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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 inside 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).

Abbreviation: EDDA, ethylenediamine(*o*-hydroxy)phenylacetic acid.

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^5-hydroxyornithine (Visca *et al.*, 1994) and the *pvdF* gene product catalyses the formylation of N^5-hydroxyornithine to give N^5-formyl-N^5-hydroxyornithine, which is present in the type I pyoverdine ($\text{pyoverdine}_{\text{PAO}}$) 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 $\text{pyoverdine}_{\text{PAO}}$ (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 *pvdJ* and *pvdK* are part of a single gene (see below), which will

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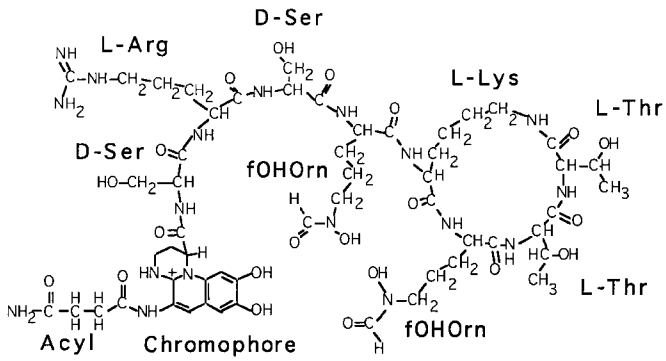
(a)



(b)

Pseudomonas species/ strain	Structure of pyoverdine peptide
P. aeruginosa PAO1	Ser-Arg-Ser-fOHOrn-c(Lys-fOHOrn-Thr-Thr) (Type I)
P. aeruginosa ATCC27853	Ser-fOHOrn-Orn-Gly-aThr-Ser-cOHOrn (Type II)
P. aeruginosa Pa6	Ser-Dab-fOHOrn-Gln-Gln-fOHOrn-Gly (Type III)
P. fluorescens 9AW	Ser-Lys-OHHis-aThr-Ser-cOHOrn
P. putida CFBP 2461	Asp-Lys-OHAsp-Ser-aThr-Ala-Thr-Lys-cOHOrn

Fig. 1. Structures of pyoverdines and pseudobactins. (a) Pyoverdine$_{PAO}$ from *P. aeruginosa* PAO1 (Abdallah, 1991). The acyl group can be a carboxylic acid or an amide (as shown). (b) The peptide components of pyoverdines and pseudobactins from different strains of *Pseudomonas* (data from Meyer, 2000). Amino acid residues that are in the D configuration are underlined. fOHOrn, N^5-Formyl-N^5-hydroxyornithine; aThr, allo-threonine; OHOrn, hydroxyornithine; c, cyclic.

be referred to here as *pvdJ*. The product of the *pvdE* gene is likely to be an ABC transporter protein and it is essential for pyoverdine synthesis (McMorran *et al.*, 1996) although the transported substrate has not been identified. A separate cluster of four genes (*pvcABCD*) at 66–70 min on the genetic map has been reported to be required for synthesis of the pyoverdine chromophore (Stintzi *et al.*, 1996, 1999). However, *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 *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 *et al.*, 1995; Wilson *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 *et al.*, 2002) and in addition expression of the *pvdS* gene is repressed in iron-rich cells (Cunliffe *et al.*, 1995; Leoni *et al.*, 1996), providing two levels of control of pyoverdine production.

The structural complexity of pyoverdine$_{PAO}$ 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 *P. aeruginosa* strain PAO1

Table 1. Genes involved in synthesis or transport of pyoverdine in *P. aeruginosa* PAO1

Gene	ORF*	Function
pvdA	PA2386	Ornithine hydroxylase (Visca *et al.*, 1994)
fpvI	PA2387	ECF sigma factor required for expression of *fpvA* (Beare *et al.*, 2003)
fpvR	PA2388	Anti-sigma factor for PvdS and FpvI (Lamont *et al.*, 2002; Beare *et al.*, 2003)
pvdF	PA2396	N^5-Hydroxyornithine transformylase (McMorran *et al.*, 2001)
pvdE	PA2397	ABC transporter (secretion) (McMorran *et al.*, 1996)
fpvA	PA2398	Ferripyoverdine receptor protein (Poole *et al.*, 1993)
pvdD	PA2399	Pyoverdine peptide synthetase (Merriman *et al.*, 1995)
pvdJ	PA2400/1	Pyoverdine peptide synthetase (Lehoux *et al.*, 2000)
pvdI	PA2402	Pyoverdine peptide synthetase (Lehoux *et al.*, 2000)
pvdS	PA2426	ECF iron sigma factor (Cunliffe *et al.*, 1995; Miyazaki *et al.*, 1995)
pvcABCD	PA2254–PA2257	Synthesis of the pyoverdine chromophore (Stintzi *et al.*, 1996, 1999)

*ORF in the *P. aeruginosa* genome sequence (http://www.pseudomonas.com; http://pseudomonas.bit.uq.edu.au).

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(Stover *et al.*, 2000) provides a new approach for identifying pyoverdine synthesis genes. In this study we identified possible pyoverdine synthesis genes in the genome sequence, mutated them, and determined the effects of the mutations on pyoverdine production. In addition, we determined which genes are present in other pseudomonads and which are restricted to strains that, like *P. aeruginosa* PAO1, make type I pyoverdine.

METHODS

Bacterial strains and growth conditions. Bacterial strains and 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* PAO1 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

Table 2. Bacterial strains and plasmids

Strain/plasmid	Description	Reference
Plasmids		
pGEM-T-Easy	Ap^R; DNA cloning vector	Promega
pEX18Tc	Tc^R; gene replacement vector	Hoang *et al.* (1998)
pEX18Gm	Gm^R; gene replacement vector	Hoang *et al.* (1998)
pUC4KINN	Ap^R Km^R; kanamycin-resistance cassette flanked by multiple restriction sites	Barany (1988)
pUC4KISS	Ap^R Km^R; kanamycin-resistance cassette flanked by multiple restriction sites	Barany (1988)
pUC18-19Km	Ap^R Km^R; kanamycin-resistance cassette flanked by multiple restriction sites	Markie *et al.* (1986)
E. coli		
MC1061	*hsdR araD139* Δ(*araABC–leu*)7679 Δ(*lac*)X74 *galU galK rpsL thi*	Casabadan & Cohen (1980)
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
P. aeruginosa		
PAO1	Pvd^+; makes type I pyoverdine	Holloway (1955); Stover *et al.* (2000)
PAO1*pvdQ*::*pEXGm*	Pvd^-; insertion of pEX18Gm in PA2385	This work
PAO1*pa2389*::*kan*	Pvd^+; insertion of Km^R cassette in PA2389	This work
PAO1*pvdP*::*pEXGm*	Pvd^-; insertion of pEX18Gm in PA2392	This work
PAO1*pvdM*::*kan*	Pvd^-; insertion of Km^R cassette in PA2393	This work
PAO1*pvdN*::*kan*	Pvd^-; insertion of Km^R cassette in PA2394	This work
PAO1*pvdO*::*kan*	Pvd^-; insertion of Km^R cassette in PA2395	This work
PAO1*pvdJ*::*kan*	Pvd^-; insertion of Km^R cassette in PA2400/1	This work
PAO1*pvdI*::*kan*	Pvd^-; insertion of Km^R cassette in PA2402	This work
PAO1*pa2411*::*kan*	Pvd^+; insertion of Km^R cassette in PA2411	This work
PAO1*pvdH*::*pEXGm*	Pvd^-; insertion of pEX18Gm in PA2413	This work
PAO1*pvdL*::*pEXGm*	Pvd^-; insertion of pEX18Gm in PA2424	This work
PAO1*pvdG*::*kan*	Pvd^-; insertion of Km^R cassette in PA2425	This work
Pa4	Pvd^+; makes type II pyoverdine	Meyer *et al.* (1997)
Pa6	Pvd^+; makes type III pyoverdine	Meyer *et al.* (1997)
58.35	Pvd^+; makes type II pyoverdine	Meyer *et al.* (1997)
58.36	Pvd^+; makes type III pyoverdine	Meyer *et al.* (1997)
58.40	Pvd^+; makes type I pyoverdine	Meyer *et al.* (1997)
59.20	Pvd^+; makes type III pyoverdine	Meyer *et al.* (1997)
59.40	Pvd^+; makes type II pyoverdine	Meyer *et al.* (1997)
59.41	Pvd^+; makes type I pyoverdine	Meyer *et al.* (1997)

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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 *Hin*dIII and *Eco*RI) corresponding to sites that were incorporated into the PCR primers, and subcloned into pEX18Tc or pEX18Gm. Kanamycin-resistance 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 heterodiploid 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).

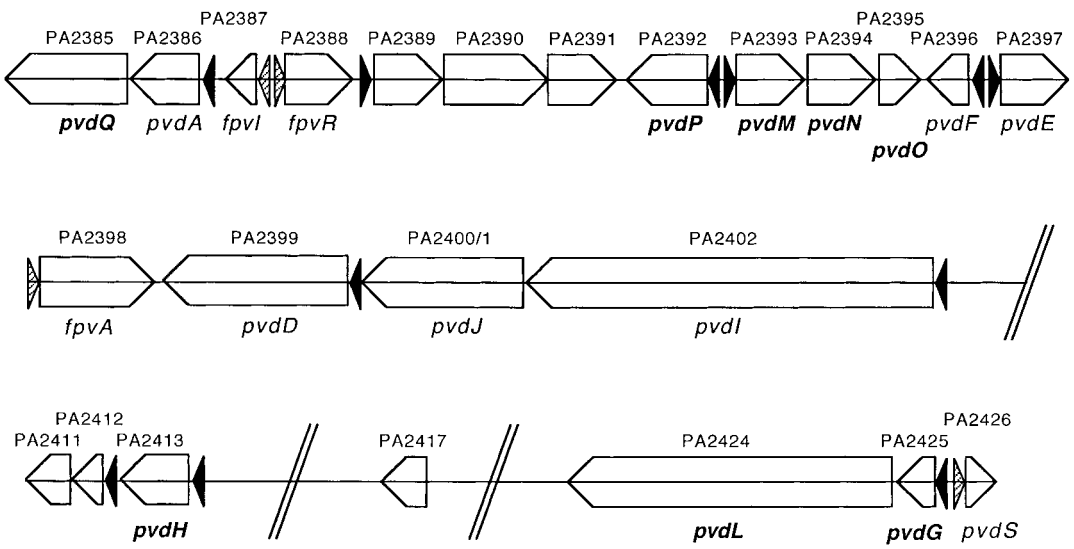

Fig. 2. The pyoverdine locus of *P. aeruginosa* PAO1. The orientations of ORFs are shown, with numbers corresponding to those in the *P. aeruginosa* genome (http://www.pseudomonas.com; http://pseudomonas.bit.uq.edu.au). Gene names are also shown, with genes that were identified in this study in bold. The positions of PvdS-dependent promoters, and likely promoters, are indicated by black arrowheads and other known promoters are indicated by hatched arrowheads.

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Table 3. Putative pyoverdine synthesis/secretion genes analysed in this study

ORF	Gene	PvdS-dependent promoter*	Function/homologous genes†	Phenotype of mutant strain‡	Phenotype of Ochsner *et al.* (2002)§
PA2385	*pvdQ*	No; may be operonic with *pvdA* (PA2386)	38 % identity with aculeacin A acylase from *Actinoplanes utahensis* (Inokoshi *et al.*, 1992)	Pvd$^-$	Pvd$^-$
PA2389		Yes	Over 30 % identity with periplasmic MFPs of RND/MFP/OMF-type efflux systems (Poole, 2001; Zgurskaya & Nikaido, 2000)	Pvd$^+$	Pvd$^+$
PA2390		Operonic with PA2389/PA2391	Over 40 % identity with RND-type transporter components of RND/MFP/OMF-type efflux systems (Poole, 2001; Zgurskaya & Nikaido, 2000)	ND	Pvd$^+$
PA2391		Operonic with PA2390/PA2391	Over 30 % identity with OMF proteins of RND/MFP/OMF-type efflux systems (Poole, 2001; Zgurskaya & Nikaido, 2000)	ND	Pvd$^+$
PA2392	*pvdP*	Yes	No known function	Pvd$^-$	Pvd$^-$
PA2393	*pvdM*	Yes	23 % identity with porcine dipeptidase (Rached *et al.*, 1990)	Pvd$^-$	Pvd$^-$
PA2394	*pvdN*	Operonic with *pvdM/O*	26 % identity with isopenicillin N epimerase from *Streptomyces clavuligerus* (Kovacevic *et al.*, 1990)	Pvd$^-$	Pvd$^-$
PA2395	*pvdO*	Operonic with *pvdM/N*	No known function	Pvd$^-$	Pvd$^-$
PA2411		Operonic with PA2412	36 % identity with thioesterase GrsT from *Bacillus brevis* (Kratzschmar *et al.*, 1989)	Pvd$^+$	Pvd$^+$
PA2412		Yes	No known function	ND	Pvd$^-$
PA2413	*pvdH*	Yes	55 % identity with diaminobutyrate ketoglutarate aminotransferase from *Acinetobacter baumannii* (Ikai & Yamamoto, 1997)	Pvd$^-$	Pvd$^-$
PA2417		No	30 % identity with PtxR transcriptional regulator (PA2258) from *P. aeruginosa* (Hamood *et al.*, 1996)	Pvd$^+$	ND
PA2424	*pvdL*	Operonic with PA2425	33 % identity with TycB peptide synthetase from *Bacillus brevis* (Mootz & Marahiel, 1997)	Pvd$^-$	Pvd$^-$
PA2425	*pvdG*	Yes	34 % identity with GrsT thioesterase from *Bacillus brevis* (Kratzschmar *et al.*, 1989)	Pvd$^-$	ND

*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-N_{16}-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.

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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 ferri-pyoverdine 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 required 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 mutated 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-sorbosone 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*,

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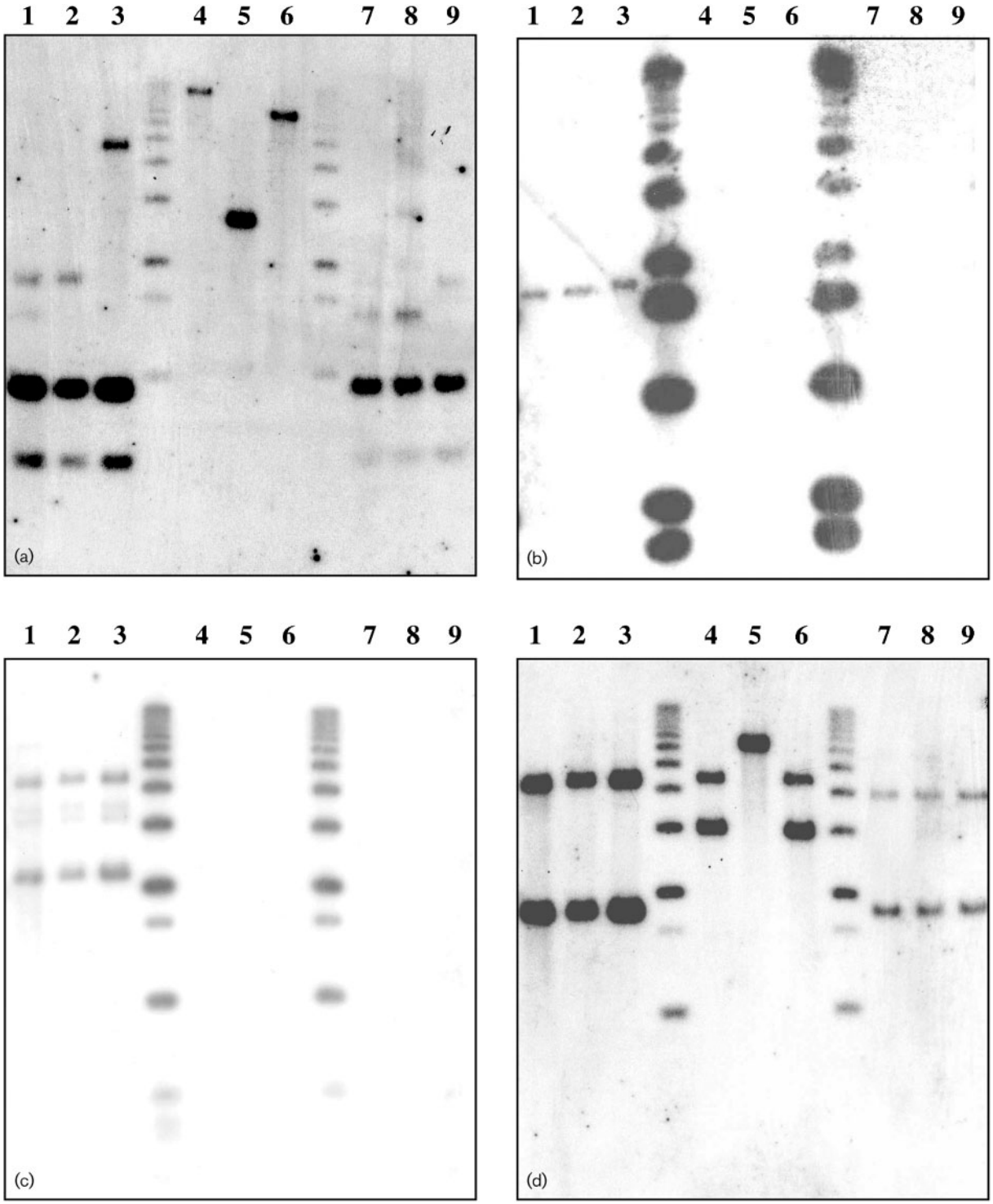

Fig. 3. Detection of *pvd* gene homologues by Southern blotting. Chromosomal DNA from *P. aeruginosa* strains producing type I pyoverdine (PAO1, 58.40 and 59.41) (lanes 1–3), type II pyoverdine (strains Pa4, 58.35 and 59.40) (lanes 4–6) and type III pyoverdine (strains Pa6, 58.36 and 59.20) (lanes 7–9) 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) *pvdN*/*pvdO*.

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Table 4. Presence of homologous genes in other fluorescent pseudomonads

ORF	Gene	*P. aeruginosa**			*P. fluorescens*†	*P. putida*†	*P. syringae*†
		Type I	Type II	Type III			
PA2385	*pvdQ*	+	+	+	56	54	53
PA2386	*pvdA*	+	+	+	74	63	33
PA2387	*fpvI*	ND	ND	ND	67	68	38
PA2388	*fpvR*	ND	ND	ND	50	55	51
PA2389		+	+	+	74	70	70
PA2390		ND	ND	ND	79	74	76
PA2391		ND	ND	ND	64	59	60
PA2392	*pvdP*	+	+	+	64	62	62
PA2393	*pvdM*	+	+	+	73	68	74
PA2394	*pvdN* }	+	+	+	60	63	62
PA2395	*pvdO* }				68	72	68
PA2396	*pvdF*	+	−	−	NS	NS	NS
PA2397	*pvdE*	+	−	−	67	67	55
PA2398	*fpvA*	ND	ND	ND	37	40	36
PA2399	*pvdD*	+	−	−	50	50	56
PA2400/1	*pvdJ*	+	−	−	52	49	51
PA2402	*pvdI*	+	−	−	50	41	42
PA2411		+	+	+	62	65	57
PA2412		ND	ND	ND	83	88	83
PA2413	*pvdH*	+	+	+	79	78	77
PA2417		ND	ND	ND	48	52	44
PA2424	*pvdL*	+	+	+	75	70	72
PA2425	*pvdG*	+	+	+	47	47	50
PA2426	*pvdS*	+	+	+	90	85	84

*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.

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.

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 $\text{pyoverdine}_{\text{PAO}}$ (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

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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* PAO1, 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 pyoverdine 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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