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Identification of type II and type III pyoverdine receptors from *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa produces, under conditions of iron limitation, a high-affinity siderophore, pyoverdine (PVD), which is recognized at the level of the outer membrane by a specific TonB-dependent receptor, FpvA. So far, for *P. aeruginosa*, three different PVDs, differing in their peptide chain, have been described (types I–III), but only the FpvA receptor for type I is known. Two PVD-producing *P. aeruginosa* strains, one type II and one type III, were mutagenized by a mini-Tn*phoA3* transposon. In each case, one mutant unable to grow in the presence of the strong iron chelator ethylenediaminedihydroxyphenylacetic acid (EDDHA) and the cognate PVD was selected. The first mutant, which had an insertion in the *pvdE* gene, upstream of *fpvA*, was unable to take up type II PVD and showed resistance to pyocin S3, which is known to use type II FpvA as receptor. The second mutant was unable to take up type III PVD and had the transposon insertion in *fpvA*. Cosmid libraries of the respective type II and type III PVD wild-type strains were constructed and screened for clones restoring the capacity to grow in the presence of PVD. From the respective complementing genomic fragments, type II and type III *fpvA* sequences were determined. When *in trans*, type II and type III *fpvA* restored PVD production, uptake, growth in the presence of EDDHA and, in the case of type II *fpvA*, pyocin S3 sensitivity. Complementation of *fpvA* mutants obtained by allelic exchange was achieved by the presence of cognate *fpvA in trans*. All three receptors posses an N-terminal extension of about 70 amino acids, similar to FecA of *Escherichia coli*, but only FpvAI has a TAT export sequence at its N-terminal end.

INTRODUCTION

Most Gram-negative bacteria with an aerobic lifestyle are confronted with the problem of iron(III) insolubility and therefore excrete iron-chelating molecules, termed siderophores (Neilands, 1995; Ratledge & Dover, 2000). Siderophores are recognized by specific receptors, which function as gated porin channels in concert with the TonB protein that energizes the receptor protein (Ratledge & Dover, 2000). This protein family is characterized by a large C-terminal domain consisting of 22 antiparallel β-strands, which form a β-barrel that spans the outer membrane (Koebnik *et al.*, 2000). In contrast to outer-membrane porins, TonB-dependent outer-membrane proteins contain an additional domain known as a 'cork' or 'plug' that transiently blocks the channel formed by the β-barrel domain and, by using energy transduced by TonB, selectively allows uptake of cognate siderophore–iron complexes (Ferguson *et al.*, 1998).

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Abbreviations: CAA, Casamino acid medium; CF, cystic fibrosis; EDDHA, ethylenediaminedihydroxyphenylacetic acid; ESI-MS/MS, electrospray-ionization tandem mass spectrometry; Gm, gentamycin; IROMP, iron-repressed outer-membrane protein; PVD, pyoverdine.

The GenBank accession numbers for the *fpvA* sequences reported in this manuscript are AF537094 and AF537095.

Pseudomonas aeruginosa produces the siderophore pyoverdine (PVD), which is composed of a dihydroxyquinoline chromophore and a variable peptide chain (Meyer, 2000). Three structurally different PVDs (with different peptide chains) have been identified from *P. aeruginos*a strains (Cornelis *et al.*, 1989; Meyer *et al.*, 1997; De Vos *et al.*, 2001), each recognized at the level of the outer membrane by a specific receptor (Cornelis *et al.*, 1989). The receptor for *P. aeruginosa* PAO1 PVD (type I PVD) has been intensively characterized using physiological, immunological and molecular approaches (among others, Poole *et al.*, 1993; Schalk *et al.*, 2002). PVD is essential for the virulence of *P. aeruginosa* in mouse models (Meyer *et al.*, 1996; Handfield *et al.*, 2000). It has been shown previously that *P. aeruginosa* strains producing type II PVD receptor are killed by pyocin S3, a *P. aeruginosa* bacteriocin (Baysse *et al.*, 1999; Michel-Briand & Baysse, 2002). Mutants that failed to produce the receptor were found to be unable to take up PVD and became resistant to pyocin S3 (Baysse *et al.*, 1999). In this work, we describe the isolation of receptor-negative mutants for type II and type III PVDs, respectively, their physiological characterization, the cloning of the respective receptors by complementation and their analysis at the molecular level.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are shown in Table 1.

Transposon mutagenesis. Mutagenesis of *P. aeruginosa* 7NSK2 (Höfte *et al.*, 1990) was done by biparental mating with the donor strain *Escherichia coli* SM10 (λ pir) containing the suicide delivery system pUT (de Lorenzo *et al.*, 1990) and the transposon mini-Tn*phoA3* as described previously (Pattery *et al.*, 1999). Transconjugants were selected on Casamino acid medium (CAA) plates supplemented with appropriate antibiotics [100 mg gentamycin (Gm) l^{-1}; 10 mg tetracycline l^{-1}]. Candidates for receptor mutants were first selected on CAA plus 0·5 mg ethylenediaminedihydroxyphenylacetic acid (EDDHA) ml^{-1}, and CAA plus 0·5 mg EDDHA ml^{-1} and PVDII (50 μM). Mutants deficient for FpvAII were confirmed by their resistance to pyocin S3 (Baysse *et al.*, 1999). The same strategy was used for the selection of PVD receptor mutants of clinical isolate *P. aeruginosa* 59.20, a type III PVD producer (Meyer *et al.*, 1997), with the exception of the pyocin S3 test.

Purification of PVDs. For growth-stimulation experiments, PVDs were partially purified from 10 ml CAA culture supernatants, while for uptake experiments they were purified by a more elaborate method, as described previously (Meyer *et al.*, 1997). The amount of PVD present in the solution was estimated by measuring the absorbance at 400 nm (Meyer *et al.*, 1997).

Physiological characterization. Growth stimulation by the different PVDs on CAA plus EDDHA was done on agar plates by streaking, parallel to the wild-type, one PVD biosynthesis mutant (as positive control) and the candidate receptor-negative mutant. Growth stimulation was recorded after one day, and the plates were photographed using a Fuji Digital camera. For more accurate analysis, growth was assessed in microtitre plates (300 μl of culture), 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).

Uptake of ^{59}Fe-labelled PVD. Uptake of the different purified ^{59}Fe-labelled PVDs was done as described previously (Munsch *et al.*, 2000).

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).

Inverse-PCR (IPCR) characterization of mutants. Genomic DNA was digested with *Pst*I (or *Sma*I or *Eco*RV) and ligations were performed according to standard methods (Sambrook *et al.*, 1989). IPCR of circularized *Pst*I-digested DNAs of the mutants was performed using the primers PhoA5 and GM1 (Table 2). Nested-PCR (NPCR) was done after the first amplification using primers PhoA4 and Gm2. IPCR and NPCR were done for 30 cycles (30 s at 94 °C for the denaturation, 30 s at 55 °C for annealing and 4 min at 72 °C for the elongation), preceded by one cycle of denaturation of 50 s, and terminated by one cycle of elongation of 10 min. The NPCR-amplified fragments were cloned in the vector pCR2.1 (TA-cloning kit; Invitrogen).

Construction of genomic libraries and complementation. A genomic library of 20–25 kb *Pst*I partially digested genome fragments of the wild-type strain 59.20 was constructed in the cosmid pRG930 (Van den Eede *et al.*, 1992), using the Gigapack III Gold kit (Stratagene). Two-thousand clones were selected on Luria–Bertani agar supplemented with spectinomycin (50 μg ml^{-1}) and streptomycin (20 μg ml^{-1}). Triparental mating between pooled clones of the bank, a helper *E. coli* strain containing plasmid pRK 2013 and mutant 59.20-18B3 was performed in order to complement strain 59.20-18B3. The complemented mutant was selected on CAA plus EDDHA. The cosmid clone 2E7 that complemented the mutant was isolated from the library and the cosmid DNA was purified.

For strain 7NSK2, a *Sau*3AI genomic library of 7NSK2 was constructed as described previously (Lim *et al.*, 1997) and screened by colony blotting (Dig-System; Roche).

Generation of Δ*fpvA* mutants by allelic exchange. The wild-type *fpvAII* gene was amplified using as the template wild-type *P. aeruginosa* 7NSK2 DNA and primers FpvAII-R1 and FpvAII-R2 (Table 2). The amplification reaction was carried out by using 50 ng of the template and TaKaRa Ex *Taq* polymerase (TaKaRa Biomedicals) in a reaction mixture of 50 μl. The PCR product was purified with the QIAQuick gel extraction Kit (QIAGEN). After the addition of dATP (Sambrook *et al.*, 1989), the PCR fragment was cloned into the *Eco*RI site of the pCR2.1 vector (Invitrogen), following the protocol supplied by the manufacturer, and one clone was selected for sequence and restriction analyses.

The constructed plasmid was digested in a unique *Sal*I restriction site and the overhangs were filled using T4 DNA polymerase according to the manufacturer's instructions (Fermentas). This plasmid was ligated to a Gm cassette (Baysse *et al.*, 2001) and transformed into DH5α cells. Plasmid DNA of one transformant was analysed by restriction digestion to ensure that the Gm cassette was inserted into the *fpvAII* gene. This DNA was used as template for PCR amplification using 50 ng of template, 5 μl of 10× PCR buffer, 10 μl Q solution (QIAGEN), 4 μl of 2·5 mM dNTPs, 1 μl of 20 μM of each primer (R1 and R2) and 2 μl Proof Start enzyme (QIAGEN) in a master mix of 50 μl.

The PCR fragment was purified using the QIAQuick gel extraction Kit (QIAGEN) according to the manufacturer's instructions. This fragment, containing the Δ*fpvAII* gene, was then ligated into

Table 1. Strains and vectors used in this study

Strain/plasmid	Characteristics	Source/reference
Strain		
Pseudomonas aeruginosa		
7NSK2	Wild-type, type II PVD	Höfte *et al.* (1990)
7NSK2-8AG5	Tn*5* mutant in *pvdE*; Gm^R	This study
7NSK2-13AB3	Tn*5* mutant in *ccmE*; Gm^R	This study
7NSK2-*fpvA*	Allelic *fpvAII* mutant; Gm^R	This study
59.20	Wild-type, type III PVD	Meyer *et al.* (1997)
59.20-24A45	Tn*5* mutant in *ccmB*; Gm^R	This study
59.20-18B3	Tn*5* mutant in *fpvA*; Gm^R	This study
59.20-*fpvA*	Allelic *fpvAIII* mutant; Gm^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
HB101	*supE44 hsd20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 syl-5 mtl-1*	Boyer & Roulland-Dussoix (1969)
CM404	HB101 with pRK2013; helper strain for conjugation	Cornelis *et al.* (1992)
Plasmid		
pRG930	Cosmid vector, wide-host-range; Sm^R	Van den Eede *et al.* (1992)
pGEM-T	Cloning vector	Invitrogen
PBluescript KS^+	Lac^+; Ap^R	Stratagene
pCRII-2.1	TA cloning vector for PCR fragments; Ap^R, Km^R	Invitrogen
pGV4692(mini-Tn*phoA3*)	Mini-Tn*phoA3* transposon with the Gm cassette on pGV4692, a derivative of pUT (Ap^R)	de Lorenzo *et al.* (1990); Pattery *et al.* (1999)
pBR325	ColE1 vector; Ap^R, Cm^R, Tc^R	Bolivar (1978)
pBBR1-MCS	Wide-host-range cloning vector	Kovach *et al.* (1994)
pBBR1-GM	Gm cassette cloned into pBBR1-MCS	Kovach *et al.* (1994)
pFPR1	pKS^+ with 4·3 kb *Eco*RI fragment from genomic DNA of *P. aeruginosa* 7NSK2-8AG5	This study
pFPR2	pKS^+ with 1·7 kb *Eco*RV–*Not*I fragment from pFPR1 subcloned in pKS^+	This study
pC7NSK2	pRG930 with 20 kb partial *Pst*I fragment of genomic DNA from 7NSK2	This study
pC59.20	pRG930 with 20 kb partial *Sau*3AI fragment of genomic DNA from 59.20	This study

*Eco*RI-restricted pBR325 and transformed into DH5α with selection of chloramphenicol- and Gm-resistant colonies. Screening of clones was done by colony PCR using primers Gm1 and Gm2. The PCR was carried out by using 4 μl of 2·5 mM dNTPs, 1·5 μl of 50 mM $MgCl_2$, 1 μl of 20 μM of each primer and 0·25 μl *Taq* polymerase (5 U μl^{-1}). Additional screening was done by restriction analysis. The selected clones were used to transform *E. coli* GJ23 cells before mobilization of the disrupted *fpvAII* gene by conjugation into *P. aeruginosa* 7NSK2. Recombinants were selected by their resistance to Gm (100 μg ml^{-1}) and spectinomycin (50 μg ml^{-1}).

The *fpvAIII* ORF (2445 bp) from *P.aeruginosa* 59.20 was similarly amplified using primers FpvAIII-F and FpvAIII-R, and cloned into pCR2.1 using the TA-cloning kit (Invitrogen). The same Gm^R cassette was inserted blunt into the *SnaB*I site (1458 bp downstream from the start of the cloned ORF). Since double crossover recombinants should be Gm^R but sensitive to tetracycline, clones not growing on tetracycline (100 μg ml^{-1}) were selected.

Sequence determination and analyses. Sequencing was realized by Eurogentec or Genome Express and the sequences were compared using the BLASTX algorithm against the NCBI database and the *P. aeruginosa* database (http://www.pseudomonas.com). For determination of ORFs, the GENE COMPARE software was utilized (Applied Maths). The multiple-sequence alignment of FpvAI, FpvAII and FpvAIII was based on a larger non-redundant CLUSTAL W alignment that included ~100 sequences from other known or putative TonB-dependent receptor proteins. The cork region was realigned using the HMMER 2 algorithm (http://hmmer.wustl.edu/) based on a profile generated from a structural alignment of *E. coli* FhuA, FepA and FecA. Subsequently, the alignment was edited

Table 2. List of primers used in this study

Name	Sequence (5′→3′)	Purpose
PhoA4	GCACCGCCGGGTGCAGTAATTAT	Inverse PCR
PhoA5	GCGGCAGTCTGATCACCCGTTA	Inverse PCR
Gm1	TGGACCAGTTGCGTGAGCGCATA	Inverse PCR
Gm2	TGTCAACTGGGTTCGTGCCTTC	Inverse PCR
Gem1	GCGGCCGCACACCGTGGAAAC	Amplification of Gm cassette
Gem2	GCAAGTTCGGCTCTAGCGCCGGCG	Amplification of Gm cassette
FpvAI-1F	CGAAGGCCAGAACTACGAGA	Multiplex PCR
FpvAI-1R	TGTAGCTGGTGTAGAGGCTCAA	Multiplex PCR
FpvAII-2F	TACCTCGACGGCCTGCACAT	Multiplex PCR
FpvAII-2R	GAAGGTGAATGGCTTGCCGTA	Multiplex PCR
FpvAIII-3F	ACTGGGACAAGATCCAAGAGAC	Multiplex PCR
FpvAIII-3R	CTGGTAGGACGAAATGCGAG	Multiplex PCR
FpvAII-R1	GGTTTCGCGCCGACCAAA	*fpvAII* gene replacement
FpvAII-R2	CCGGATGCCTTACCAGTT	*fpvAII* gene replacement
FpvAIII-F	ATGCCAACAGCACATGCGGTTT	*fpvAIII* gene replacement
FpvAIII-R	TCAGTAGCTATAGGTAAGCGTG	*fpvAIII* gene replacement
FpvAIIISDF	GGAGAAAAACATGCAACAGCAC	*fpvAIII* cloning

manually to ensure optimal gap placement and correct alignment of highly conserved residues.

Subtractive hybridization. Suppression subtractive hybridization was carried out using the CLONTECH PCR-Select Bacterial Genome Subtraction Kit (Clontech) as recommended by the supplier and as described recently (Parsons *et al.*, 2002). The subtraction library was screened by sequencing of PCR amplicons from individual clones. Sequences obtained were used in BLAST searches at the *Pseudomonas* Genome Project web-site (http://www.pseudomonas.com) to determine their presence or absence from the PAO1 genome. Tester-specific sequences were further analysed using BLASTN and BLASTX searches of the database using the NCBI web-site (http://www.ncbi.nlm.nih.gov).

Electrospray-ionization tandem mass spectrometry (ESI-MS/MS) and N-terminal sequence analysis. Gel pieces were washed with 50 mM ammonium bicarbonate (pH 7·8)/acetonitrile (60 : 40) for 1 h at room temperature. The solution was removed and the bands were vacuum-dried for 25 min in a SpeedVac (Savant Instruments). Gel pieces were rehydrated in 12 μl of modified sequencing-grade trypsin (Promega; 12 ng μl^{-1} in 50 mM ammonium bicarbonate) at 4 °C for 1 h. Excess trypsin solution was removed and the gel pieces suspended in 20–30 μl of 50 mM ammonium bicarbonate and incubated overnight at 37 °C. Eluted peptides were concentrated and de-salted using C_{18} Zip-Tips (Millipore) and were eluted in 1–2 μl of 50 % methanol/1 % formic acid directly into borosilicate nanoelectrospray needles (Micromass). ESI-MS/MS was performed using a Q-Tof hybrid quadrupole/orthogonal-acceleration time-of-flight (TOF) mass spectrometer (Micromass). Nanoelectrospray needles containing the sample were mounted in the source and stable flow was obtained using capillary voltages of 900–1200 V. Precursor ion scans were performed to detect mass : charge (m/z) values for peptides within the mixture. The m/z value of each individual precursor ion was selected for fragmentation and collided with argon gas using collision energies of 18–30 eV. Fragment ions (corresponding to the loss of amino acids from the precursor peptide) were recorded and processed using MASSLYNX version 3.4 (Micromass). Amino acid sequences were deduced by the mass differences between *y*- or *b*-ion 'ladder' series using the program MASSSEQ (Micromass) and confirmed by manual interpretation. N-terminal Edman sequencing was performed as described previously (Nouwens *et al.*, 2000).

Multiplex PCR for the identification of *fpvAI*, *fpvAII* and *fpvAIII*. Six primers were designed for the simultaneous amplification of the different *fpvA* genes: primers 1F and 1R for the amplification of a 326 bp fragment corresponding to *fpvAI*; primers 2F and 2R for the amplification of an 897 bp fragment corresponding to *fpvAII*; and primers 3F and 3R for the amplification of a 506 bp fragment corresponding to *fpvAIII*. The following conditions were used: first a denaturation at 94 °C for 3 min, followed by 30 cycles with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, and terminating with a last cycle at 72 °C for 10 min.

Pyocin S3 susceptibility assay. A total cell lysate containing pyocin S3 was prepared from *E. coli* DH5α(pYS3.3) and tested for bactericidal activity against *P. aeruginosa* strains as described by Duport *et al.* (1995).

RESULTS

Isolation of PVD utilization mutants from type II and type III PVD-producing strains

Strain 7NSK2, a plant rhizosphere isolate (Höfte *et al.*, 1990) and type II PVD producer, was chosen because it was easily mutagenized with transposons. For similar reasons, we chose strain 59.20, a clinical isolate and type III PVD producer (Meyer *et al.*, 1997). Out of 1500 7NSK2 mutants analysed, 50 were found to be unable to grow in the presence of the strong iron chelator EDDHA. Only two of them (7NSK2-8AG5 and 7NSK2-13AB3) were unable to grow in the presence of EDDHA and type II PVD. One of these (7NSK2-13AB3) was still sensitive to pyocin S3, while the other (7NSK2-8AG5) had acquired resistance to pyocin S3. For strain 59.20, 3000 candidates were analysed, and 25 were found to be unable to grow in the presence

of EDDHA. Again, two mutants (59.20-18B3 and 59.20-24A45) were unable to grow in the presence of EDDHA and type III PVD.

Molecular characterization of the mutants unable to grow in the presence of the cognate PVD

The 7NSK2 and 59.20 PVD utilization mutants were analysed by inverse-PCR. The sequence flanking the transposon end was compared with the *P. aeruginosa* Genome Project database (http://www.pseudomonas.com) using the BLASTX program. The S3-sensitive mutant 7NSK2-13AB3 had an insertion into a gene with high similarity to *ccmE* (*PA1483*), a gene encoding a haem chaperone involved in *c*-type cytochrome biogenesis (Thöny-Meyer, 1997; Goldman & Kranz, 2001). Interestingly, mutant 59.20-24A45 had the transposon inserted into a gene with high similarity to *ccmB* (*PA1476*), also involved in *c*-type cytochrome biogenesis, and part of the *ccmABCDEFGH* operon (Thöny-Meyer, 1997; Goldman & Kranz, 2001). In this case, the identity was 98 % at the amino acid level between residues 22 and 88 of CcmB.

The 7NSK2 PVD utilization mutant 7NSK2-8AG5 had an insertion in a gene showing similarity (23 identical amino acids out of 25) with the *pvdE* gene *PA2397* (Fig. 1), upstream of *fpvA*, encoding an ABC transporter needed for PVD biosynthesis (McMorran *et al.*, 1996). The 59.20 utilization mutant 59.20-18B3 had an insertion in a gene matching the *fpvA* gene *PA2398* (Fig. 1).

Physiological characterization of the receptor-negative mutants from *P. aeruginosa* 7NSK2 and 59.20

The 7NSK2-*pvdE* mutant failed to produce PVD, became totally resistant to pyocin S3 (results not shown) and failed to grow in CAA in the presence of EDDHA and the cognate PVD (Table 3). Analysis of outer-membrane proteins by SDS-PAGE shows one iron-repressed outer-membrane protein (IROMP) of 80 kDa missing in the *pvdE* mutant (results not shown). Uptake of ^{59}Fe-labelled PVD is completely abolished in the 7NSK2-*pvdE* mutant (9282 ± 529 c.p.m. incorporated after 5 min for the wild-type vs 54 ± 50 c.p.m. for the *pvdE* mutant). All these observations confirmed that the *pvdE* mutant does not produce the type II PVD receptor.

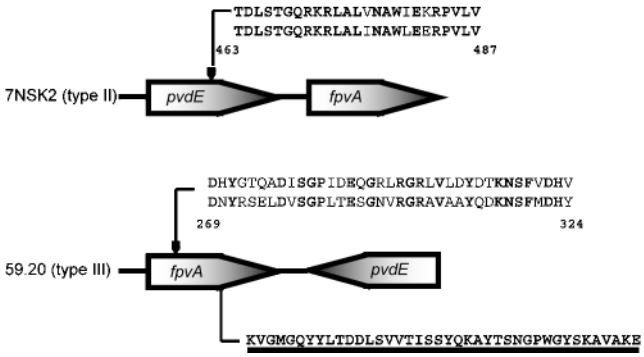

Fig. 1. Schematic representation of the *fpvA* and *pvdE* genes in *P. aeruginosa* strains 7NSK2 (type II PVD) and 59.20 (type III PVD). The positions of the Tn*5* insertions are indicated by triangles, and the sequences above are an alignment with the corresponding PAO1 sequence below (http://www.pseudomonas.com). The underlined sequence under the *fpvAIII* gene represents the translated DNA sequence obtained after subtractive hybridization from a CF epidemic strain (Cheng *et al.*, 1996). The GenBank accession numbers for the sequences reported in this manuscript are AF537094 (*fpvAIII*) and AF537095 (*fpvAII*).

Table 3. Growth of wild-type type II and type III PVD producers and their corresponding mutants

The genes shown in parentheses represent the cognate *fpvA in trans*. Optical density for all values was measured at 600 nm, and data are the means of three replica cultures in the Bio-Screen; the range of deviation was between 0 and 0·04.

Time	Medium*	Strain							
		7NSK2	7NSK2-*pvdE*	7NSK2-*fpvA*	7NSK2-*pvdE* (*fpvAII*)	7NSK2-*fpvA* (*fpvAII*)	59.20	59.20-*fpvA*	59.20-*fpvA* (*fpvAIII*)
24 h	CAA	0·59	0·60	0·53	0·59	0·53	0·64	0·63	0·50
	EDDHA	0·63	0·07	0·06	0·08	0·61	0·38	0·13	0·32
	PVDII	0·90	0·09	0·07	0·93	0·82	0·08	0·08	0·52
	PVDIII	0·44	0·07	0·07	0·11	0·08	0·97	0·13	0·81
36 h	CAA	0·57	0·63	0·48	0·62	0·51	0·74	0·71	0·59
	EDDHA	0·82	0·09	0·1	0·07	0·81	0·62	0·08	0·59
	PVDII	0·95	0·11	0·07	0·91	1·06	1·02	0·07	0·81
	PVDIII	0·71	0·15	0·1	0·12	0·63	1·07	0·12	0·89

*EDDHA, CAA medium with 0·5 mg EDDHA ml^{-1}; PVDII and PVDIII, CAA medium with 0·5 mg EDDHA ml^{-1} and 50 μM purified PVD.

Mutant 59.20-18B3 showed strongly reduced PVD production and could not grow in the presence of EDDHA with or without type III PVD (Table 1). An IROMP of about 80 kDa was also clearly less abundant than for the wild-type (results not shown). Type III ^{59}Fe-labelled PVD uptake was also strongly reduced in the case of this *fpvA* mutant (25 960 ± 3142 c.p.m. for the wild-type vs 2068 ± 467 c.p.m. for the *fpvA* mutant). Uptake of the other *P. aeruginosa* siderophore, pyochelin, was unaffected in both mutants (results not shown).

Complementation of the two receptor-negative mutants

A 7NSK2 genomic library was screened using a digoxigenin-labelled fragment corresponding to a partial sequence of *fpvAII* obtained from the sequence downstream of the interrupted *pvdE* gene present on the inverse-PCR fragment. The 59.20 library was directly screened in search for a clone complementing the *fpvAIII* mutation (see Methods). From each library, one cosmid clone (pC7NSK2 from the 7NSK2 library and pC59.20 from the 59.20 library) was selected that, after transfer to the respective 7NSK2-*pvdE* and 59.20-*fpvA* mutants, restored the capacity to grow in the presence of EDDHA and the cognate PVD (results not shown). Furthermore, the complemented 7NSK2-*pvdE* mutant regained pyocin S3 sensitivity (results not shown).

Sequence determination of *fpvAII* and *fpvAIII*

Since both pC7NSK2 and pC59.20 complemented all mutant phenotypes, we looked for the *fpvAII* and *fpvAIII* genes on the corresponding complementing cosmids. Primers were therefore designed to sequence each *fpvA* gene directly on cosmid DNA as described in Methods. Briefly, 2848 and 2740 nt fragments containing the *fpvAII* and *fpvAIII* ORFs were sequenced from pC7NSK2 and pC59.20, respectively. BLAST searches (Altschul *et al.*, 1997) of FpvAII and FpvAIII peptide sequences against the nr protein database (http://www.ncbi.nlm.nih.gov/) showed that, like FpvAI, these proteins shared 25–31 % identity over the majority of their length with approximately 100 other known or putative TonB-dependent outer-membrane proteins. FpvAII has 808 residues, while FpvAI and FpvAIII have 815. Using the PSI-BLAST algorithm, the highest level of identity was found between FpvAII and a putative TonB-dependent receptor (GenBank accession no. AAL45999.1) from *Agrobacterium tumefaciens* (45 % identity, 60 % similarity), while FpvAIII was most similar (37 % identity, 54 % similarity) to the ferric pseudobactin PbuA receptor from *Pseudomonas fluorescens* M114 (Morris *et al.*, 1994).

Unlike FpvAI, FpvAII and FpvIII do not end with a phenylalanine, as is customary, but with a tryptophan in the case of FpvAII and a tyrosine in the case of FpvAIII. However, these are relatively conservative changes and several of the 269 putative TonB-dependent receptor proteins, which make up Pfam TonB_boxC domain alignment (http://pfam.wustl.edu), similarly have terminal tryptophan or tyrosine residues.

Interestingly, analysis of the N-terminal sequences shows that FpvAI has a typical twin-arginine translocase (TAT) secretion signal (**RR**A**F** motif), as recently demonstrated by Ochsner *et al.* (2002a), while the two other PVD receptors seem rather to have classical secretion signal sequences (results not shown). Finally, analysis of the sequence downstream of *fpvaIII* showed that the *pvdE* gene in this case was transcribed in the opposite orientation (Fig. 1).

Identification of type III *fpvA* from a cystic fibrosis (CF) epidemic strain by subtractive hybridization

Independently from our transposon mutagenesis approach, suppression subtractive hybridization was used to identify genes specific to a *P. aeruginosa* CF epidemic strain from Liverpool (Cheng *et al.*, 1996; McCallum *et al.*, 2001, 2002; Parsons *et al.*, 2002). One sequence of 341 bp was translated in all six reading frames and one perfect alignment was found with a peptide corresponding to FpvAIII (shown in bold in Fig. 1a), suggesting a high degree of conservation of *fpvAIII*.

Peptide sequencing of FpvAII

We used N-terminal sequence analysis and ESI-MS/MS to confirm the identity of the 80 kDa IROMP that was absent from outer-membrane preparations from strain 7NSK2-*fpvA* (see below). The corresponding band was cut from two SDS-PAGE gels. The N-terminal sequence was XAQKIQFD (where X could not be identified), which corresponds to the sequence EAQKIQFD (residues 44–51) deduced from the translated nucleotide sequence of *fpvAII*. The band was also subjected to an in-gel tryptic digest prior to internal sequence analysis using ESI-MS/MS. Eight peptides varying between 6 and 15 aa in length were sequenced (results not shown). A total of 86 aa, equalling sequence coverage of 10·6 %, were determined and all matched the deduced amino acid sequence from *fpvAII* (results not shown).

Generation of 7NSK2 and 59.20 *fpvA* mutants by allelic exchange and complementation by the respective *fpvA* genes

The *fpvA* gene in strains 7NSK2 and 59.20 was inactivated by allelic exchange using a Gm cassette (see Methods). In parallel, both *fpvA* genes were PCR-amplified and cloned in the broad-host-range plasmid pBBR-1MCS (Kovach *et al.*, 1994) in order to complement the respective *fpvA* mutants.

Both *fpvA* mutants had a strongly reduced PVD production, in contrast with the 7NSK2-*pvdE* mutant, which produced no PVD (results not shown). Fig. 2(a) shows that 7NSK2-*fpvA* lacks an IROMP of 80 kDa (lane 2 vs lane 1). The situation was less clear in the case of 59.20, although one IROMP of about 80 kDa was less abundant in the

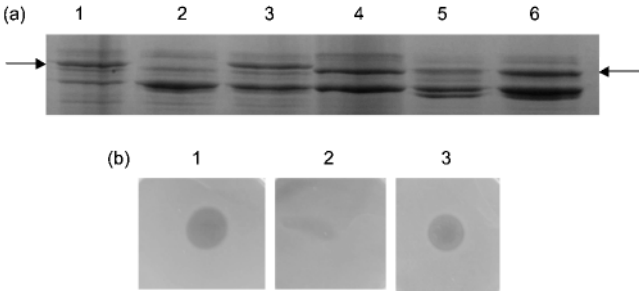

Fig. 2. (a) SDS-PAGE (10% polyacrylamide) of outer-membrane proteins from *P. aeruginosa* strains 7NSK2 (lanes 1–3) and 59.20 (lanes 4–6) grown in CAA. Lanes: 1 and 4, wild-type; 2 and 5, *fpvA* mutants; 3 and 6, complemented *fpvA* mutants with cognate *fpvA in trans*. The molecular masses were calculated by comparison with the migration of a pre-stained standard (10–250 kDa; Bio-Rad). The 80 kDa IROMPs described in the text are indicated by arrows. (b) Sensitivity to pyocin S3 of 7NSK2 (panel 1), 7NSK2-*fpvA* (panel 2) and 7NSK2-*fpvA* with *fpvAII in trans* (panel 3).

case of the *fpvAIII* mutant (compare lanes 4 and 5). Complementation by the homologous *fpvA* gene *in trans* resulted in both cases in the reappearance (for 7NSK2-*fpvA*) or much stronger intensity (for 59.20-*fpvA*) of this particular IROMP on the gel (Fig. 2a, lanes 3 and 6). It should also be noted that one IROMP, of about 70 kDa, is produced by the mutant 59.20 and the complemented mutant, but not by the wild-type. Fig. 2(b) shows the pyocin S3 sensitivity test for 7NSK2 (panel 1), 7NSK2-*fpvA* (panel 2) and 7NSK2-*fpvA* with *fpvAII in trans* (panel 3). As expected, pyocin S3 sensitivity is restored in the complemented *fpvA* mutant.

For both strains, growth of the wild-type, *fpvA* mutant and *fpvA* mutant with the cognate *fpvA* gene *in trans* was measured after 24 and 36 h (Table 3). The different media tested were CAA, CAA plus EDDHA, CAA plus EDDHA and type II PVD, and CAA plus EDDHA and type III PVD. The growth of strain 59.20 was partially inhibited in the presence of EDDHA while this was not the case for 7NSK2, especially when comparing the optical density values after 24 h. The growth of strain 7NSK2 in the presence of EDDHA was strongly stimulated by its cognate PVD, but inhibited by type III PVD, while, unexpectedly, both PVDs stimulated the growth of strain 59.20. Growth of the two *fpvA* mutants was observed only in CAA medium. The *fpvA* gene *in trans* restored growth in the presence of EDDHA partially (59.20-*fpvA*) or fully (7NSK2-*fpvA*). Again, type III PVD was inhibitory for the complemented 7NSK2-*fpvA* mutant during the first 24 h, while type II PVD clearly stimulated the complemented 59.20-*fpvA* mutant, even after 24 h. After 36 h cultivation, growth of complemented 7NSK2-*fpvA* was observed in the presence of the heterologous PVD, probably because of restored production of sufficient amounts of the homologous PVD.

The 7NSK2-*pvdE* mutant could be complemented with *fpvAII in trans* for growth in the presence of EDDHA only when type II PVD was present in the medium.

Conservation of the different *fpvA* genes among *P. aeruginosa* clinical and environmental isolates

Primers were developed in order to amplify, from each *fpvA*, a fragment of different size (326 bp for *fpvAI*, 897 bp for *fpvAII* and 506 bp for *fpvAIII*) using a multiplex PCR setting. Fig. 3 shows the result of a typical PCR amplification, done on *P. aeruginosa* CF isolates. Out of 21 independent sporadic CF isolates (each with a different PFGE type), 13 were found to contain the gene for *fpvAII*, five for *fpvAI* and three for *fpvAIII*. All isolates from an epidemic strain from Liverpool (Cheng *et al.*, 1996; McCallum *et al.*, 2001, 2002) gave amplification of a DNA fragment corresponding to *fpvAIII* (lane 5, upper gel; lanes 6–10, lower gel). One transmissible strain from Manchester (Jones *et al.*, 2001) gave an amplicon corresponding to type II *fpvA* (lane 11, lower gel). In each case, only one amplicon was observed.

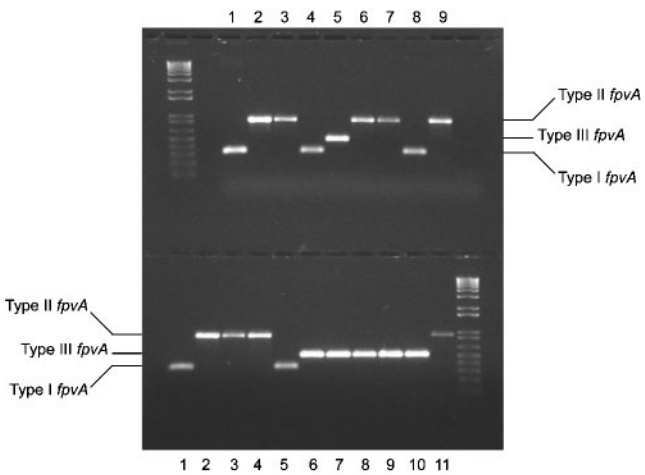

Fig. 3. Multiplex PCR amplification of *fpvA* genes from different *P. aeruginosa* CF isolates. Upper gel lanes 1–4 and 6–9 and lower gel lanes 1–4 contain amplicons from CF non-epidemic strain isolates. Upper gel lane 5 and lower gel lanes 6–10 contain amplicons from different isolates of the Liverpool CF epidemic strain. Other amplicons included in the figure are from PAO1 (lower gel, lane 5) and a strain from Manchester reported as transmissible (lower gel, lane 11). Upper gel (far left) and lower gel (far right) contain 1 kb Plus DNA ladder (Life Technologies). The distribution of *fpvA* types is: type I, upper gel lanes 1, 4 and 8, lower gel lanes 1 and 5; type II, upper gel lanes 2, 3, 6, 7 and 9, lower gel lanes 2–4 and 11; type III, upper gel lane 5, lower gel lanes 6–10. The marker is a 100 bp ladder.

DISCUSSION

P. aeruginosa is an important nosocomial pathogen as well as the main cause of lung infections among CF patients (Govan & Deretic, 1996; Bodey *et al.*, 1983). In mouse models, the production of siderophores, particularly PVD, was found to have a major impact on the virulence of this bacterium (Meyer *et al.*, 1996; Takase *et al.*, 2000a, 2000b; Handfield *et al.*, 2000). These reports suggest that PVD-mediated iron uptake could represent an interesting target for drug or vaccine development. The presence of three PVD-receptor-mediated iron uptake systems among *P. aeruginosa* strains (Cornelis *et al.*, 1989; Meyer *et al.*, 1997; De Vos *et al.*, 2001) necessitated the characterization of the two remaining receptors for type II and type III PVDs. Interestingly, type II FpvA is known to be the port of entry for pyocin S3, a *P. aeruginosa* bacteriocin causing the death of bacteria producing this particular siderophore receptor (Baysse *et al.*, 1999).

Using transposon mutagenesis, combined with a screening in the presence of EDDHA and the cognate PVD, we were able to obtain mutants unable to utilize these siderophores. Two mutations affecting the utilization of PVDs were found in cytochrome *c* biogenesis genes (Thöny-Meyer, 1997; Goldman & Kranz, 2001), *ccmB* in the case of 59.20, and *ccmE* in the case of 7NSK2. We have previously shown that mutations in another *ccm* gene, *ccmC*, affect the production, maturation and utilization of PVD in *P. fluorescens* (Gaballa *et al.*, 1996, 1998), and that this effect could be due to a reduction in the oxidative power of the periplasm (Baysse *et al.*, 2002). For each strain, one mutant was obtained that showed defective production of FpvA, as judged by different criteria, but in the case of 7NSK2 the transposon inserted in *pvdE*, upstream of *fpvA*. Absence of this gene is the first time that an insertion into *pvdE* has been shown to affect not only PVD production (McMorran *et al.*, 1996) but also PVD uptake via the FpvA receptor. Complementation of the *pvdE* mutation with *fpvAII in trans* was possible only in the presence of the cognate PVD. Probably, the defect in the production of the FpvA receptor in a *pvdE* mutant is due to the almost complete absence of PVD in the medium and lack of auto-induction of the receptor (Lamont *et al.*, 2002; Visca *et al.*, 2002). PvdE is a membrane protein of the family of ABC transporters, but its function has been unknown until now (McMorran *et al.*, 1996). Recently, Ochsner *et al.* (2002b) demonstrated that *fpvA* expression depends on a promoter upstream of the gene that contains a binding motif for the alternative sigma factor PvdS needed for the transcription of different PVD biosynthesis genes (Wilson *et al.*, 2001), confirming earlier observations (McMorran *et al.*, 1996). This finding seems to exclude the possibility that an insertion into the *pvdE* gene could have a polar effect on the transcription of *fpvA*. Furthermore, in the case of *fpvAIII*, the *pvdE* gene is transcribed in the opposite orientation (Fig. 1a).

Although the 59.20-*fpvA* mutant was clearly deficient for the uptake of its cognate PVD, absence of the corresponding 80 kDa protein could not be unambiguously evidenced by SDS-PAGE. Furthermore, the *fpvAIII* mutant, whether complemented or not, produces a new IROMP of 70 kDa. It has been shown before that in the absence of PVD production, the profile of IROMPs on SDS-PAGE can be altered (Höfte *et al.*, 1993; Mossialos *et al.*, 2000). It is therefore possible that other IROMPs, including one of the same molecular mass as FpvAIII, are produced by the *fpvAIII* mutant. All three receptors have an N-terminal periplasmic extension, also found in other PVD or pseudobactin receptors (Shen *et al.*, 2002) such as PbuA (GenBank accession no. Q08017), PupA (GenBank accession no. P25184) and PupB (GenBank accession no. P38047). This N-terminal domain has recently been shown to interact with another protein, FpvR, an anti-sigma factor that sequesters PvdS (Lamont *et al.*, 2002; Visca *et al.*, 2002). By interacting with the siderophore, the conformation of the receptor changes, allowing PvdS to be liberated from FpvR (Lamont *et al.*, 2002). This explains why receptor-negative mutants produce little PVD compared to the wild-type (Lamont *et al.*, 2002; Shen *et al.*, 2002). Since our *fpvAII* and *fpvAIII* mutants also show strongly decreased PVD production, we assume that a similar mechanism is at work in the corresponding *P. aeruginosa* strains.

An intriguing observation is the capacity of strain 59.20 to utilize, besides its cognate type III PVD, type II PVD, via the FpvAIII receptor. Until recently, it was generally assumed that receptors for PVDs or pseudobactins had a strict specificity for the cognate PVD (Hohnadel & Meyer, 1988; Cornelis *et al.*, 1989). However, it was found that the receptor for type II *P. aeruginosa* PVD also recognizes two other PVDs, from *P. fluorescens* PL7 and PL8 (Meyer *et al.*, 1999, 2002). The efficiency of the uptake of type II PVD by FpvAIII, if any, must however be low since no significant incorporation of ^{59}Fe-labelled PVDII by strain 59.20 could be observed (results not shown). Of course, the possibility that PVDII is not taken up via FpvAIII but can interact with the receptor and, by the above-described mechanism, trigger the production of the cognate PVDIII by the strain, thereby promoting growth, cannot be ruled out.

The availability of the three *P. aeruginosa fpvA* sequences allowed us to look for their conservation among different clinical isolates, using a PCR approach, similar to what was done for the genes encoding the outer-membrane lipoproteins OprI and OprL (De Vos *et al.*, 1997). A PCR set-up has already been described for the amplification of the *fpvA* gene corresponding to type I PVD (Al-Samarrai *et al.*, 2000). Each strain tested so far seems to produce only one single receptor (Fig. 3). So far, no negative results have been obtained using the multiplex PCR, meaning that the receptors are fairly conserved. This simple test allows the rapid identification of *P. aeruginosa* isolates that fail to produce PVD, as frequently observed in the case of CF strains (De Vos *et al.*, 2001).

In conclusion, the identification of the three PVD receptors

characteristic of all *P. aeruginosa* strains tested so far should help us to identify domains involved in the interaction with the respective PVDs. It will also be interesting in the future to investigate whether FpvAII and FpvAIII, like FpvAI, use the mechanism of PVD-recycling (Folschweiller *et al.*, 2000; Schalk *et al.*, 2001, 2002). Identification of loops interacting with PVD or with pyocin S3 (in the case of FpvAII) is also an interesting goal for coming research. Also, given the importance of PVD for the virulence of *P. aeruginosa*, and with the perspective of developing a vaccine against this pathogen, identification of protective epitopes would be an important goal to meet.

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