-producing strains are able to utilize type I pyoverdine (De Vos *et al.*, 2001; Pirnay *et al.*, 2002). Here we demonstrate that this capacity to utilize type I ferripyoverdine as the sole source of iron is conferred by FpvB. Previous reports on heterologous pyoverdine uptake in fluorescent pseudomonads are often explained by structural identity with the homologous pyoverdine leading to promiscuous uptake by the cognate pyoverdine receptor (Meyer *et al.*, 2002b). Our results also clearly suggest that FpvA III is such a promiscuous receptor since it confers to the bacterium the ability to grow in the presence of type II and type III pyoverdines (Fig. 3). FpvB also confers some ability to utilize type III pyoverdine (Fig. 3b). Conversely, the type II pyoverdine receptor seems to be more specific for type II pyoverdine.

The discovery of FpvB, a previously undescribed pyoverdine receptor, has implications for *P. aeruginosa* biology that are not necessarily restricted to pyoverdine uptake alone. A second pyoverdine receptor in *P. aeruginosa* could also be involved in entry of pyocins as demonstrated for the type II pyoverdine receptor and pyocin S3 (Baysse *et al.*, 1999). In the case of pyocin Sa there are indications (Smith *et al.*, 1992) that an *fpvA* mutant shows a reduced sensitivity, but not a complete resistance to the killing by Sa pyocin, suggesting that this bacteriocin could use a second type I pyoverdine receptor (perhaps FpvB) as gate of entry into the cell.

ACKNOWLEDGEMENTS

This research was supported in part by a grant from the National Institutes of Allergy and Infectious Diseases (AI15940) to M. L. V. and by a grant OZR from the VUB to P. C.; S. A. B. is the recipient of a Medical Research Council (MRC) fellowship.

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