Identification and characterization of novel pyoverdine synthesis genes in *Pseudomonas aeruginosa*

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Fluorescent pseudomonads secrete yellow-green siderophores named pyoverdines or pseudobactins. These comprise a dihydroxyquinoline derivative joined to a type-specific peptide and, usually, a carboxylic acid or amide. In *Pseudomonas aeruginosa* strain PAO1, six genes that encode proteins required for pyoverdine synthesis (*pvd* genes) have been identified previously. Expression of all of these genes requires an alternative sigma factor PvdS. The purpose of this research was to identify other genes that are required for pyoverdine synthesis in *P. aeruginosa* PAO1. Fourteen candidate genes were identified from the PAO1 genome sequence on the basis of their location in the genome, the functions of homologues in other bacteria, and whether their expression was likely to be PvdS-dependent. The candidate genes were mutated and the effects of the mutations on pyoverdine production were determined. Eight new *pvd* genes were identified. The presence of homologues of *pvd* genes in other strains of *P. aeruginosa* was determined by Southern blotting and in other fluorescent pseudomonads by interrogation of genome sequences. Five *pvd* genes were restricted to strains of *P. aeruginosa* that make the same pyoverdine as strain PAO1, suggesting that they direct synthesis of the type-specific peptide. The remaining genes were present in all strains of *P. aeruginosa* that were examined and homologues were present in other *Pseudomonas* species. These genes are likely to direct synthesis of the dihydroxyquinoline moiety and the attached carboxylic acid/amide group. It is likely that most if not all of the genes required for pyoverdine synthesis in *P. aeruginosa* PAO1 have now been identified and this will form the basis for a biochemical description of the pathway of pyoverdine synthesis.

INTRODUCTION

Fluorescent pseudomonads secrete yellow-green fluorescent siderophores termed pyoverdines or pseudobactins (Fig. 1). These enable acquisition of Fe(III) ions from the environment (reviewed by Meyer & Stintzi, 1998) and also serve as signalling molecules controlling gene expression in the bacterial cells (Lamont *et al.*, 2002; Visca *et al.*, 2002). A large number of pyoverdines and pseudobactins have been characterized and all comprise a shared dihydroxyquinoline chromophore joined to an acyl (carboxylic acid or amide) group and a short (6–12 amino acid) type-specific peptide (Fig. 1) (reviewed by Budzikiewicz, 1993; Meyer, 2000). Pyoverdines/pseudobactins produced by a single strain all have the same peptide but they may differ in the nature of the acyl group. Strains of *Pseudomonas* can utilize heterologous pyoverdines and pseudobactins for iron acquisition and the spectrum of ferrisiderophores that can be used forms the basis of a strain identification method termed siderotyping (Meyer *et al.*, 2002).

The pyoverdines that are produced by strains of *P. aeruginosa* are classified into three types (I–III) that are distinguished by their peptides (Fig. 1) (Meyer *et al.*, 1997). The genes and enzymes that are required for synthesis of pyoverdine are best characterized in the type I strain PAO1. Most of the pyoverdine synthesis genes that have been identified in this strain are at about 47 min on the genetic map (Ankenbauer *et al.*, 1986; Hohnadel *et al.*, 1986; Rombel & Lamont, 1992; Stintzi *et al.*, 1996; Tsuda *et al.*, 1995) and these genes are listed in Table 1. The *pvdA* gene encodes an enzyme that catalyses synthesis of N^\delta^-hydroxoyrithnine (Visca *et al.*, 1994) and the *pvdF* gene product catalyses the formylation of N^\delta^-hydroxoyrithnine to give N^\delta^-formyl-N^\delta^-hydroxoyrithnine, which is present in the type I pyoverdine (pyoverdine\textsubscript{PAO}) made by *P. aeruginosa* PAO1 (McMorran *et al.*, 2001). The product of the *pvdD* gene is a peptide synthetase that directs incorporation of two L-threonine residues into the peptide of pyoverdine\textsubscript{PAO} (Merriman *et al.*, 1995; Ackerley *et al.*, 2003). The *pvdIJK* gene products also have the characteristics of peptide synthetases (Lehoux *et al.*, 2000); resequencing of *P. aeruginosa* PAO1 DNA shows that *pvdF* and *pvdK* are part of a single gene (see below), which will
be referred to here as \textit{pvdJ}. The product of the \textit{pvdE} gene is likely to be an ABC transporter protein and it is essential for pyoverdine synthesis (McMorran \textit{et al.}, 1996) although the transported substrate has not been identified. A separate cluster of four genes (\textit{pvcABCD}) at 66–70 min on the genetic map has been reported to be required for synthesis of the pyoverdine chromophore (Stintzi \textit{et al.}, 1996, 1999). However, \textit{pvc} mutants are able to make pyoverdine in some growth media so that these genes are not essential for pyoverdine synthesis (P. Cornelis & U. Ochsner, personal communication).

Expression of all of the pyoverdine-synthesis genes that have been characterized to date requires an alternative sigma factor protein, PvdS (reviewed by Visca \textit{et al.}, 2002). Promoters that are recognized by RNA polymerase containing PvdS contain a sequence motif, the IS box, at about 33 bp from the transcription start sites and this forms part of the promoter sequence (Rombel \textit{et al.}, 1995; Wilson \textit{et al.}, 2001). A second sequence CGT at about –10 bp is also required for promoter recognition by PvdS (S. Tsao, M. J. Wilson & I. L. Lamont, unpublished data). The activity of PvdS is regulated post-translationally by an anti-sigma factor FpvR (Lamont \textit{et al.}, 2002) and in addition expression of the \textit{pvdS} gene is repressed in iron-rich cells (Cunliffe \textit{et al.}, 1995; Leoni \textit{et al.}, 1996), providing two levels of control of pyoverdine production.

The structural complexity of pyoverdine\textsubscript{PAO} suggests that the biosynthetic pathway will involve a number of enzymes in addition to those that have been identified to date. The sequence of the genome of \textit{P. aeruginosa} strain PAO1

\begin{table}[h]
\centering
\begin{tabular}{|l|l|}
\hline
\textbf{Gene} & \textbf{ORF}\textsuperscript{*} & \textbf{Function} \\
\hline
\textit{pvdA} & PA2386 & Ornithine hydroxylase (Visca \textit{et al.}, 1994) \\
\textit{fpvI} & PA2387 & ECF sigma factor required for expression of \textit{fpvA} (Beare \textit{et al.}, 2003) \\
\textit{fpvR} & PA2388 & Anti-sigma factor for PvdS and FpvI (Lamont \textit{et al.}, 2002; Beare \textit{et al.}, 2003) \\
\textit{pvdF} & PA2396 & \textit{N}^{5}\text{-Hydroxyornithine} transformylase (McMorran \textit{et al.}, 2001) \\
\textit{pvdE} & PA2397 & ABC transporter (secretion) (McMorran \textit{et al.}, 1996) \\
\textit{fpvA} & PA2398 & Ferripyoverdine receptor protein (Poole \textit{et al.}, 1993) \\
\textit{pvdD} & PA2399 & Pyoverdine peptide synthetase (Merriman \textit{et al.}, 1995) \\
\textit{pvdJ} & PA2400/1 & Pyoverdine peptide synthetase (Lehoux \textit{et al.}, 2000) \\
\textit{pvdI} & PA2402 & Pyoverdine peptide synthetase (Lehoux \textit{et al.}, 2000) \\
\textit{pvdS} & PA2426 & ECF iron sigma factor (Cunliffe \textit{et al.}, 1995; Miyazaki \textit{et al.}, 1995) \\
\textit{pvcABCD} & PA2254–PA2257 & Synthesis of the pyoverdine chromophore (Stintzi \textit{et al.}, 1996, 1999) \\
\hline
\end{tabular}
\caption{Genes involved in synthesis or transport of pyoverdine in \textit{P. aeruginosa} PAO1}
\textsuperscript{*ORF in the \textit{P. aeruginosa} genome sequence (http://www.pseudomonas.com; http://pseudomonas.bit.uq.edu.au).}
\end{table}
Plasmids used in this study are listed in Table 2. Escherichia coli was grown in Luria (L-) broth (Sambrook et al., 2000) and P. aeruginosa in L-broth or King’s B broth (King et al., 1954) at 37°C with aeration for liquid cultures. Media were solidified by the addition of agar (1.5%) and supplemented with antibiotics or with the iron-chelating compound ethylenediamine (o-hydroxy) phenylacetic acid (EDDA) as described previously (McMorran et al., 2001). Gentamicin was added to a final concentration of 4 μg ml⁻¹ (E. coli) and 20 μg ml⁻¹ (P. aeruginosa) where required.

**Molecular biology methods.** Plasmid DNA was prepared using the High Pure Plasmid Isolation kit (Roche) and genomic DNA was prepared from P. aeruginosa as described by Chen & Kuo (1993). DNA was amplified from P. aeruginosa DNA by PCR using primers designed from the P. aeruginosa PA01 genome sequence (http://www.pseudomonas.com); details of primers are available on request. Restriction digestion, gel electrophoresis and DNA cloning were done by standard methods (Sambrook et al., 2000) with cloning into pGEM-T Easy carried out using the protocol recommended by the manufacturer (Promega). All plasmid constructs were verified by DNA sequencing (Centre for Gene Research, University of Otago, Dunedin). Sequencing a cloned PCR fragment spanning the junction of PA2400 and PA2401 in the P. aeruginosa genome showed that a GC base-pair was missing from the genome sequence at position 2669175 and when this was included PA2400 and PA2401 form a single reading frame PA2400/1 (pvdJ). Southern blotting was carried out by standard methods (Sambrook et al., 2000), using as probes

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**Table 2. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T-Easy</td>
<td>Ap⁺; DNA cloning vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pEX18Tc</td>
<td>Tc⁺; gene replacement vector</td>
<td>Hoang et al. (1998)</td>
</tr>
<tr>
<td>pEX18Gm</td>
<td>Gm⁺; gene replacement vector</td>
<td>Hoang et al. (1998)</td>
</tr>
<tr>
<td>pUC4KINN</td>
<td>Ap⁺ Km⁺; kanamycin-resistance cassette flanked by multiple restriction sites</td>
<td>Barany (1988)</td>
</tr>
<tr>
<td>pUC4KISS</td>
<td>Ap⁺ Km⁺; kanamycin-resistance cassette flanked by multiple restriction sites</td>
<td>Barany (1988)</td>
</tr>
<tr>
<td>pUC18-19Km</td>
<td>Ap⁺ Km⁺; kanamycin-resistance cassette flanked by multiple restriction sites</td>
<td>Markie et al. (1986)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC1061</td>
<td><code>hsdR araD139 Δ(araABC-leu)7679 Δ(lac)X74 galU galK rpsL thi</code></td>
<td>Casabudan &amp; Cohen (1980)</td>
</tr>
<tr>
<td>DH5α</td>
<td><code>supE44 ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</code></td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA01</td>
<td>Pvd⁺; makes type I pyoverdine</td>
<td>Holloway (1955); Stover et al. (2000)</td>
</tr>
<tr>
<td>PA01pvdQ::pEXGm</td>
<td>Pvd⁺; insertion of pEX18Gm in PA2385</td>
<td>This work</td>
</tr>
<tr>
<td>PA01pvdA2389::kan</td>
<td>Pvd⁺; insertion of Km⁺ cassette in PA2389</td>
<td>This work</td>
</tr>
<tr>
<td>PA01pvdB::pEXGm</td>
<td>Pvd⁺; insertion of pEX18Gm in PA2392</td>
<td>This work</td>
</tr>
<tr>
<td>PA01pvdM::kan</td>
<td>Pvd⁺; insertion of Km⁺ cassette in PA2393</td>
<td>This work</td>
</tr>
<tr>
<td>PA01pvdN::kan</td>
<td>Pvd⁺; insertion of Km⁺ cassette in PA2394</td>
<td>This work</td>
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<tr>
<td>PA01pvdO::kan</td>
<td>Pvd⁺; insertion of Km⁺ cassette in PA2395</td>
<td>This work</td>
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<tr>
<td>PA01pvdJ::kan</td>
<td>Pvd⁺; insertion of Km⁺ cassette in PA2400</td>
<td>This work</td>
</tr>
<tr>
<td>PA01pvdL::kan</td>
<td>Pvd⁺; insertion of Km⁺ cassette in PA2402</td>
<td>This work</td>
</tr>
<tr>
<td>PA01pvdG::kan</td>
<td>Pvd⁺; insertion of Km⁺ cassette in PA2425</td>
<td>This work</td>
</tr>
<tr>
<td>Pa4</td>
<td>Pvd⁺; makes type II pyoverdine</td>
<td>Meyer et al. (1997)</td>
</tr>
<tr>
<td>Pa6</td>
<td>Pvd⁺; makes type III pyoverdine</td>
<td>Meyer et al. (1997)</td>
</tr>
<tr>
<td>58.35</td>
<td>Pvd⁺; makes type II pyoverdine</td>
<td>Meyer et al. (1997)</td>
</tr>
<tr>
<td>58.36</td>
<td>Pvd⁺; makes type III pyoverdine</td>
<td>Meyer et al. (1997)</td>
</tr>
<tr>
<td>58.40</td>
<td>Pvd⁺; makes type I pyoverdine</td>
<td>Meyer et al. (1997)</td>
</tr>
<tr>
<td>59.20</td>
<td>Pvd⁺; makes type III pyoverdine</td>
<td>Meyer et al. (1997)</td>
</tr>
<tr>
<td>59.40</td>
<td>Pvd⁺; makes type II pyoverdine</td>
<td>Meyer et al. (1997)</td>
</tr>
<tr>
<td>59.41</td>
<td>Pvd⁺; makes type I pyoverdine</td>
<td>Meyer et al. (1997)</td>
</tr>
</tbody>
</table>
radiolabelled PCR fragments or cloned restriction fragments corresponding to individual genes, except that *pvdN* and *pvdO* were part of the same PCR fragment. Membranes were washed at 65 °C in 0.1% SDS/0.1 × SSC prior to autoradiography.

**Gene disruptions in *P. aeruginosa***. PCR fragments (1–0.1–5 kb) corresponding to genes to be mutated were cloned into pGEM T-Easy, excised using restriction enzymes (usually *Hind*III and EcoRI) corresponding to sites that were incorporated into the PCR primers, and subcloned into pEX18Tc or pEX18Gm. Kanamycin-resistant cassettes were then cloned into restriction sites within the target genes unless the cloned fragment was internal to the gene to be mutated. pEX constructs were transferred into *P. aeruginosa* PAO1 by triparental conjugation using the helper plasmid pRK2013 as described previously (McMorran *et al.*, 1996) with selection for transconjugants in which plasmid DNA had integrated into the chromosome of *P. aeruginosa* by homologous recombination. For heterodiplid strains in which conjugation gave rise to bacteria containing both a wild-type and a mutant (kanamycin-disrupted) gene, plasmid DNA containing the wild-type gene was cured from the bacteria by subculture in L-broth containing kanamycin, followed by sucrose-selection for plasmid-lacking strains (Hoang *et al.*, 1998).

**DNA from all recombinant *P. aeruginosa*** strains was analysed by PCR and Southern blotting to ensure that the intended mutations had been generated.

**Phenotypic analysis of bacteria.** *P. aeruginosa* strains were analysed for production of pyoverdine by growth on King’s B agar and on agar supplemented with EDDA; EDDA prevents the growth of *P. aeruginosa* strains that are unable to make or take up (ferri)pyoverdine (Ankenbauer *et al.*, 1986), and pyoverdine gives a yellow-green pigmentation around *Pvd* + colonies (King *et al.*, 1954). Pyoverdine production was quantified by growing cultures of bacteria in King’s B broth as described previously (McMorran *et al.*, 2001).

**Computational analysis.** DNA sequences were obtained from the *P. aeruginosa* genome project (http://www.pseudomonas.com) and the *P. aeruginosa* genome database (http://pseudomonas.bit.uq.edu.au). Sequences were manipulated using Seqed (Devereux *et al.*, 1984) and analysed using NLDNA and Codonuse as described previously (Merriman *et al.*, 1995). Database searches and analysis of the genomes of other fluorescent pseudomonads were carried out at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) with BLAST algorithms.

**RESULTS AND DISCUSSION**

**Identification of candidate pyoverdine synthesis and transport genes**

The *pvd* mutations identified previously spanned a region of the *P. aeruginosa* PAO1 genome extending from approximately *pvdA* (PA2386) to *pvdS* (PA2426) (Tsuda *et al.*, 1995) (Fig. 2). ORFs in this part of the genome were identified as part of the *P. aeruginosa* genome sequencing project (Croft *et al.*, 2000; Stover *et al.*, 2000). For all of these ORFs, codon usage was found to be similar to that of other *P. aeruginosa* genes (Grocock & Sharp, 2002; West & Iglewski, 1988) (data not shown). Two approaches were taken to identify ORFs in this region of the genome that may contribute to pyoverdine synthesis or transport. Firstly, the ORFs were screened to identify those that are preceded by a probable PvdS-dependent promoter, or may be part of an operon that is preceded by a probable PvdS-promoter, and so are likely to be co-expressed with pyoverdine synthesis genes. Secondly, BLAST searches were carried out to determine whether homologous genes are involved in synthesis of siderophores or other secondary metabolites. This resulted in the identification of 14 previously uncharacterized genes that may be required for pyoverdine synthesis or transport (Table 3).

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**Fig. 2.** The pyoverdine locus of *P. aeruginosa* PAO1. The orientations of ORFs are shown, with numbers corresponding to those in the *P. aeruginosa* genome (http://www.pseudomonas.com; http://pseudomonas.bit.uq.edu.au). Gene names are also shown, with genes that were identified in this study in bold. The positions of PvdS-dependent promoters, and likely promoters, are indicated by black arrowheads and other known promoters are indicated by hatched arrowheads.
<table>
<thead>
<tr>
<th>ORF</th>
<th>Gene</th>
<th>PvdS-dependent promoter*</th>
<th>Function/homologous genes†</th>
<th>Phenotype of mutant strain†</th>
<th>Phenotype of Ochsner et al. (2002)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA2385</td>
<td>pvdQ</td>
<td>No; may be operonic with pvdA (PA2386)</td>
<td>38% identity with aculeacin A acylase from <em>Actinoplanes utahensis</em> (Inokoshi et al., 1992)</td>
<td>Pvd⁻</td>
<td>Pvd⁻</td>
</tr>
<tr>
<td>PA2389</td>
<td></td>
<td>Yes</td>
<td>Over 30% identity with periplasmic MFPs of RND/MFP/OMF-type efflux systems (Poole, 2001; Zgurskaya &amp; Nikaido, 2000)</td>
<td>Pvd⁺</td>
<td>Pvd⁺</td>
</tr>
<tr>
<td>PA2390</td>
<td></td>
<td>Operonic with PA2389/PA2391</td>
<td>Over 40% identity with RND-type transporter components of RND/MFP/OMF-type efflux systems (Poole, 2001; Zgurskaya &amp; Nikaido, 2000)</td>
<td>ND</td>
<td>Pvd⁺</td>
</tr>
<tr>
<td>PA2391</td>
<td></td>
<td>Operonic with PA2390/PA2391</td>
<td>Over 30% identity with OMF proteins of RND/MFP/OMF-type efflux systems (Poole, 2001; Zgurskaya &amp; Nikaido, 2000)</td>
<td>ND</td>
<td>Pvd⁺</td>
</tr>
<tr>
<td>PA2392</td>
<td>pvdP</td>
<td>Yes</td>
<td>No known function</td>
<td>Pvd⁻</td>
<td>Pvd⁻</td>
</tr>
<tr>
<td>PA2393</td>
<td>pvdM</td>
<td>Yes</td>
<td>23% identity with porcine dipeptidase (Rached et al., 1990)</td>
<td>Pvd⁻</td>
<td>Pvd⁻</td>
</tr>
<tr>
<td>PA2394</td>
<td>pvdN</td>
<td>Operonic with pvdM/O</td>
<td>26% identity with isopenicillin N epimerase from <em>Streptomyces clavuligerus</em> (Kovacevic et al., 1990)</td>
<td>Pvd⁻</td>
<td>Pvd⁻</td>
</tr>
<tr>
<td>PA2395</td>
<td>pvdO</td>
<td>Operonic with pvdM/N</td>
<td>No known function</td>
<td>Pvd⁻</td>
<td>Pvd⁻</td>
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<tr>
<td>PA2411</td>
<td></td>
<td>Operonic with PA2412</td>
<td>36% identity with thioesterase GrsT from <em>Bacillus brevis</em> (Kratzschmar et al., 1989)</td>
<td>Pvd⁺</td>
<td>Pvd⁺</td>
</tr>
<tr>
<td>PA2412</td>
<td></td>
<td></td>
<td>No known function</td>
<td>ND</td>
<td>Pvd⁻</td>
</tr>
<tr>
<td>PA2413</td>
<td>pvdH</td>
<td>Yes</td>
<td>55% identity with diaminobutyrate ketoglutarate aminotransferase from <em>Acinetobacter baumannii</em> (Ikai &amp; Yamamoto, 1997)</td>
<td>Pvd⁻</td>
<td>Pvd⁻</td>
</tr>
<tr>
<td>PA2417</td>
<td></td>
<td>No</td>
<td>30% identity with PtxR transcriptional regulator (PA2258) from <em>P. aeruginosa</em> (Hamood et al., 1996)</td>
<td>Pvd⁺</td>
<td>ND</td>
</tr>
<tr>
<td>PA2424</td>
<td>pvdL</td>
<td>Operonic with PA2425</td>
<td>33% identity with TycB transcriptional regulator from <em>Bacillus brevis</em> (Mootz &amp; Marahiel, 1997)</td>
<td>Pvd⁻</td>
<td>Pvd⁻</td>
</tr>
<tr>
<td>PA2425</td>
<td>pvdG</td>
<td>Yes</td>
<td>34% identity with GrsT thioesterase from <em>Bacillus brevis</em> (Kratzschmar et al., 1989)</td>
<td>Pvd⁻</td>
<td>ND</td>
</tr>
</tbody>
</table>

*PvdS-dependent promoters were predicted on the basis of a strong match (at least 7 out of 8 bases with appropriate spacing) with the PvdS-dependent promoter consensus sequence TAAAT-N₁₆-CGT (Rombel et al., 1995; Wilson et al., 2001; S. Tsao, M. J. Wilson & I. L. Lamont, unpublished data). 'Operonic' indicates gene pairs for which the intergenic distance is less than 30 bp and there is no evidence of an intergenic promoter; pvdL is 78 bp downstream of pvdG and these genes are very probably expressed as an operon (Mossialos et al., 2002). pvdQ is 123 bp downstream from pvdA, with no promoters identified within the intergenic region, so these genes may also be operonic.

†Homologues were identified by BLAST searches at the NCBI database (www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi); the percentage amino acid identity is shown. MFP, membrane fusion protein; OMF, outer-membrane factor; RND, resistance-nodulation-division.

‡Mutant strains of *P. aeruginosa* PAO1 were analysed for production of pyoverdine as described in Methods.

§Phenotype reported by Ochsner et al. (2002) for *P. aeruginosa* PAO1 with a mutation in the gene shown.
Mutational analysis of candidate genes

Mutations were introduced into eleven of the genes listed in Table 3 as described in Methods. Three of the mutant strains (in ORFs PA2389, PA2411 and PA2417) retained the ability to make pyoverdine. These mutants were able to grow in the presence of EDDA, indicating that uptake of ferripyoverdine was also unaffected by the mutations. The remaining eight mutant strains did not make any detectable pyoverdine and were also unable to grow in the presence of the iron-chelating compound EDDA (Table 3). These phenotypes indicate that the corresponding genes are essential for pyoverdine synthesis and they were assigned the names pvdG–pvdQ (Table 3, Fig. 2). Three of these genes (pvdM, pvdN and pvdG) are predicted to be in operons upstream of other pvd genes (see Fig. 2) and it is possible that mutations in these genes cause a Pvd− phenotype because of polar effects on expression of the downstream gene(s). However, we have named these pvd genes, as different genes within a biosynthetic operon invariably encode products that contribute to the same biochemical pathway.

While this manuscript was being prepared, another study described mutations in many of the genes characterized in this study (Ochsner et al., 2002). The phenotypes obtained by these researchers are listed in Table 3 and are consistent with those described here. The role of pvdL in pyoverdine synthesis has also been described very recently (Mossialos et al., 2002).

A total of 15 pvd genes have now been identified that are essential for pyoverdine synthesis in P. aeruginosa PAO1 and it is likely that most, if not all, of the genes that are essential for pyoverdine synthesis in this strain are now known. An early study mapped two mutations that affected pyoverdine synthesis to a locus at 23 min on the recalibrated genetic map of P. aeruginosa PAO1 (Hohnadel et al., 1986) but further study of these mutants has not been reported. The pvc genes lie at about 66–70 min on the genetic map (Stintzi et al., 1996), about 240 kb away from pvdS. All other pvd mutations, including all of the 24 mutations identified by Tsuda et al. (1995), have been mapped to the 47 min region of the P. aeruginosa chromosome that corresponds to the part of the genome represented in Fig. 2. We have not mutagenized the 17 genes in this interval that did not meet our criteria for candidate pyoverdine synthesis genes and so cannot exclude the possibility that they contribute to pyoverdine synthesis. However, two of these genes (PA2403 and PA2407) were mutated by Ochsner et al. (2002) and the mutant bacteria retained the ability to make pyoverdine. In addition, the sequences of several of these genes suggest that they have functions other than pyoverdine synthesis. For example, PA2414 has 55% sequence identity with L-sorbose dehydrogenase from Acetobacter liquefaciens and PA2416 has 55% identity with a periplasmic trehalase from E. coli (data not shown). A number of mutations in this part of the genome did not affect pyoverdine synthesis (Tsuda et al., 1995).

Detection of pvd gene homologues in other Pseudomonas strains

The three different kinds of pyoverdines (types I–III) that are made by strains of P. aeruginosa all have the same dihydroxyquinoline component and are distinguished by the compositions of their peptides (Meyer et al., 1997; Meyer, 2000) (Fig. 1). It is likely that synthesis of the shared dihydroxyquinoline group, with its attached carboxylic acid or amide, has the same biosynthetic pathway in all strains and that the enzymes for this are encoded by orthologous genes in different strains. In contrast, type-specific genes probably direct synthesis of the peptides that distinguish the different pyoverdines.

Pyoverdine synthesis genes from P. aeruginosa PAO1 were used as hybridization probes with genomic DNA from other P. aeruginosa strains that produce type I, type II or type III pyoverdine (Fig. 3, Table 4). Hybridizations were carried out under conditions of high stringency to ensure that only very similar DNA sequences would hybridize. Some of the probes hybridized with DNA from all of the strains tested, indicating that orthologous genes are present in all of these strains. Other genes were only present in type I strains.

Partial or complete genome sequences are available for strains of P. fluorescens, P. putida and P. syringae. The predicted sequences of the products of P. aeruginosa pvd genes were used in BLAST searches in order to identify homologues in other Pseudomonas species; the results are shown in Table 4. Many of the genes that were detected by hybridization in all strains of P. aeruginosa (PA2389, pvdP, the pvdM–pvdO operon, PA2411, pvdH, pvdL and pvdS) had homologues with over 60% sequence identity in the other species. These are likely to be orthologues of the P. aeruginosa genes and to have common functions in all of the fluorescent pseudomonads. In addition, pvdQ and pvdG, which were detected in all P. aeruginosa strains, had homologues in the other species but with lower amounts of sequence similarity and these may also be orthologues. PvdS is an ECF sigma factor that is required for expression of other pyoverdine synthesis genes. The other pvd genes that are present in all fluorescent pseudomonads are most likely to be required for synthesis of the dihydroxyquinoline and amide/carboxylic acid moiety that is present in all pyoverdines. The pvdL gene-product has all of the characteristics of a peptide synthetase and corresponding genes are present in different strains of P. aeruginosa, and other pseudomonads (Table 4). Synthesis of the dihydroxyquinoline chromophore of pyoverdines is known to require amino acid precursors (Baysse et al., 2002; Budzikiewicz, 1993). It therefore seems likely that PvdL catalyses synthesis of a peptide that is modified by other enzymes (encoded by the shared pvd genes) to form the dihydroxyquinoline derivative coupled to an amide/carboxylic acid that is present in pyoverdines. Similar conclusions were reached by Mossialos et al. (2002) in an independent study of pvdL.

Genes that are present in only some strains (pvdA, pvdD,
Fig. 3. Detection of *pvd* gene homologues by Southern blotting. Chromosomal DNA from *P. aeruginosa* strains producing type I pyoverdine (PAO1, 58.40 and 59.41) (lanes 1–3), type II pyoverdine (strains Pa4, 58.35 and 59.40) (lanes 4–6) and type III pyoverdine (strains Pa6, 58.36 and 59.20) (lanes 7–9) was digested with *Pst*I. Following electrophoresis, the DNA was analysed by Southern blotting using the following *pvd* genes from *P. aeruginosa* PAO1 as probes: (a) *pvdA*; (b) *pvdF*; (c) *pvdJ*; (d) *pvdNi*pvd*O*.
pvdF, pvdI and pvdJ in *P. aeruginosa* PAO1) most likely direct synthesis of the peptide components of pyoverdines. This has been demonstrated biochemically for the products of pvdA and pvdF that catalyse hydroxylation of ornithine and formylation of hydroxyornithine, respectively, to generate N\(^5\)-formyl-N\(^5\)-hydroxyornithine, which is present in the peptide of type I pyoverdine (McMorran et al., 2001; Visca et al., 1994). Homologues of pvdA have been shown to be present in a strain of *P. aeruginosa* that makes type II pyoverdine and also in strains of *P. fluorescens* and *Burkholderia cepacia*, though not in a strain of *P. putida* (Visca et al., 1994). A homologue of pvdA from *Pseudomonas* sp. B10 complemented a pvdA mutation in *P. aeruginosa* PAO1 (Ambrosi et al., 2000), showing that it is an orthologue of pvdA. In this study, homologues of pvdA were present in all strains of *P. aeruginosa* that were tested, and also in *P. fluorescens* and *P. putida*, though not *P. syringae* (Table 4). However homologues of pvdF were not found outside *P. aeruginosa* type I strains and synthesis of formylhydroxyornithine must involve a different biosynthetic process in other strains/species that incorporate this compound into pyoverdine or pseudobactin.

Table 4. Presence of homologous genes in other fluorescent pseudomonads

<table>
<thead>
<tr>
<th>ORF</th>
<th>Gene</th>
<th><em>P. aeruginosa</em></th>
<th><em>P. fluorescens</em></th>
<th><em>P. putida</em></th>
<th><em>P. syringae</em></th>
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<tbody>
<tr>
<td></td>
<td>Type I</td>
<td>Type II</td>
<td>Type III</td>
<td>Type I</td>
<td>Type II</td>
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*The presence of corresponding genes in strains of *P. aeruginosa* producing type I, type II or type III pyoverdine was detected by Southern blotting (Fig. 3). ND, Not determined.

†Homologous genes were identified in *P. fluorescens* Pf-5, *P. putida* KT2440 and *P. syringae* by BLAST searches using the translations of the ORFs. The percentage of identical amino acids for the most similar protein is shown. NS, No significant homologues detected.

pvdD, pvdI and pvdJ were also detected by hybridization only in strains of *P. aeruginosa* making type I pyoverdine. These genes are thought to encode peptide synthetases (Lehoux et al., 2000; Merriman et al., 1995), a family of proteins with many conserved sequence features (Marahiel et al., 1997). PvdD directs incorporation of two L-threonine residues into pyoverdinePAO (Ackerley et al., 2003) and pvdI and pvdJ are very likely to encode peptide synthetases that direct incorporation of the remaining six amino acids into the peptide of type I pyoverdines. Homologues of these gene products are present in the other fluorescent *Pseudomonas* species but the levels of sequence identity (41–56 %) were no higher than those of paralogues of pvdD, pvdI and pvdJ in the *P. aeruginosa* genome (data not shown). The homologues in the other species are very likely to encode peptide synthetases that direct incorporation of different amino acids into pyoverdines/pseudobactins, or other secondary metabolites.

The pvdE gene product has all the characteristics of an ABC-type transporter protein (McMorran et al., 1996) although its substrate(s) has not been identified. Homologues were
not detected by Southern blotting in other strains of
P. aeruginosa, suggesting that the substrate is strain-specific
although homologues are present in other fluorescent pseudomonads. ABC transporter proteins that have many
shared sequence features may have different substrates
(Higgins, 1992, 2001). It remains to be determined whether
the PvdE homologues present in other species transport
the same substrate as in P. aeruginosa PA01, or whether they transport a different substrate with the sequence
similarities reflecting shared structural features.

In conclusion, the research described here has identified
eight previously undescribed genes that are required for
synthesis of pyoverdine. Analysis of the distribution of
pyoverdine synthesis genes amongst fluorescent pseudomonads, along with analysis of their sequences, indicates
their possible roles in the biochemical pathway of pyover-
dine synthesis. This will provide the basis for biochemical
characterization of individual enzymes and a complete
description of the pathway of pyoverdine synthesis.

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aeruginosa PA01 genome and the US DOE Joint Genome Institute and
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Pseudomonas genome sequences.

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