

# Characterization and Genetic Manipulation of Peptide Synthetases in *Pseudomonas aeruginosa* PAO1 in Order to Generate Novel Pyoverdines

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## Summary

**PvdD**, a nonribosomal peptide synthetase (NRPS) of *Pseudomonas aeruginosa* PAO1, incorporates two L-threonines into the siderophore pyoverdine. A *pvdD* mutant did not synthesize pyoverdine and lacked a high Mr iron-regulated cytoplasmic protein (IRCP). Analysis of other IRCPs and the *P. aeruginosa* genome enabled the remaining pyoverdine NRPSs to be identified. The *pvdD* mutation could be complemented in *trans*, enabling design of plasmid-based systems for the generation of novel pyoverdines. Introduction of a truncated *pvdD* gene resulted in attenuated forms of pyoverdine, and introduction of L-threonine-incorporating NRPSs from other organisms restored pyoverdine production to mutant cells. This is the first successful rational in vivo modification of NRPS modules outside of *Bacillus subtilis*. The systems employed did not allow incorporation of other residues into pyoverdine, indicating that there are multiple elements contributing toward substrate specificity in NRPSs.

## Introduction

Fluorescent pseudomonads survive iron limitation by secreting pyoverdines, mixed hydroxamate-catecholate siderophores that sequester iron from the environment and are then taken up by the bacteria by specific receptors [1, 2]. The pyoverdine secreted by *P. aeruginosa* strain PAO1 can also act as a signaling molecule that regulates gene expression in the bacteria [3]. A large number of pyoverdines have been characterized, and all consist of an invariant chromophore joined to an acyl (carboxylic acid or amide) group and a short (6 to 12 amino acid) type-specific peptide (reviewed in [4]). The pyoverdine secreted by *P. aeruginosa* PAO1 is shown in Figure 1A.

The pyoverdine peptide and chromophore are thought to be derived from amino acid precursors that are assembled by nonribosomal peptide synthetases (NRPSs) with other enzymes catalyzing additional reactions to complete the mature pyoverdines [5–10]. NRPSs are multimodular enzymes, with each module governing the insertion of a single amino acid into the peptide product. This process is defined by the multiple carrier model [11], which describes a coordinated stepwise series of amino- to carboxyl-terminal transpeptidation reactions.

Domains within each NRPS module provide active sites for the recognition and adenylation activation of a substrate amino acid (adenylation, or A, domains); transfer of this activated intermediate to a phosphopantetheinyl cofactor (bound by a thiolation, or T, domain); and peptide bond formation (catalysed by a condensation, or C, domain), so that the activated residue is incorporated into the growing peptide chain. Usually, the final module in a multienzyme complex also has a thioesterase (TE) domain that cleaves the nascent peptide from this terminal NRPS and may also be required for catalysis of internal cyclization reactions [12]. We have characterized a pyoverdine peptide synthetase from *P. aeruginosa* PAO1, PvdD, that is a bimodular enzyme containing each of these domain types [5]. PvdD catalyses incorporation of two L-threonine residues at the C terminus of the pyoverdine peptide (Figure 1B). Other pyoverdine peptide synthetases have been characterized using mutational and bioinformatic approaches [6–9].

The semiautonomous activity of individual domains within a module and of individual modules within a multienzyme complex offers potential for the rational alteration of peptide products by manipulation of NRPSs. Of particular interest is the potential for engineering novel antibiotics and other secondary metabolites with industrial applications. To date, attempts to synthesize novel compounds by NRPS domain or module swapping have met with only limited success. Novel AT domain regions have been introduced into the seventh and second modules, respectively, of the *Bacillus subtilis* surfactin synthetase NRPS multienzyme complex [13, 14]. These experiments resulted in the synthesis of novel surfactins that contained the anticipated single residue alterations. However, surfactin biosynthesis was significantly reduced relative to wild-type. Much higher levels of recombinant surfactin production were obtained when a module from the lichenysin synthetases of *Bacillus licheniformis* was introduced into highly homologous modules of the *B. subtilis* surfactin synthetase complex [15]. The novel multienzyme complex had a Glu-Gln alteration in substrate specificity and generated the predicted novel surfactin product. Lichenysin and surfactin are structurally similar, and this may have contributed to the success of this module swapping experiment.

Other rationally modified surfactins have been obtained by relocation of the TE domain [16, 17] and by module deletion [18, 19]. However, to date, rational modification of NRPS modules in vivo has been confined to a single model system: the surfactin synthetases of *B. subtilis*. In this study, we describe manipulation of the *pvdD* gene of *P. aeruginosa* in order to generate novel pyoverdines.

## Results

### Generation and Characterization of a *P. aeruginosa* PAO1 *pvdD* Mutant

A *pvdD* deletion mutant was generated in which the wild-type gene was replaced with a mutant copy lacking

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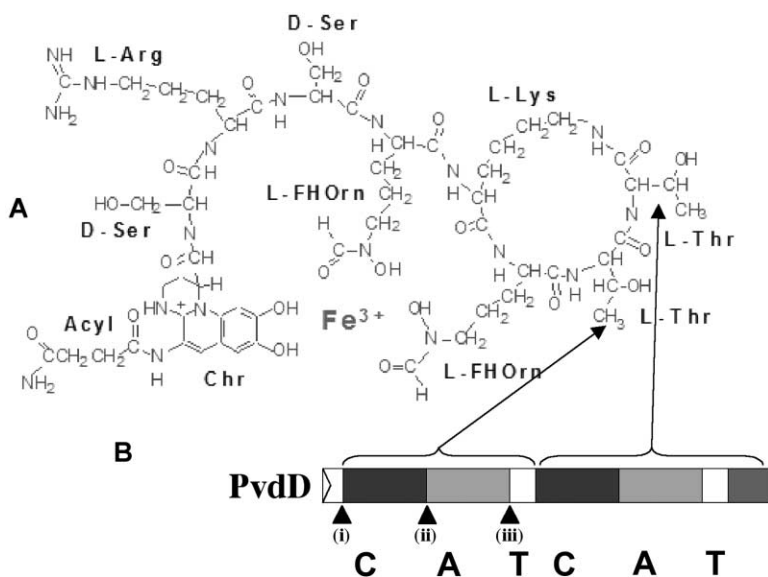


Figure 1. Pyoverdine and PvdD

(A) Pyoverdine Pa from *P. aeruginosa* PAO1 complexed with ferric iron [44]. L-FHOrn, L-N<sup>5</sup>-formyl-N<sup>6</sup>-hydroxyornithine; D-Ser, D-serine; L-arg, L-arginine; L-lys, L-lysine; L-thr, L-threonine; Chr, chromophore.

(B) The domain structure of PvdD. The modules are defined by the CAT domain repeats, and the second module also contains a thioesterase domain. The arrows indicate the L-Thr residues incorporated by each module. The black triangles indicate insertion points for domain swapping experiments conducted in this study.

6.5 kb of the 7.3 kb gene. The identity of the mutant was confirmed by Southern blotting (data not shown). Unlike wild-type *P. aeruginosa*, the *pvdD* mutant did not secrete pyoverdine on agar (Figure 2A; Table 1) or in liquid culture (Figure 2B). Like other *pvd* mutants [6], but unlike wild-type *P. aeruginosa*, the *pvdD* mutant strain was unable to grow when the iron-chelating compound ethylene-diamine-di(o-hydroxyphenylacetic acid) (EDDA) was included in the growth medium (Table 1). The *pvdD* mutant was able to utilize exogenous pyoverdine to grow on agar containing EDDA, indicating that uptake of ferripyoverdine was not affected (Figure 2C).

#### PvdD Is an IRCP

Large (180–600 kDa) iron-regulated cytoplasmic proteins (IRCPs) that are made by fluorescent pseudomonads have been identified and are proposed to be peptide synthetases that contribute to pyoverdine synthesis [10]. IRCPs were prepared from the *P. aeruginosa pvdD* mutant and wild-type bacteria. IRCP bands of approxi-

mately 550, 480, 290, and 250 kDa were observed for the wild-type as reported previously, but the *pvdD* mutant lacked the protein of approximately 290 kDa (Figure 3). This is consistent with the 273 kDa size of PvdD that was predicted from sequence data [20]. The presence of plasmid pPVDD1 that carries the cloned *pvdD* gene restored the 290 kDa IRCP to the *pvdD* mutant and increased the amounts of this protein in wild-type bacteria (Figure 3), showing that the 290 kDa IRCP is indeed PvdD. These findings confirm that at least one of the IRCPs is a peptide synthetase that is required for pyoverdine synthesis.

The *P. aeruginosa* PAO genome contains two open reading frames, *pvdI* (PA2400) and *pvdJ* (PA2402), immediately upstream of *pvdD* (PA2399), which encode probable peptide synthetases that are required for pyoverdine synthesis [6, 8, 9]. *pvdI* and *pvdJ* encode proteins of 568 kDa and 240 kDa, respectively, that are likely to correspond to the IRCPs of approximately 550 kDa and 250 kDa (Figure 3). On the basis of colinearity (defined

Table 1. Fluorescence and Growth of Modified *P. aeruginosa* Strains

Strain <sup>b</sup>	Gene Expressed	Fluorescence <sup>a</sup>	Growth on KB Agar + EDDA
Wild-type	NA	+++++	yes
<i>pvdD</i> mutant	NA	–	no
<i>pvdD</i> (pPVDD1)	<i>pvdD</i> high copy number	+++	yes
<i>pvdD</i> (pPVDD2)	<i>pvdD</i> low copy number	++++	yes
Wild-type (pPVDD1)	<i>pvdD</i> high copy number	+++	yes
Wild-type (pPVDD2)	<i>pvdD</i> low copy number	++++	yes
<i>pvdD</i> (pMOD1)	<i>pvdD</i> module 1	–	no
<i>pvdD</i> (pMOD2)	<i>pvdD</i> module 2	+++	no
<i>pvdD</i> (pMOD1, pMOD2)	<i>pvdD</i> modules 1 and 2	+++	limited
<i>pvdD</i> (pDAX12)	<i>pvdD</i> A swap control	+++	yes
<i>pvdD</i> (pDAX12::syrB-A)	<i>pvdD</i> + <i>syrB</i> Adom	++	yes
<i>pvdD</i> (pDAX12::snbC-A)	<i>pvdD</i> + <i>snbC</i> Adom	+	yes
<i>pvdD</i> (pDAX13)	<i>pvdD</i> CA swap control	+++	yes
<i>pvdD</i> (other pDAX12/13)	other swap constructs	–	no

<sup>a</sup>The “+” signs indicate fluorescence on King’s B agar following overnight growth; the number of + signs indicates the intensity relative to wild-type, with “+++++” indicating maximal fluorescence and “–” indicating no detectable fluorescence (see Figures 2 and 5).

<sup>b</sup>The bacterial strain (wild-type *P. aeruginosa* PAO1 or *P. aeruginosa pvdD*) and plasmids that are present are listed.

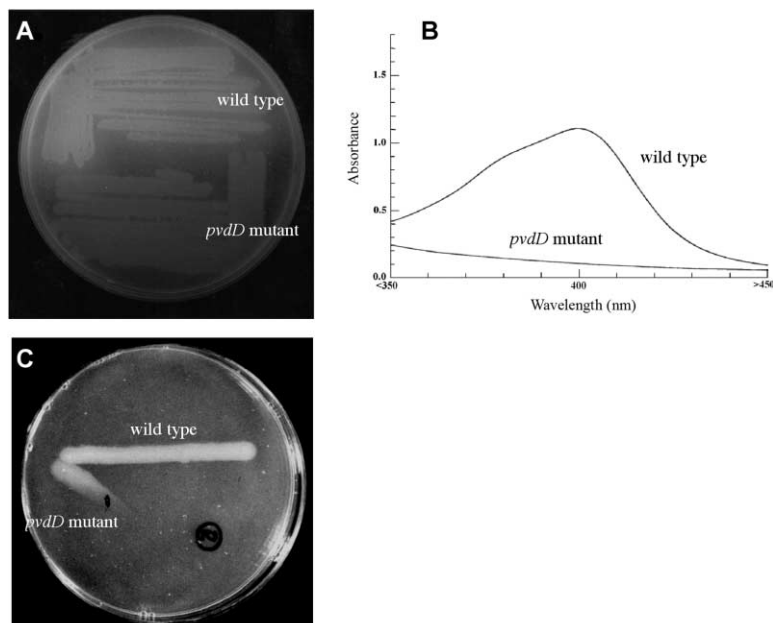


Figure 2. Phenotype of the *pvdD* Mutant  
(A) Wild-type and *pvdD* mutant cells grown on iron-limiting King's B agar, viewed under UV light. The mutant cells lack the characteristic fluorescence conferred by pyoverdine secretion.  
(B) Scanning spectrophotometric traces of wild-type and *pvdD* mutant King's B culture supernatant, as described in Experimental Procedures. The pyoverdine present in the wild-type supernatant has an absorbance maximum of 403 nm.  
(C) *pvdD* mutant cells streaked near wild-type on King's B agar containing the iron-chelating agent EDDA are only able to grow within the pyoverdine-containing zone around these cells, indicating that they are able to utilize, but not synthesize, pyoverdine.

in [21, 22]), the *pvdI* gene is predicted to encode the synthetase modules that incorporate (in order) D-serine, L-arginine, D-serine, and L-N<sup>5</sup>-formyl-N<sup>5</sup>-hydroxyornithine, while the product of the *pvdJ* gene is predicted to direct incorporation of L-lysine and L-N<sup>5</sup>-formyl-N<sup>5</sup>-hydroxyornithine [8] (Figure 3). Consistent with this possibility, sequence analysis shows that the first and the third modules of PvdI have epimerization domains that would convert L-serine to the D-serine that is present in pyoverdine. Furthermore, the A domains of modules that are proposed to activate identical amino acid substrates (modules 1 and 3 of PvdI [serine] and modules 4 of PvdI and 2 of PvdJ [N<sup>5</sup>-formyl-N<sup>5</sup>-hydroxyornithine]) are 98% and 99% identical to one another, respectively.

Substrate binding interactions predicted from tertiary structural models, as previously described for PvdD [5] and PvdL [7], were also consistent with the colinear substrate assignment (data not shown).

A further likely pyoverdine peptide synthetase encoded by the *pvdL* gene (PA2424) is a protein of 479 kDa that is probably the 480 kDa IRCP band (Figure 3). This protein is likely to govern synthesis of the pyoverdine chromophore [7, 8].

#### Complementation of the *pvdD* Mutation

pPVDD1 that carries *pvdD* was tested for complementation of the *pvdD* mutation. *P. aeruginosa pvdD*(pPVDD1) cells were able to synthesize pyoverdine and to grow

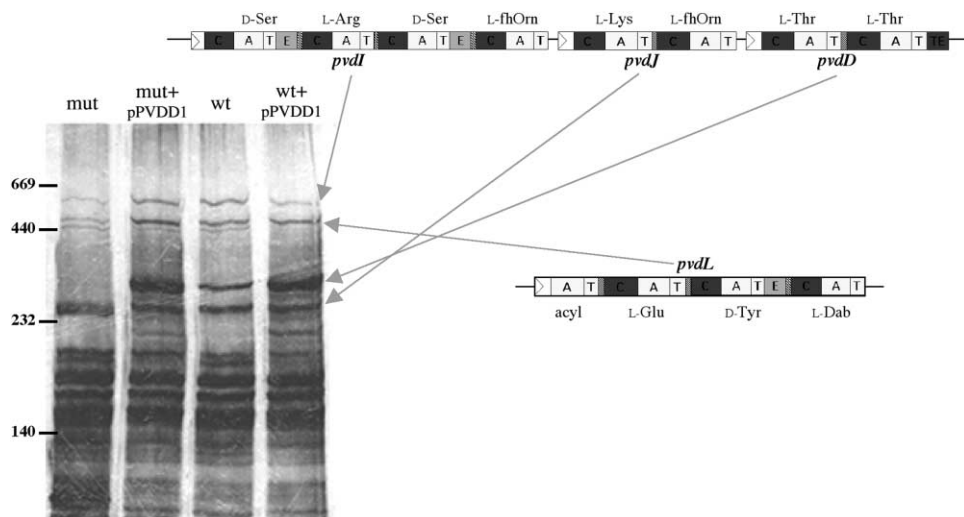


Figure 3. IRCP Band Analysis

The four IRCP bands are indicated by arrows that correlate them with the genes that are thought to encode them. mut, *pvdD* mutant; wt, wild-type PAO1; Ser, serine; Arg, arginine; fhOrn, N<sup>5</sup>-formyl-N<sup>5</sup>-hydroxyornithine; Thr, threonine; Glu, glutamate; Tyr, tyrosine; Dab, 2,4-diaminobutyric acid; acyl, unusual module with high homology to acyl CoA ligases [7]; C, condensation domain; A, adenylation domain; T, thiolation domain; TE, thioesterase domain.

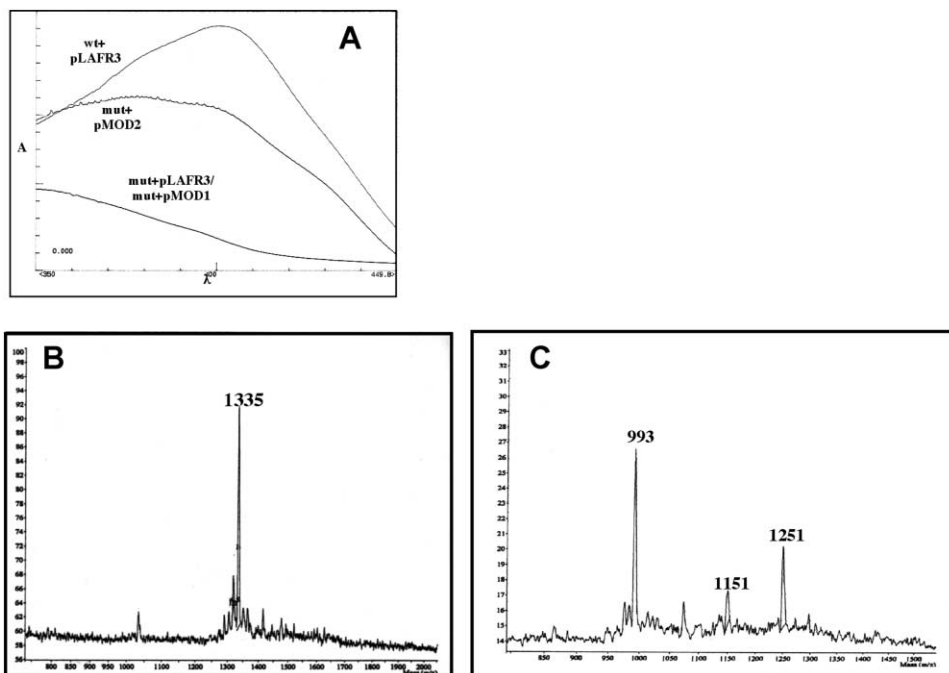


Figure 4. Spectral Analysis of *pvdD*::*pMOD2* Cells

(A) Scanning spectrophotometric analysis. Wt + pLAFR3, *P. aeruginosa*(pLAFR3); mut + pMOD2, *P. aeruginosa pvdD*(pMOD2); mut + pLAFR3, *P. aeruginosa pvdD*(pLAFR3); mut + pMOD1, *P. aeruginosa pvdD*(pMOD1). The last two strains gave identical traces.  
 (B) MALDI-ToF analysis of pyoverdine from *P. aeruginosa* PAO1. The molecular mass of the major peak is indicated.  
 (C) MALDI-ToF analysis of pyoverdine-like compounds from supernatants of *P. aeruginosa pvdD*(pMOD2) cultures. The molecular mass of each peak is indicated.

on agar containing EDDA (Table 1). They were also able to support growth of a pyoverdine-deficient *pvdF* mutant of *P. aeruginosa* in cross-feeding experiments in the presence of EDDA. However, *pvdD*(pPVDD1) cells secreted less pyoverdine than wild-type bacteria (Table 1). Spectral analysis of culture supernatants revealed a pyoverdine concentration of around 150  $\mu$ M for the *pvdD*(pPVDD1) cells, compared with about 400  $\mu$ M for the wild-type.

Supernatants of cultures of wild-type *P. aeruginosa* containing pPVDD1 also contained about 150  $\mu$ M of pyoverdine, indicating that pPVDD1 reduced the amount of pyoverdine made by the cells. This was most likely due to overexpression of *pvdD* (Figure 3) resulting from the high copy number of the pUCP22 vector (20 to 30 per cell [23]). The *pvdD*-containing DNA fragment was subcloned from pPVDD1 into pLAFR3, a broad host range plasmid with a copy number of about three [24]. The resulting plasmid (pPVDD2) resulted in pyoverdine secretion at amounts comparable to wild-type levels (around 300  $\mu$ M; Table 1). These results show that plasmid-borne *pvdD* can complement the *pvdD* mutation, although complementation is less effective at higher gene copy number.

#### Effects of Deleting a *pvdD* Module

We tested the effects of introducing pMOD1 (containing a single module of *pvdD*) and pMOD2 (containing a module equivalent to the second module of *pvdD* and the thioesterase domain) into the *P. aeruginosa pvdD* mutant.

*P. aeruginosa pvdD* carrying pMOD1 did not secrete any pyoverdine-like pigment or fluoresce under UV light, and these bacteria did not grow in the presence of EDDA (Table 1). In contrast, *P. aeruginosa pvdD*(pMOD2) cells did secrete pigment and had some UV fluorescence (Table 1). Spectrophotometric analysis indicated that the pigment secreted by these cells was similar but not identical to wild-type pyoverdine (Figure 4A). *P. aeruginosa pvdD*(pMOD2) cells were unable to grow on agar containing EDDA (Table 1), indicating that the pyoverdine-like pigment that was secreted was not an effective siderophore. The pigment produced by *P. aeruginosa pvdD*(pMOD2) was purified and analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectral analysis, along with wild-type pyoverdine (Figures 4B and 4C). Wild-type pyoverdine has a molecular mass of approximately 1335 Da. The mass of the largest molecular species present in the *P. aeruginosa pvdD*(pMOD2) sample was 1251 Da. Allowing for a 0.1% error, this is consistent with the presence of a pyoverdine that lacks a threonine residue (101 Da) due to the absence of a threonine-incorporating module from the pMOD2 plasmid and can no longer cyclise the internal lysine side chain with the carboxyl terminus, gaining a water equivalent (18 Da).

The *P. aeruginosa pvdD*(pMOD2) sample also contains smaller molecular species (993 and 1151 Da; Figure 4C). The 1151 Da product corresponds to the mass of "pyoverdine" lacking both threonine residues, and the 993 Da product to a "pyoverdine" lacking both threo-

nines and also the L-N<sup>5</sup>-formyl-N<sup>5</sup>-hydroxyornithine residue immediately upstream. Thus, it appears that multiple attenuated species of pyoverdine were present. This conclusion is supported by the results of an HPLC amino acid composition analysis. Wild-type pyoverdine contained the amino acids Ser, Arg, Lys, fhOrn, and Thr in a ratio of approximately 2:1:1:2:2, respectively, and this is consistent with its structure (Figure 1). The products purified from the *P. aeruginosa pvdD*(pMOD2) culture supernatant gave an approximately 2:1:1:1<sup>1/2</sup>:1<sup>1/2</sup> ratio of the same amino acids, consistent with many of the molecules lacking threonine and some of them also lacking L-N<sup>5</sup>-formyl-N<sup>5</sup>-hydroxyornithine.

When pMOD1 and pMOD2 were both present in the *P. aeruginosa pvdD* mutant, the bacteria fluoresced under UV light and grew to a very limited extent on agar containing EDDA (Table 1), indicating that a functional pyoverdine was synthesized. Culture supernatant gave an identical spectral trace to that observed for the *pvdD*(pMOD2) cells (Figure 4A), with elevated absorption evident in the 350–400 nm region (data not shown). The MALDI-ToF spectral trace for pigment purified from *P. aeruginosa pvdD*(pMOD1/pMOD2) cells was similar to that for pigment purified from the *pvdD*(pMOD2) strain (Figure 4C), except that a very faint peak at 1337 Da was evident, and this was consistent with low amounts of wild-type pyoverdine being synthesized by these bacteria (data not shown). These results indicate that in *P. aeruginosa pvdD*(pMOD1/pMOD2) the two modules of PvdD expressed from the different plasmids were capable of associating in vivo to form an active bimodular PvdD synthetase, although levels of pyoverdine production were greatly reduced relative to wild-type.

#### Expression of Heterologous Peptide Synthetase Modules in the *pvdD* Mutant

The results described above indicate that the pyoverdine synthesis and secretion systems are tolerant of quite substantial changes being introduced into the peptide moiety. This suggested that the pyoverdine synthetase system of *P. aeruginosa* might be a good model system for carrying out domain swapping experiments to generate novel peptide products. The initial strategy employed to test this was to generate hybrid *pvdD* genes containing A domain sequences from other peptide synthetase genes and determine whether these hybrid genes enabled production of pyoverdine-like molecules by *pvdD* mutant cells.

The module chosen for alteration was the first of the two L-threonine activating modules of PvdD, as manipulation of the second PvdD module could potentially inhibit the activity of the associated thioesterase. Novel restriction sites were engineered into pPVDD1 (giving plasmid pDAX12) such that they bordered the A1 and A10 motifs that effectively define the peptide synthetase adenylation region ([25]; Figure 1, triangles (ii) and (iii)). The criteria for selecting the locations of these sites were, first, that they be close to but outside the A1 and A10 motifs; second, that introduction of the novel restriction sequences have a minimal impact upon the primary protein sequence (Figure 5A); and finally, that the novel restriction sites not be generated within re-

gions that are likely to be important in the overall tertiary structure of the domain, i.e., outside  $\alpha$  helix or  $\beta$  sheet regions that might be disrupted by the insertion of foreign domain sequences (as predicted from a 3D model based on the solved GrsA structure [5, 25]). These sites were used to excise the native *pvdD* sequence and replace it with DNA corresponding to the threonine-incorporating A domains from *snbC* of *Streptomyces pristinaespiralis* and *syrB* of *Pseudomonas syringae*, a cysteine- and a valine-incorporating A domain from *acvA* of *Penicillium chrysogenum* and a proposed serine-incorporating domain from the *P. aeruginosa pvdI* gene (Table 2). Clustalw alignment was used to ensure that the introduced A domains were equivalent to the excised regions of *pvdD* and would be expressed in-frame (as illustrated for the *snbC* A domain in Figure 5A).

The *P. aeruginosa pvdD* mutant carrying the recombinant plasmids was tested for the production of pyoverdine-like compounds (Table 1). The phenotype of *P. aeruginosa pvdD*(pDAX12) was indistinguishable from *pvdD*(pPVDD1) cells, indicating that the introduced restriction sites had not impaired the function of the *pvdD* gene. The presence of the threonine-incorporating domains from *S. pristinaespiralis* or *P. syringae* also restored pyoverdine production to *pvdD* mutant cells (Table 1; Figures 5B and 5C). The pDAX12::*syrB*-A construct was more effective than pDAX12::*snbC*-A in restoring pyoverdine production, and the levels of pyoverdine production with either construct were significantly lower than with pDAX12 (Figures 5B and 5C). MALDI-ToF mass spectral analysis confirmed that *P. aeruginosa pvdD*(pDAX12::*syrB*-A) produced a compound with the same mass as pyoverdine (Figure 5D). *P. aeruginosa pvdD*(pDAX12::*snbC*-A) cells did not secrete sufficient pyoverdine for MALDI-ToF analysis to be performed.

*P. aeruginosa pvdD* containing pDAX12::*val*-A, pDAX12::*cys*-A, or pDAX12::*ser*-A did not secrete any pyoverdine-like products or grow in the presence of EDDA (Table 1). There is some evidence that C domains contribute to the substrate selectivity of peptide synthetase modules, displaying low selectivity for an incoming “donor” amino acid residue but much greater selectivity for the “acceptor” residue that is activated by the A domain of the same module [26–28]. It was therefore possible that the pDAX12::*val*-A, pDAX12::*cys*-A, and pDAX12::*ser*-A constructs did not give rise to novel pyoverdine products because the PvdD C domain in the hybrid proteins was not compatible with the introduced A domains. To test this hypothesis, a plasmid (pDAX13) was constructed to enable the introduction of heterologous C and A domain units together into PvdD. In pDAX13, an introduced NotI restriction site is present downstream of the A10 motif (as in Figure 5A), and an introduced XbaI site is present just before the C1 condensation motif (as defined in [29]; site [j] in Figure 1). C-A regions were exchanged into pDAX13 from the same genes that were used for the A domain swaps, except for *syrB*, which does not have a C domain.

*P. aeruginosa pvdD* containing pDAX13 was phenotypically identical to *pvdD*(pPVDD1) and *pvdD*(pDAX12) (Table 1). However, none of the recombinant constructs containing foreign CA inserts allowed *P. aeruginosa pvdD* to synthesize discernible levels of fluorescent pig-

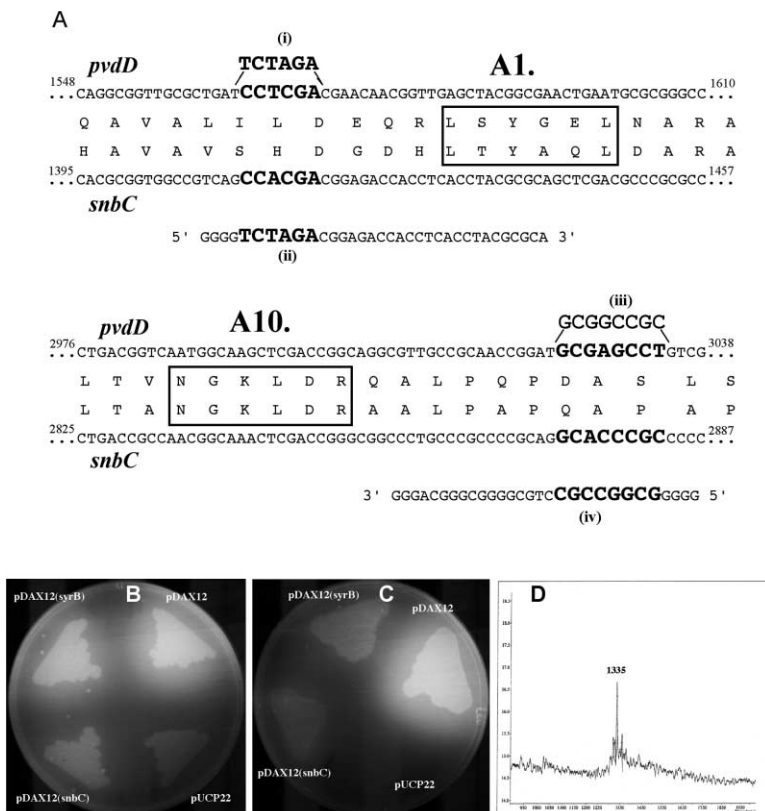


Figure 5. Strategy for Introduction of Novel A Domains

(A) Nucleotide and protein sequence alignments for *pvdD* and *snbC* are shown around the A1 sequence motif and the A10 sequence motif. A novel XbaI site (i) was introduced upstream of the A1 motif (boxed), and a novel NotI site (iii) was created downstream of the A10 motif (boxed) in pDAX12. The equivalent regions of alternative A domains were defined by protein and nucleotide alignment, as indicated for *snbC*. PCR primers were designed to amplify these regions, incorporating the appropriate restriction sequences into the product [as for *snbC* primers SnbCAUp (ii) and SnbCADown (iv)] for cloning into pDAX12.

(B) *P. aeruginosa pvdD* mutant cells containing the plasmid constructs indicated, streaked onto King's B agar and viewed under UV light.

(C) As for (A), but on King's B agar containing the iron-chelating agent EDDA.

(D) MALDI-ToF analysis of pyoverdine purified from *P. aeruginosa pvdD*(pDAX12::SyrB-A) culture supernatant.

ment. Bacteria containing these constructs were unable to grow on agar containing EDDA (Table 1).

## Discussion

The purpose of the research described here was to investigate the suitability of the pyoverdine synthesis system of *P. aeruginosa* PAO1 for manipulation of peptide synthetases and generation of novel products. To this end, we have shown that a mutation in the *pvdD* gene can be complemented by a plasmid-borne form of *pvdD* and by versions of *pvdD* in which part of the *P. aeruginosa* gene was replaced by segments of threonine-incorporating NRPSs from other species. Additionally, the presence of only a single module of *pvdD* resulted in synthesis of a form of pyoverdine from which a threonine residue was absent. Preliminary experiments involving the introduction of peptide synthetase domains that direct the incorporation of alternative amino acids were unsuccessful, and further research will be required to address this issue.

For most peptide synthetases, the order in which modules are encoded in the chromosome corresponds to the order of amino acid residues in the peptide product (the colinearity rule; [21, 22]), and genome analysis indicated that the same is likely to be true for pyoverdine. However, complementation in *trans* with the plasmid-borne *pvdD* gene shows that this genomic organization is not essential for pyoverdine synthesis. The *pvdD* mutation could also be complemented when the two modules of PvdD were encoded by fragments of *pvdD* that were present on different plasmids. This is consistent

with earlier findings that absolute colinearity is not essential for syringomycin synthesis by *P. syringae* [30] or surfactin synthesis by *B. subtilis* [31] and that four independently purified yersiniabactin synthetase proteins of *Yersinia pestis* can reconstitute a complete multienzyme complex in vitro [32]. Colinearity of NRPS-encoding DNA with the peptide products presumably provides a metabolic or evolutionary advantage but is not essential for peptide synthesis.

The *pvdD* mutant and also the mutant containing pMOD1 that encodes a module of PvdD did not secrete any pyoverdine-like products, most likely because these strains lack the TE domain that is essential for release of the nascent pyoverdine from the final NRPS. Likewise, deletion of the surfactin synthetase TE domain prevented *B. subtilis* from secreting surfactin [16]. It has been shown that mutations in TE domains result in the accumulation of enzyme bound peptide intermediates [33, 34].

The presence of a single module of *pvdD* (on plasmid pMOD2) resulted in the secretion of a mixture of compounds with similar spectrophotometric properties to pyoverdine but with lower molecular weights. One of these had a mass corresponding to a form of pyoverdine with only seven amino acid residues in the peptide, lacking one of the carboxy-terminal threonines. The smaller forms may have arisen because of excess thioesterase activity resulting in premature cleavage of the nascent pyoverdine peptide from the NRPS complex, perhaps due to the higher copy number of the plasmid DNA relative to the chromosome, and/or the pMOD2 module lacking important structural elements for effec-

Table 2. Bacteria and Plasmids Used in This Study

Strain/Plasmid	Relevant Genotype and Phenotype	Reference
<i>E. coli</i>		
DH5 $\alpha$	<i>supE44 <math>\Delta</math>lacU169 (<math>\emptyset</math>80 lacZ DM5) hsdR17</i>	a
<i>P. aeruginosa</i>		
PAO1	wild-type Pvd <sup>+</sup>	b
PAO1 <i>pvdD</i>	<i>pvdD</i> Pvd <sup>-</sup>	c
PAO1 OT11 <i>pvdF</i>	<i>leu-1 pro-1 Sm<sup>R</sup> pvdF Km<sup>R</sup> Pvd<sup>-</sup></i>	d
Plasmid		
pSOT1	<i>P. aeruginosa</i> pyoverdine DNA in Sall site of pUC9	e
pSUP202	mobilizable ColE1 plasmid	f
pDFA3	pSUP202::kan <sup>R</sup> + PstI-bordered <i>pvdD</i> deletion (pSOT1-derived)	c
pUCP22	<i>lacI<sup>R</sup> lacZ(<math>\alpha</math>-fragment) aacC1 ColE1 ori RO1600 ori Ap<sup>R</sup> Gm<sup>R</sup></i>	g
pPVDD1	pUCP22 + Bam-Sal <i>pvdD ex</i> pSOT1	c
pLAFR3	<i>cos mob Tc<sup>R</sup></i>	h
pPVDD2	pLAFR3 + Bam-Sal <i>pvdD ex</i> pSOT1	c
pPROEX1::mod1	<i>lacI<sup>R</sup> ori Ap<sup>R</sup> pTrc pvdD(module 1)</i>	i
pSF2	pGEM-T (Promega) containing <i>oriT</i> and <i>rep</i> Ap <sup>R</sup>	j
pMOD1	pPROEX1::mod1 + <i>mob</i> (ex pSF2)	c
pUC18 $\Delta$ Sma	pUC18 lacking SmaI, KpnI, and SstI sites from the MCS	c
pUC18 $\Delta$ Sma::pvdD	pUC18 $\Delta$ Sma + Bam-Sal <i>pvdD ex</i> pSOT1	c
pMOD2	pPVDD1 (single <i>pvdD</i> module deletion)	c
pDAX10	pUC18 $\Delta$ Sma::pvdD-derivative	c
pDAX11	pDAX10-derivative	c
pDAX12	pPVDD1(Xba/Not flanking Adom1) A domain swapping vector	c
pVRC1210	T7-RNA polymerase-based expression vector + <i>snbC</i> Ap <sup>R</sup>	k
pPEN510	pUC19 carrying the <i>acvA</i> synthetase gene	l
pYM101	pUC19 carrying the <i>syrB,C,D</i> gene cluster	m
pDAX12::cys-A	pDAX12 + <i>acvA</i> (mod2; cys-specifying) mod1 A domain swap	c
pDAX12::ser-A	pDAX12 + <i>pvdI</i> (mod1; ser-specifying) mod1 A domain swap	c
pDAX12::snbC-A	pDAX12 + <i>snbC</i> (mod1; thr-specifying) mod1 A domain swap	c
pDAX12::syrB-A	pDAX12 + <i>syrB</i> (mod9; thr-specifying) mod1 A domain swap	c
pDAX12::val-A	pDAX12 + <i>acvA</i> (mod3; val-specifying) mod1 A domain swap	c
pDAX13	pPVDD1(Xba/Not flanking CA <sub>dom1</sub> ) CA-swapping vector	c
pDAX13::cys-CA	pDAX13 + <i>acvA</i> (mod2; cys-specifying) mod1 CA domain swap	c
pDAX13::ser-CA	pDAX13 + <i>pvdI</i> (mod1; ser-specifying) mod1 CA domain swap	c
pDAX13::snbC-CA	pDAX13 + <i>snbC</i> (mod1; thr-specifying) mod1 CA domain swap	c
pDAX13::val-CA	pDAX13 + <i>acvA</i> (mod3; val-specifying) mod1 CA domain swap	c

References: a, GIBCO BRL; b, [42]; c, this study; d, [46]; e, [20]; f, [47]; g, [23]; h, [24]; i, [5]; j, H.P. Schweizer; k, [48]; l, [49]; m, [50].

tive association with the upstream synthetase PvdJ. Alternatively, the single-threonine form of pyoverdine is not cyclised and consequently may be susceptible to spontaneous or proteolytic cleavage of amino acid residues from the carboxy terminus. Minimal module substitutions at position 2 of the surfactin synthetase complex gave rise to multiple surfactin-like products, the size of these products being consistent with one or more residues having been lost from the surfactin peptide chain [14]. Thus, it must be considered that manipulation of peptide synthetase templates may result in the generation of inherently unstable peptide products. The *pvdD*(pMOD2) cells secreting the attenuated forms of pyoverdine were unable to grow on agar containing EDDA, indicating that these compounds could not act as siderophores. It remains to be determined whether these compounds were unable to chelate Fe<sup>3+</sup> ions or whether *P. aeruginosa* lacks the ability to take up the resulting complexes.

*pvdD* mutant cells containing versions of *pvdD* in which the A domain of module 1 was replaced by the A domains of two threonine-incorporating NRPSs from other species produced functional pyoverdine, although in lower amounts than obtained with native *pvdD*. The SncA domain gave the lower amounts of pyoverdine

production, despite this domain having greater similarity to the PvdD A domain than SyrB (54% identity and 75% similarity for SncC/PvdD as opposed to 38% identity and 68% similarity for SyrB/PvdD as determined by Clustalw alignment). These results indicate that peptide synthetase modules with very strong sequence similarity that activate the same amino acid substrate may nonetheless act in slightly different fashions.

However, recombinant constructs designed to incorporate alternative residues into the pyoverdine peptide (pDAX12::cys-A, pDAX12::val-A, and pDAX12::ser-A) failed to restore pigment production to *pvdD* mutant cells. This was most likely due to one or more of the following: failure of the introduced A domains to activate an amino acid substrate; inability of the introduced A domains to interact effectively with the PvdD T domain; or inability of the PvdD C domain to catalyze peptide bond formation between the incoming L-N<sup>5</sup>-formyl-N<sup>5</sup>-hydroxyornithine and the novel residue activated by the introduced A domains.

The last possibility was considered the most likely, as there is some evidence that C domains display selectivity for the residue activated by the A domain immediately downstream [26, 27]. This proposal initially appears to conflict with the successful results of the AT

domain swaps for the *B. subtilis* surfactin synthetase system [13, 14]. However, for both studies, the recombinant synthetases exhibited markedly decreased rates of lipopeptide biosynthesis, suggesting that “intermodular interactions might be disturbed” [14]. Later, the same group proposed that an editing function of the C domains might have promoted an incompatibility of the C and A domains in their artificial junctions, explaining why the reported fusions had decreased activity and why other fusion constructs had displayed no activity at all [35]. The yield of recombinant surfactin was significantly increased by carrying out domain swaps that included a section of C domain as well as the AT domains swapped previously [15].

To allow for possible C domain incompatibility, hybrid genes were constructed in which the CA domains of the first module of PvdD were replaced by the CA domains of other peptide synthetases. However, none of the CA hybrid genes, including one (pDAX13::snbC-CA) containing a threonine-incorporating A domain, restored production of pyoverdine-like compounds to the *pvdD* mutant. This indicates that, at least for *snbC*, introduction of a novel C domain actually inhibited pyoverdine synthesis. One explanation is that C domains may not only display selectivity for cognate acceptor residues but also for donor residues, and the SnbC C domain was unable to utilize the incoming L-N<sup>5</sup>-formyl-N<sup>5</sup>-hydroxyornithine substrate provided by the pyoverdine synthetase system. Sequence alignments [27] indicate that C domains do cluster according to donor-residue specificity. Alignment of pyoverdine peptide synthetase C domain sequences (data not shown) also support this proposal: the two L-N<sup>5</sup>-formyl-N<sup>5</sup>-hydroxyornithine activating modules are 99% identical across their A domain sequences but only 26% identical across their C domains; whereas the first of these modules shares 58% identity across the C domain region with the L-arginine activating module of PvdI, these latter two domains both receiving an incoming D-serine donor residue. Neither of these C domains have more than 28% identity with any of the pyoverdine synthetase C domains that are predicted to receive L-amino acid donor residues. Furthermore, the C domains of the first module of PvdD and the first module of PvdJ, which both receive incoming L-N<sup>5</sup>-formyl-N<sup>5</sup>-hydroxyornithine donor residues, are 70% identical with one another. The successful domain swaps that included sections of C domains as well as the AT domain regions [15] do not preclude C domains having a proofreading activity. The foreign lichenysin synthetase modules from *B. licheniformis* that were introduced into the surfactin synthetases in *B. subtilis* accept identical donor residues to the original surfactin modules. Also, C domain regions that confer acceptor residue specificity may be located downstream of the HHxxxDG swap site used previously [15], while those that define donor residue specificity may be upstream of this site at the N terminus of a C domain. This is consistent with domain organization in PvdD; a disproportionate 54 of the 105 nonidentical residues in the 430 residue C domains of the two PvdD modules are within the first 120 amino acids of their primary sequences.

A second possible reason for the failure of the CA-hybrid genes (and the altered-specificity A domain hybrids) to give rise to pyoverdine-like compounds is that

T domains may also play a role in determining the substrate specificity of an individual module, and the introduced CA sequences may have been incompatible with the native PvdD T domain. It has been shown that the phosphopantetheinylated T domain of the valine-activating SrfB1 module of surfactin synthetase could be aminoacylated by the cognate A domain but not by the aspartic acid-activating A domain of SrfB2 [36]. Furthermore, in sequence alignments, unrelated T domains show a degree of clustering according to the amino acid substrate that is activated by their associated A domain [27]. It has also been proposed that, rather than direct substrate recognition by T domains, there may be an optimal interaction between paired A and T domains such that the 4'-PP cofactor bound to a given T domain may have greater accessibility to the residue activated by a native A domain partner than a nonnative A domain partner [37]. This may be one explanation for SnbC being less effective than SyrB at replacing the PvdD A domain, despite its greater shared amino acid identity. Likewise, suboptimal T domain interactions may have contributed to the failure of the CA-swapping experiments described in this paper.

Thus, there are two likely reasons why the CA-swapping strategy might have proved unsuccessful. Each of these reasons is also compatible with the observations made for the A domain swapping experiments. The “suboptimal T domain interaction” hypothesis would explain why foreign A domain hybrids recognizing the same amino acid substrate produced different levels of pyoverdine, and the “C domain donor residue specificity” hypothesis would explain why these hybrid modules still managed to produce some pyoverdine, as opposed to the CA domain hybrids. The original “C domain acceptor residue specificity” hypothesis of Belshaw et al. [26] then completes the picture, explaining why none of the constructs carrying A domains of altered specificity were able to restore pigment production to the *pvdD* mutant cells.

## Significance

So far as we are aware, this work is the first report of the specific modification of a nonribosomally synthesized peptide *in vivo* outside of *B. subtilis*. The demonstrated ability of modified pyoverdines to be secreted and readily purified as clearly visible pigments, coupled with the availability of a plasmid-borne complementation system, suggests that the pyoverdine synthetases of *P. aeruginosa* may provide an excellent model system for studying domain and module swapping of peptide synthetase enzymes. Plasmid-based expression will greatly facilitate the engineering of modified synthetase genes and will enhance the feasibility of ultimately establishing combinatorial peptide synthetase libraries [38, 39]. It will also facilitate further research aimed at identifying the precise parameters that enable engineered hybrid synthetases to be fully active.

## Experimental Procedures

### Bacterial Strains, Plasmids, Chemicals, and Media

The bacterial strains and plasmids employed in this study are listed in Table 2. *Escherichia coli* was grown in Luria broth (LB; GIBCO



BRL). *P. aeruginosa* was cultured in Brain Heart Infusion broth (BHI; GIBCO BRL) or in King's B broth [40] or succinate medium [41] for iron-limited conditions. Plates were prepared with 1.5% (w/v) agar (GIBCO BRL). Ethylene-diamine-di(o-hydroxyphenylacetic acid) (EDDA) and antibiotics were used at the same concentrations as previously [6, 46].

#### DNA Methods

To generate a *pvdD* deletion mutant, a 6.5 kb *XhoI*-*ClaI* fragment internal to the *pvdD* gene was excised from plasmid pSOT1 and the free ends blunt-ended and fused. *PstI* digestion then released a 3.0 kb DNA fragment with approximately 1.5 kb of DNA either side of the fusion junction. This DNA fragment was cloned into the suicide plasmid pSUP202 along with a kanamycin cassette (external to the homologous region) to give pDFA3. pDFA3 was introduced into *P. aeruginosa* PAO1 by conjugation [6], and recombinants in which plasmid integration had occurred by homologous recombination with the chromosomal *pvdD* were selected using kanamycin and tetracycline. Subsequent plasmid excision and loss of the wild-type *pvdD* gene was detected by loss of antibiotic resistance on replica plates and a loss of pyoverdine production. Southern blotting [43] was used to confirm that the expected genetic events had occurred.

PCR was performed using the Expand High Fidelity PCR system (Roche Molecular Biochemicals). Details including primer sequences are available as Supplemental Data. Chromosomal DNA from *P. aeruginosa* was used as template for *pvdD* or *pvdI* PCRs; plasmids pYM101, pVCR1210, and pPEN510 (Table 2) were used as templates for the *syrB*, *snbC*, and *acvA* PCRs, respectively. Plasmids that were constructed in this work are listed in Table 2. Details of their construction are available as Supplemental Data. For all plasmid constructs, DNA sequencing was carried out across fusion sites (DNA sequencing facility, Centre for Gene Research, University of Otago) in order to ensure that the intended constructs had been obtained.

#### IRCP Analysis

IRCPs were prepared according to the method of Georges and Meyer [10]. Proteins were solubilized in 0.125 M Tris-HCl (pH 6.8), used in SDS-PAGE on 5% acrylamide gels with 30  $\mu$ g total protein loaded per lane, and detected by silver staining.

#### Assays and Spectral Analysis

To assay pyoverdine production in liquid media, cells were grown for 16 hr in King's B and then diluted to an OD<sub>600</sub> of 2.5. One milliliter aliquots were then pelleted, and the supernatant was collected, diluted 1:5 with dH<sub>2</sub>O, and the absorbance from 350 to 450 nm was measured in a Shimadzu scanning spectrophotometer. Pyoverdine concentration was determined at the absorbance maximum of 403 nm (Figure 2), where the extinction coefficient  $\epsilon = 1.4 \times 10^4$  L.mol<sup>-1</sup>.cm<sup>-1</sup> [44]. MALDI-ToF mass spectrometric analysis was performed by the Protein Microchemistry Facility of the University of Otago, using a Finigan Lasermat 2000. Amino acid composition analysis was also performed by the Protein Microchemistry Facility.

#### Pyoverdine Purification

Pyoverdine (or pyoverdine-like compounds) was purified from *P. aeruginosa* strains as described by Meyer et al. [45]. Pyoverdine solutions were freeze dried, the dry weight of the pyoverdine was measured, and was ddH<sub>2</sub>O added to give a final concentration of 4 mg.ml<sup>-1</sup>.

#### Supplemental Data

Supplemental Data including details of PCR and the construction of plasmids can be found at <http://www.chembiol.com/cgi/content/full/11/7/971/DC1/>.

#### Acknowledgments

D.F.A. was supported by a PostGraduate scholarship from the Health Research Council of New Zealand. This research was supported in part by a grant from the University of Otago. We are very grateful to Denis Thibaut, Dennis Gross, Geoffrey Turner, and Herbert Schweizer for providing plasmid constructs and cloned peptide synthetase genes.

Received: November 13, 2003

Revised: April 27, 2004

Accepted: April 28, 2004

Published: July 23, 2004

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