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

Magaly de Chial, 1† Bart Ghysels, 1† Scott A. Beatson, 2 Valérie Geoffroy, 3 Jean Marie Meyer, 3 Theresa Pattery, 1 Christine Baysse, 1 Patrice Chablain, 1 Yasmin N. Parsons, 4 Craig Winstanley, 4 Stuart J. Cordwell 5 and Pierre Cornelis 1

1Flanders Interuniversity Institute of Biotechnology (VIB6), Laboratory of Microbial Interactions, Vrije Universiteit Brussel, Building E, room 6.6, Pleinlaan 2, B-1050 Brussels, Belgium
2MRC Functional Genetics Unit, Department of Human Anatomy and Genetics, University of Oxford, South Parks Road, Oxford OX1 3QX, UK
3Laboratoire de Microbiologie et de Génétique, Université Louis Pasteur, UPRES-A 7010, F-67000 Strasbourg, France
4Department of Medical Microbiology and Genitourinary Medicine, University of Liverpool, Duncan Building, Liverpool L69 3GA, UK
5Australian Proteome Analysis Facility, Sydney, Australia 2109

*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-TnphoA 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 possess 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).

Abbreviations: CAA, Casamino acid medium; CF, cystic fibrosis; EDDHA, ethylenediaminedihydroxyphenylacetic acid; ES/−MS/MS, electrospray-ionization tandem mass spectrometry; Gm, gentamycin; IROMP, iron-repressed outer-membrane protein; PVD, pyoverdine.
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. aeruginosa 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-TnpphoA3 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 analyses, 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 ⁵⁹Fe-labelled PVD. Uptake of the different purified ⁵⁹Fe-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 PstI (or Smal or EcoRV) and ligations were performed according to standard methods (Sambrook et al., 1989). IPCR of circularized PstI-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 PstI partially digested genome fragments of the wild-type strain 59.20 was constructed in the cosmid pRH930 (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 Sau3AI genomic library of 7NSK2 was constructed as described previously (Lim et al., 1997) and screened by colony blotting (Dig-System; Roche).

Generation of ΔfpvA 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 EcoRI 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 SalI 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 ΔfpvAII gene, was then ligated into...
Table 1. Strains and vectors used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7NSK2</td>
<td>Wild-type, type II PVD</td>
<td>Höfte et al. (1990)</td>
</tr>
<tr>
<td>7NSK2-8AG5</td>
<td>Tn5 mutant in pvdE; GmR</td>
<td>This study</td>
</tr>
<tr>
<td>7NSK2-13AB3</td>
<td>Tn5 mutant in ccmE; GmR</td>
<td>This study</td>
</tr>
<tr>
<td>7NSK2-fpvA</td>
<td>Allelic fpvAII mutant; GmR</td>
<td>This study</td>
</tr>
<tr>
<td>59.20</td>
<td>Wild-type, type III PVD</td>
<td>Meyer et al. (1997)</td>
</tr>
<tr>
<td>59.20-24A45</td>
<td>Tn5 mutant in ccmB; GmR</td>
<td>This study</td>
</tr>
<tr>
<td>59.20-18B3</td>
<td>Tn5 mutant in fpvA; GmR</td>
<td>This study</td>
</tr>
<tr>
<td>59.20-fpvA</td>
<td>Allelic fpvAII mutant; GmR</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (80 lacZAM15 recA</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td></td>
<td>hsdR17 recA1 endA1 gyrA96 thi-1 relA1)</td>
<td></td>
</tr>
<tr>
<td>GJ23</td>
<td>JC2692(pGI28) (R6Kad11); KmR, SmR, TeR</td>
<td>Van Haute et al. (1983)</td>
</tr>
<tr>
<td>SM10 (λ pir)</td>
<td>thi-1 thr leu tnaA lacY supE recA::RP4-2 tc::Mu; λ pir; KmR</td>
<td>Herrero et al. (1990)</td>
</tr>
<tr>
<td>Top 10F</td>
<td>F’ [lacIΔ7 Tn10 (TetR)] mcrαΔ(ntr–hsdRMS–mcrBC)Δ80</td>
<td>Invitrogen</td>
</tr>
<tr>
<td></td>
<td>lacZAM15A lacX74 recA1 araD139 Δ(ara–leu)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7697 galU galK rpsL (StrR) endA1 supG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>supE44 hsd20 (rM mB) recA13 ara-14 proA2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>lacY1 galK2 rpsL20 syl-5 mtl-1</td>
<td></td>
</tr>
<tr>
<td>HB101</td>
<td>Pseudomonas aeruginosa</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
</tr>
<tr>
<td></td>
<td>CM404</td>
<td>Cornelis et al. (1992)</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRG930</td>
<td>Cosmid vector, wide-host-range; SmR</td>
<td>Van den Eede et al. (1992)</td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>PBluescript KS+</td>
<td>Lac+; ApR</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pCR1-2.1</td>
<td>TA cloning vector for PCR fragments; ApR, KmR</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pGV4692(mini-TnphoA3)</td>
<td>Mini-TnphoA3 transposon with the Gm cassette</td>
<td>de Lorenzo et al. (1990); Pattery et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>on pGV4692, a derivative of PUT (ApR)</td>
<td></td>
</tr>
<tr>
<td>pBR325</td>
<td>ColE1 vector; ApR, KmR, TeR</td>
<td>Bolivar (1978)</td>
</tr>
<tr>
<td>pBRR1-MCS</td>
<td>Wide-host-range cloning vector</td>
<td>Kovach et al. (1994)</td>
</tr>
<tr>
<td>pBRR1-GM</td>
<td>Gm cassette cloned into pBRR1-MCS</td>
<td>Kovach et al. (1994)</td>
</tr>
<tr>
<td>pFPR1</td>
<td>pKS+ with 4-3 kb EcoRI fragment from genomic DNA of P. aeruginosa 7NSK2</td>
<td>This study</td>
</tr>
<tr>
<td>pFPR2</td>
<td>pKS+ with 1-7 kb EcoRV–NotI fragment from pFPR1 subcloned in pKS+</td>
<td>This study</td>
</tr>
<tr>
<td>pC7NSK2</td>
<td>pRG930 with 20 kb partial PstI fragment of genomic DNA from 7NSK2</td>
<td>This study</td>
</tr>
<tr>
<td>pC59.20</td>
<td>pRG930 with 20 kb partial Sau3A1 fragment of genomic DNA from 59.20</td>
<td>This study</td>
</tr>
</tbody>
</table>

EcoRI-restricted pBRR325 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 MgCl2, 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 GmR cassette was inserted blunt into the SmalI site (1458 bp downstream from the start of the cloned ORF). Since double crossover recombinants should be GmR 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 FpvAII, FpvAIII and FpvAIV 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.
bands were vacuum-dried for 25 min in a SpeedVac (Savant) for 1 h at room temperature. The solution was removed and the gel pieces suspended in 20–30 ml of 50 mM ammonium bicarbonate (pH 7.8)/acetonitrile (60 : 40) at 4˚C for 1 h. Excess trypsin solution was removed and the gel pieces suspended in 20–30 ml of modified ammonium bicarbonate and incubated overnight at 37˚C. Eluted peptides were concentrated and de-salted using C18 Zip-Tips (Millipore) and were eluted in 1–2 ml 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 of an argon gas beam 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, fpvAll 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 fpvAll; 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 (*PA2398*), 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.

**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.

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

*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.
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 fpvAII 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 fpvAll and fpvAllII

Since both pC7NSK2 and pC59.20 complemented all mutant phenotypes, we looked for the fpvAll and fpvAllIII genes on the respective 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 fpvAll and fpvAllIII ORFs were sequenced from pC7NSK2 and pC59.20, respectively. BLAST searches (Altschul et al., 1997) of PfpAll and PfpAllIII 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. FpvAll has 808 residues, while FpvAI and FpvAllIII have 815. Using the PSI-BLAST algorithm, the highest level of identity was found between FpvAll and a putative TonB-dependent receptor (GenBank accession no. AAL45999.1) from Agrobacterium tumefaciens (45 % identity, 60 % similarity), while FpvAllIII was most similar (37 % identity, 54 % similarity) to the ferric pseudobactin PbuA receptor from Pseudomonas fluorescens M114 (Morris et al., 1994).

Unlike FpvAI, FpvAll and FpvAllIII do not end with a phenylalanine, as is customary, but with a tryptophan in the case of FpvAllII and a tyrosine in the case of FpvAllIII. 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 FpvAlI has a typical twin-arginine translocase (TAT) secretion signal (RRRAF 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 fpvAllIII 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 FpvAllIII (shown in bold in Fig. 1a), suggesting a high degree of conservation of fpvAllIII.

Peptide sequencing of FpvAllII

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 fpvAllII. 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 fpvAllII (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
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 fpvAII 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.

For both strains, growth of the wild-type, fpvA mutant and fpvA mutant with the cognate 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.

**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 ard (10–250 kDa; Bio-Rad). The 80 kDa IROMPs described in the text are indicated by arrows. (c) Sensitivity to pyocin S3 sensitivity is restored in the complemented fpvA mutant.

**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.

http://mic.sgmjournals.org

IP: 54.70.40.11
On: Thu, 29 Nov 2018 08:34:36
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 fpvAll 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 fpvAllI, 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 fpvAllI 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 FpvAllI, are produced by the fpvAllI 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 fpvAll and fpvAllI 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 FpvAllI receptor. Until recently, it was generally assumed that receptors for PVDs or pseudobactins had a strict specificity for the cognate PVD (Hohndel & 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 FpvAllI, 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 FpvAllI 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 FpvAll and FpvAllI, like FpvAl, 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 FpvAll) 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.

**ACKNOWLEDGEMENTS**

This work was supported by the Alphonse & Jean Forton Fund Against Cystic Fibrosis. M. De Chial is a recipient of a Ph. D. fellowship from the Panamanian government. We are grateful to Dr Patrick Plesiat for sending us isolates from France. S. A. Beaton is supported by a fellowship from the Royal Commission for the Exhibition of 1851. We thank Chris Ponting for helpful discussions regarding this study.

**REFERENCES**


M. de Chial and others


New pyoverdine receptors from *P. aeruginosa*


