Pyoverdines are siderophores that are secreted by fluorescent pseudomonads and are efficient iron-scavenging compounds (6, 19). Over 50 pyoverdines are known, and all of these contain a dihydroxyquinoline-type chromophore; this is attached to a strain-specific peptide that contains unusual amino acids, such as D-isomers and amino acids that are not usually found in biomolecules, and an acyl group that varies depending on the growth conditions (Fig. 1). Ferri-pyoverdine complexes are recognized by receptor proteins located at the surfaces of the cells, and the iron is taken up by the bacteria in an energy-dependent process (reviewed in reference 27). Pyoverdines contribute to the ability of P. aeruginosa to cause infection (17, 32).

Strains of P. aeruginosa secrete one of three pyoverdine siderophores (types I to III) (18). Genes and enzymes required for pyoverdine synthesis in strain PAO, which secretes type I pyoverdine, have been characterized experimentally (1, 4, 12, 15, 16, 21, 23, 31, 34, 35). This has revealed a biosynthetic pathway in which a pyoverdine precursor is assembled by nonribosomal peptide synthetases (NRPSs), with other enzymes providing the unusual amino acid substrates for the NRPSs and modifying the synthetases (NRPSs), with other enzymes providing the unusual amino acid substrates for the NRPSs and modifying the synthetase (NRPSs) (types I to III) (18). Genes and enzymes required for pyoverdine synthesis (see below) and analysis revealed the presence of the pvdYII gene flank ed by the pvdS and pvdX genes (Fig. 2), as is found in other strains that produce type II pyoverdine. Alignment of the pvdS and pvdX gene sequences shows a very high (>99%) degree of nucleotide sequence identity (Fig. 2 and data not shown), as has been found for other genes that are present in multiple strains of P. aeruginosa (10, 30). The amount of sequence similarity changes abruptly (at the end of pvdX) or slightly more gradually (towards the end of pvdS) (Fig. 2) so that there is no significant sequence similarity in the intergenic regions or between the pvdY genes. The genetic mechanisms that led to pvdY replacing (or being replaced by) pvdYII during the evolution of P. aeruginosa genomes are not clear.

The similarity of pvdYII to siderophore synthesis genes (see below) and its linkage to pvdS raised the possibility that pvdYII is required for pyoverdine synthesis by Pa4. This hypothesis was tested by engineering a Pa4 mutant strain in which the wild-type allele was replaced by a mutant allele. The XbaI-HindIII restriction fragment containing pvdYII was excised from the pGEM-T Easy clone and subcloned into pEX18Gm (8) using standard methods (26). A promoterless lacZ gene

Characterization of a Gene Encoding an Acetylase Required for Pyoverdine Synthesis in Pseudomonas aeruginosa

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Strains of Pseudomonas aeruginosa secrete one of three pyoverdine siderophores (types I to III). We have characterized a gene, pvdYII (for the pvdY gene present in type II P. aeruginosa strains), that is only present in strains that make type II pyoverdine. A mutation in pvdYII prevented pyoverdine synthesis. Bioinformatic, genetic, and biochemical approaches indicate that the PvdYII enzyme catalyzes acetylation of hydroxynor- thine. Expression of pvdYII is repressed by the presence of iron and upregulated by the presence of type II pyoverdine. Characterization of pvdYII provides insights into the molecular basis for production of different pyoverdines by different strains of P. aeruginosa.

Pyoverdines are siderophores that are secreted by fluorescent pseudomonads and are efficient iron-scavenging compounds (6, 19). Over 50 pyoverdines are known, and all of these contain a dihydroxyquinoline-type chromophore; this is attached to a strain-specific peptide that contains unusual amino acids, such as D-isomers and amino acids that are not usually found in biomolecules, and an acyl group that varies depending on the growth conditions (Fig. 1). Ferri-pyoverdine complexes are recognized by receptor proteins located at the surfaces of the cells, and the iron is taken up by the bacteria in an energy-dependent process (reviewed in reference 27). Pyoverdines contribute to the ability of P. aeruginosa to cause infection (17, 32).

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In strain PAO, the pvdS gene is adjacent to a gene, pvdY, that is of unknown function, although a mutation in pvdY resulted in reduced pyoverdine synthesis (23). pvdY is also adjacent to pvdX (Fig. 2), a gene of unknown function. The same arrangement of genes has been demonstrated in strains of P. aeruginosa that make other pyoverdines (29). In strains that make type II pyoverdine, the pvdY gene (unlike the pvdS and pvdX genes) has very little sequence similarity to the pvdY gene in strains that make type I or type III pyoverdines, and it is not alignable by pairwise alignment (29) (L. W. Martin, M. Wallace, and I. L. Lamont, unpublished data).

The pvdY gene present in type II strains is referred to here as pvdYII. The aim of this research was to investigate the function of pvdYII in P. aeruginosa strain Pa4 that makes type II pyoverdine (18). We first amplified a DNA fragment carrying the gene from genomic DNA from P. aeruginosa strain Pa4 by PCR using suitable primers (5'-CCCTCTAGACAGGAA CTG GCC GTC TCT CG-3' and 5'-GGAAGCCTCTGAAGCTG ATCCACACCTC T-3', with introduced XbaI and HindIII restriction sites shown in boldface). This gave rise to a product of about 1.5 kb that was cloned into pGEM-T Easy (Promega) using the manufacturer's protocol. DNA sequencing (Allan Wilson Centre Genome Service, Palmerston North, New Zealand) and analysis revealed the presence of the pvdYII gene flanked by the pvdS and pvdX genes (Fig. 2), as is found in other strains that produce type II pyoverdine. Alignment of the pvdS and pvdX gene sequences shows a very high (>99%) degree of nucleotide sequence identity (Fig. 2 and data not shown), as has been found for other genes that are present in multiple strains of P. aeruginosa (10, 30). The amount of sequence similarity changes abruptly (at the end of pvdX) or slightly more gradually (towards the end of pvdS) (Fig. 2) so that there is no significant sequence similarity in the intergenic regions or between the pvdY genes. The genetic mechanisms that led to pvdY replacing (or being replaced by) pvdYII during the evolution of P. aeruginosa genomes are not clear.

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cassette was excised from pZ1918 (28) using SphI and was cloned into the unique SphI site in pvdYII in the pEXGm plasmid; two alleles were obtained, with lacZ in the sense (pvdYII::lacZ) or antisense (pvdYII::Zcal) orientation. Wild-type pvdYII in strain Pa4 was then replaced with the mutant alleles using methods described elsewhere (8). Replacement of the wild-type gene by the mutant alleles was confirmed by Southern blotting (data not shown).

The resulting pvdYII mutants failed to make detectable pyoverdine and were unable to grow in the presence of the iron-chelating compound ethylenediamine(ortho-hydroxy)phenylacetic acid (EDDHA) that inhibits growth of Pvd- mutants (12) (Table 1), showing that pvdYII is indeed required for pyoverdine synthesis by Pa4. The wild-type pvdYII gene was subcloned as a HindIII-XbaI fragment from pGEM-T Easy into the miniCTX2 vector and then integrated into the chromosome of the Pa4 pvdYII::lacZ mutant strain as described previously (9). This restored the ability of the bacteria to make pyoverdine and to grow on medium containing EDDHA (Table 1), confirming that the inability of the mutant to synthesize pyoverdine was due to the absence of functional pvdYII.

Expression of pyoverdine synthesis genes in P. aeruginosa strain PAO is dependent on the alternative sigma factor PvdS (13, 20), and it is likely that the same is true in other strains of P. aeruginosa. The pvdYII open reading frame in strain Pa4 is preceded by a DNA sequence (TAAAT-N16-CGT) that is present in PvdS-dependent promoters (23, 36, 37), so that it is very likely that expression of pvdYII is dependent at least in part on PvdS. The Pa4 pvdYII mutant contains a pvdYII::Zcal reporter gene fusion, and this was used to investigate gene expression using the same methods as those described previously (11, 37). In three independent experiments, bacteria grown in Kings B broth gave 3,137 U of β-galactosidase (standard deviation [SD], 306 U). Assays with the Pa4 pvdYII::lacZ strain, in which the lacZ reporter gene is in the antisense orientation to pvdYII, gave 29 U of β-galactosidase, consistent with lacZ expression in the Pa4 pvdYII::lacZ strain being dependent on the pvdYII promoter. Growth of the Pa4 pvdYII::Zcal strain in Kings B broth containing FeCl₃ (60 μg ml⁻¹) gave 568 U (SD, 88 U), showing that iron results in repression of pvdYII gene expression in strain Pa4. This reflects Pvd gene expression in strain PAO which is strongly down-

FIG. 1. Type I and II pyoverdines. (A) Type I pyoverdine (5). (B) Type II pyoverdine (33). Chr, dihydroxyquinoline-type chromophoric group; L-(CHO)OHOrn, 6-N-formyl-6-N-hydroxyornithine; L-cHOOrn, N-hydroxy-cyclo-ornithine; R, succinyl, succinamide, or α-ketoglutaryl residue. Figure adapted from reference 18, with permission.

FIG. 2. Organization of pvdXYS genes. The organization of the pvdX, pvdY, and pvdS genes from P. aeruginosa strain Pa4 (top) and strain PAO (bottom) is shown. The genes are not drawn to scale; the pvdY protein-coding sequence is 468 bp, and the pvdYII protein-coding sequence is 912 bp. The stop codons for pvdX and pvdS are in bold.
regulated in iron-replete cells due to the Fur protein that represses expression of pvdS (22).

In P. aeruginosa strain PAO, PvdS-dependent gene expression is lower in Pvd- mutants than in wild-type bacteria and can be increased to wild-type levels by the addition of pyoverdine (11). This is due to a pyoverdine-responsive transmembrane regulatory system that controls the activity of PvdS. The addition of type II pyoverdine to Pa4 pvdY::lacZ bacteria growing in Kings B broth resulted in 5,861 U of β-galactosidase (SD, 1,001 U), an increase in pvdY::lacZ gene expression relative to bacteria without added pyoverdine. This is consistent with the existence of a pyoverdine-inducible signaling pathway, although the increase in gene expression was less than twofold. This is less than that seen with the pvdF gene in strain PAO, where the increase in expression is about fivefold (11), or with the pslA pyoverdine biosynthesis gene in P. fluorescens strain B10 (3), although it should be noted that expression of the pvdA gene in P. aeruginosa PAO1 did not show increased expression when pyoverdine was added (3).

The predicted product of pvdYII is a 39-kDa protein. BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) showed that this protein has highest sequence similarities with acetyl transferases that are involved in siderophore synthesis. These include IucB that is required for aerobactin synthesis in Escherichia coli (7) (41% amino acid identity), RhdB, a putative acetyltransferase involved in rhizobactin synthesis in Sinorhizobium meliloti (14) (52% amino acid identity), and an acetylase that is involved in siderophore synthesis in P. fluorescens (21) (69% amino acid identity). IucB and the P. fluorescens enzyme have been shown to catalyze acetylation of hydroxylation groups to form iron-binding hydroxamate end groups.

The pyoverdine synthesized by strain Pa4 does not contain acetyl hydroxamate groups but contains a cyclic hydroxamate (Fig. 1). We hypothesized that pvdYII catalyzes acetylation of N-hydroxyornithine as part of the process of synthesis of this hydroxamate.

Type I pyoverdine contains two formyl hydroxamate groups, and synthesis of these is catalyzed by the PvdF enzyme, which formylates a hydroxornithine precursor (15). As PvdF and pvdYII may catalyze synthesis of different hydroxamate groups (formyl- or acetyl-hydroxamate, respectively), we tested the possibility that pvdF from strain PAO could substitute for pvdYII from strain Pa4 and vice versa. A 1.5-kb DNA fragment containing the pvdF gene from P. aeruginosa PAO was amplified by PCR using primers flanking the gene (5’-GGCTTCTAG ATCATATGTTCTCCCAGA, with an introduced XbaI site shown in boldface, and 5’-ACGGCAACGTCTACTCGAG-3′), cloned into pGEM-T Easy, and then subcloned into the mini-CTX2 vector. This construct was then integrated into the chromosomes of PAO pvdF and Pa4 pvdYII mutants strains, and the mini-ctx::pvdYII construct was integrated into the chromosome of the PAO pvdF mutant. The resulting strains were tested for the ability to synthesize pyoverdine (Table 1). The ctx::pvdF construct restored the ability of the PAO pvdF mutant to make pyoverdine, but the ctx::pvdF construct did not enable synthesis of pyoverdine by the Pa4 pvdYII mutant.

Pyoverdine produced by the PAO pvdF::lacZ strain, as well as that made by PAO pvdF::pvdF and Pa4 pvdYII::pvdF, was purified as described previously (18). The pyoverdines were analyzed at the Protein Microchemistry Facility of the University of Otago by matrix-assisted laser desorption ionization–time of flight mass spectrometry (average of at least five runs) using a Thermo Finnigan LCQ Deca 2000 in conjunction with an alpha-cyano-4-hydroxycinnamic acid matrix and the internal calibrants Bradycinin (1,060.2 Da) and Renin substrate (1,759.0 Da); in addition, for the pyoverdine produced by the PAO pvdF::pvdF strain, electrospray mass spectrometry was performed using a Thermo Finnigan LCQ Deca. The pyoverdine produced by PAO pvdF::pvdF was 1,336 Da, and that produced by Pa4 pvdYII::pvdF was 1,094 Da. These are the same as type I and II pyoverdines (5, 33), showing that complementation of the genes resulted in synthesis of wild-type pyoverdines. The PAO pvdF::pvdF strain had a mass of 1,364 Da by mass spectrometry. This is consistent with a form of type I pyoverdine in which the formyl pvdYII residues have been replaced by acetyl hydroxynornithine, which would increase the Mr by 28. This pyoverdine was also analyzed by electrospray mass spectrometry, which gave an Mr of 1,362.5.

Collectively, these data provide strong evidence that pvdYII is required for synthesis of type II pyoverdine by P. aeruginosa strain Pa4 and that it catalyzes acetylation of hydroxyornithine. The pvdYII gene is restricted to strains of P. aeruginosa that make type II pyoverdine (29), and its characterization advances our understanding of the molecular basis for production of different pyoverdines by different strains of P. aeruginosa. Type II pyoverdine does not contain acetyl hydroxynornithine but does contain a terminal cyclized hydroxynornithine (Fig. 1). Our current model is that acetyl hydroxynornithine is incorporated into a peptide precursor of type II pyoverdine by the relevant NRPS, with the acetyl group being removed during release of the peptide from the NRPS and concomitant cyclization of the hydroxynornithine. Alternatively, the substrate of the NRPS may be ornithine itself, with pvdYII catalyzing its acetylation following incorporation into the precursor peptide but prior to the release of the peptide from the NRPS. In either case, absence of acetyl hydroxynornithine.

| Strain       | Pyoverdine synthesis | Growth with EDDHAa |
|--------------|----------------------|---------------------|
| PAO          | ++                   | +                   |
| PAO pvdF     |                      |                     |
| PAO pvdF::pvdF | ++                  | ++                  |
| PAO pvdF::pvdYII | ++               | ++                   |
| Pa4          | ++                   | ++                   |
| Pa4 pvdYII::lacZ |                | + NDb                |
| Pa4 pvdYII::Zcad |     |                      |
| Pa4 pvdYII::pvdF |                |                     |
| Pa4 pvdYII::pvdYII |             | ++                   |

a Pyoverdine synthesis (as indicated by production of a fluorescent yellow-green pigment on Kings B agar and by the presence of a characteristic absorbance spectrum in Kings B broth) was detected as described previously (12, 15). ++, large amounts of pyoverdine were synthesized; +, significant amounts of pyoverdine were synthesized; –, no detectable pyoverdine was synthesized.

b Bacterial growth was recorded after growth at 37°C on Kings B agar containing the iron-chelating compound ethylenediamine(–hydroxy)phenylacetic acid (EDDHA) (200 μg ml–1). ++, good growth after 24 h of incubation; +, no detectable growth after 24 h of incubation; –, no growth detected.

c ND, not determined.

TABLE 1. Synthesis of pyoverdines by strains of P. aeruginosa used in this study

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thine is likely to preclude the cyclization of the terminal hydroxyornithine derivative and release of the precursor peptide from the NRPS. A requirement for acetyl hydroxyornithine prior to cyclization of the ornithine residue would explain the observation that $pdvY_{11}$ could complement a $pdvF$ mutation in $P. aeruginosa$ PAO but $pdvF$ could not complement a $pdvY_{11}$ mutation.

**Nucleotide sequence accession numbers.** The Pa4 $pdvY_{11}$ sequence reported here has been assigned the GenBank accession number DQ328792.

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