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Nucleotide Sequence of *pvdD*, a Pyoverdine Biosynthetic Gene from *Pseudomonas aeruginosa*: PvdD Has Similarity to Peptide Synthetases

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Pseudomonas aeruginosa **secretes a fluorescent siderophore, pyoverdine, when grown under iron-deficient conditions. Pyoverdine consists of a chromophoric group bound to a partly cyclic octapeptide. As a step toward understanding the molecular events involved in pyoverdine synthesis, we have sequenced a gene, *pvdD*, required for this process. The gene encodes a 2,448-residue protein, PvdD, which has a predicted molecular mass of 273,061 Da and contains two highly similar domains of about 1,000 amino acids each. The protein is similar to peptide synthetases from a range of bacterial and fungal species, indicating that synthesis of the peptide moiety of pyoverdine proceeds by a nonribosomal mechanism. The *pvdD* gene is adjacent to a gene, *fpvA*, which encodes an outer membrane receptor protein required for uptake of ferripyoverdine.**

Pseudomonas aeruginosa is an opportunistic pathogen which infects injured, immunodeficient, or otherwise compromised patients. The bacteria secrete a siderophore, pyoverdine, which is likely to play an important role in infection by competing with transferrin for iron in order to overcome the iron-withholding defense mechanism present in mammals (3, 11, 42). Almost all isolates of *P. aeruginosa* from infected patients secrete pyoverdine (9, 22, 29), and it has been shown that pyoverdine is present in the sputa of cystic fibrosis patients infected with *P. aeruginosa* (21). A mutant of *P. aeruginosa* which is unable to synthesize pyoverdine showed reduced virulence in an animal model of infection (24).

Pyoverdine from *P. aeruginosa* PAO is a water-soluble, yellow-green fluorescent compound. It consists of a dihydroxyquinoline chromophoric group linked to an eight-residue partly cyclic peptide (D-Ser–L-Arg–D-Ser–L-N^5-OH-Orn–L-Lys–L-N^5-OH-Orn–L-Thr–L-Thr) via the N-terminal serine, with a small dicarboxylic acid attached to the chromophore. Iron complexation is thought to occur through oxygen atoms present on the dihydroxyquinoline and two hydroxamate units supplied by the L-N^5-OH-Orn residues. Several *Pseudomonas* species produce similar compounds, variously called pyoverdines or pseudobactins, all of which have the same dihydroxyquinoline group but differ in the nature of the attached peptide (reviewed in references 1 and 7), and it is likely that all of these are synthesized by similar mechanisms. Synthesis of the chromophoric group begins with condensation of D-tyrosine and L-2,4-diaminobutyric acid (7), with glutamic acid being the precursor of the amide-linked dicarboxylic acid (50). It has been suggested that biosynthesis of the peptide moiety of pyoverdine occurs by a nonribosomal mechanism (30).

Little is known about the molecular nature of enzymes involved in the biosynthesis of pyoverdine in *P. aeruginosa* or any other pseudomonad. Recently, *pvdA*, which encodes an enzyme (L-ornithine N^5-oxygenase) responsible for catalyzing the hydroxylation of ornithine, an early step in pyoverdine biosynthesis in *P. aeruginosa*, has been characterized at the molecular level (55). The *pbsC* gene, which is involved in synthesis of pseudobactin by *Pseudomonas* sp. strain M114, has also been sequenced (2). To gain further insight into the molecular mechanism of pyoverdine biosynthesis, we have determined the nucleotide sequence of the *pvdD* (pyoverdine synthetase D) gene from *P. aeruginosa.*

Nucleotide sequence of pSOT1 and identification of *pvdD*. We have previously shown that restriction fragments D and E of pSOT1 (Fig. 1) are part of a locus required for synthesis of pyoverdine (34, 35). To gain insight into the nature of the corresponding pyoverdine biosynthetic gene(s), the DNA sequence of fragments B through F of pSOT1 was determined. DNA fragments to be sequenced were subcloned into M13mp18 or mp19 (60), using standard procedures (4), with *Escherichia coli* TG1 (16) used as the host strain. Double-strande M13 DNA was prepared (25), and partial deletions of the cloned fragments were generated by using exonuclease III (Erase-a-Base system; Promega) or by using restriction enzymes which cleaved the DNA within the insert and within the M13 polylinker. Oligonucleotide primers were used to sequence regions not covered by these strategies; these were synthesized by using a model 180B DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.) or were purchased from Macromolecular Resources (Colorado State University). Single-stranded DNA of M13 subclones was prepared (5) and sequenced, using a *Taq* DNA polymerase sequencing kit (Amersham) or Sequenase Version 2.0 (United States Biochemical, Cleveland, Ohio), both with 7-deaza-dGTP. Sequence ambiguities were resolved by using the Sequenase Version 2.0 kit with dITP instead of 7-deaza-dGTP. A model 373A automated DNA sequencer (Applied Biosystems) was used to sequence across all of the cloning sites in pSOT1. Ninety-four percent of the sequence data were obtained from both strands. The remaining single-stranded data were obtained from at least two gels and were clear and unambiguous. The DNA sequence was analyzed on a MicroVaxII computer system by using various softwares: Ssedit (48), Vtutin (47), Codonuse (45), NLDNA (48), Map_Zap (41), Diagon (44), Dbextract (46), and the

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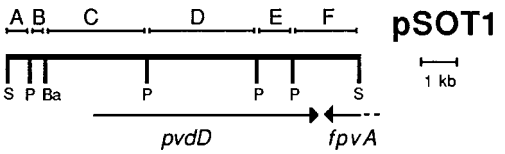


FIG. 1. (A) Clone pSOT1. pSOT1 is the largest (~9.6-kb) *Sal*I fragment of λOT1 (35) cloned into pUC9 (54) and contains DNA required for pyoverdine synthesis (34, 35). Restriction fragments are labelled by using the same nomenclature as previously (36). The locations and orientations of two genes (*pvdD* and *fpvA*) sequenced in this study are shown. Ba, *Bam*HI; P, *Pst*I; S, *Sal*I.

FoldRNA program from the version 7 UNIX of the Genetics Computer Group package (12).

The sequenced DNA contained a large open reading frame (ORF) of 7,374 bp (Fig. 2). Downstream from this ORF and oriented in the opposite direction was a partial ORF of 792 bp. Both ORFs had patterns of codon usage which are very similar to those of other genes from *P. aeruginosa* (58), indicating that they are likely to encode proteins.

Mutations mapping to restriction fragments D and E, which are entirely contained within the 7.4-kb ORF, prevent synthesis of pyoverdine by *P. aeruginosa* (34, 35). This finding shows that this gene is required for synthesis of pyoverdine, and it has been named *pvdD*, following the assignment of *pvdA*, *pvdB*, and *pvdC* to other genes involved in synthesis of pyoverdine (55). Analysis of codon usage shows that translation is likely to start at a GTG codon (Fig. 2), which nonetheless results in methionine being incorporated as the first amino acid in the protein (18). This is six bases downstream from a Shine-Dalgarno sequence which is complementary to seven of the eight bases at the 3′ end of 16S rRNA. The *pvdD* gene encodes a protein of 2,448 residues with a predicted molecular mass of 273,061 Da.

Two transcriptional start sites have been identified upstream from *pvdD* (36) (Fig. 2). Two inverted repeat sequences downstream from *pvdD* could function as transcriptional terminators for the gene. Both are capable of forming stem-loop structures; one is present 21 bp downstream from the predicted TGA termination codon (Fig. 2) and comprises an 8-bp stem and 4-bp loop ($\Delta G = -14.8$ kJ/mol, as calculated by using the FoldRNA program), and the second is 122 bp downstream from the termination codon (Fig. 2) and consists of a 12-bp stem and 3-bp loop ($\Delta G = -19.1$ kJ/mol). Both inverted repeats are followed by multiple thymidine nucleotides; this is characteristic of transcription termination sequences which function independently of the Rho termination factor (59). Transcription of *pvdD* could be terminated at either of these sites, giving a transcript of about 7.5 kb. The size of the transcript corresponding to *pvdD* was previously estimated to be 5 kb following Northern (RNA) analysis (36), but the sequence of the gene indicates that this is an underestimate, presumably due to the high degree of instability of this transcript (36).

Direct repeats within *pvdD*. Diagon analysis indicated that the *pvdD* gene contains two direct repeats of about 3 kb each. The repeats span bases 139 to 3180 and 3331 to 6363 of the sequence presented in Fig. 2 and are 91.8% identical. The deduced amino acid sequences of the repeats are 90.7% identical and 94.7% similar (Fig. 3), with one three-amino-acid insertion in the first repeat 83 residues from the N-terminal end. The C-terminal regions of the repeats (amino acids 407 to 1040) were different at only seven positions, with four of the differences representing conserved changes. In contrast, there were tracts of relatively different sequence in the N-terminal parts of the repeats.

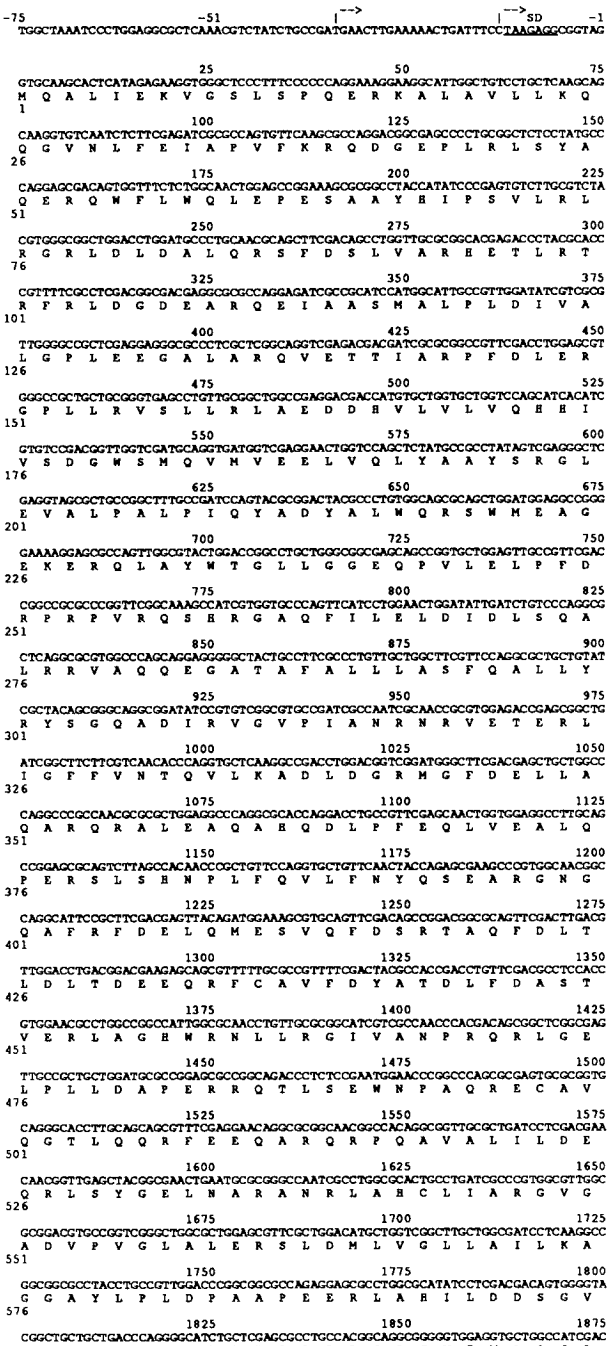
Identification of proteins similar to PvdD. The GenBank translated nucleic acid database (version 83) was searched by using FastA (32) for proteins showing sequence similarity to the first repeat of PvdD (1,013 amino acids). The protein showing greatest similarity was the product of the *pbsC* gene from *Pseudomonas* sp. strain M114, and this is discussed below. Significant similarity was also found to a family of enzymes including GrsB (53), TycA (57), SrfA (10), AcvA, PcbA, and PcbB (8, 27, 40), EntF (37), and AngR (14, 52). These enzymes are peptide synthetases, with the last two being involved in siderophore synthesis. After PbsC, the protein showing greatest similarity was GrsB, which is a well-characterized peptide synthetase from *Bacillus brevis*. This protein contains four domains, each responsible for the adenylation of a specific amino acid and its subsequent incorporation into the peptide antibiotic gramicidin (53). Each domain was similar to each of the repeats within PvdD, and an alignment of the PvdD repeats with the domains of GrsB is shown in Fig. 3.

The mechanism of nonribosomal peptide synthesis by GrsB and other peptide synthetases has been studied (reviewed in references 26 and 56). Each domain of a peptide synthetase recognizes a specific amino acid and activates this substrate by adenylation. It is thought that the amino acyladenylate is then covalently attached to the enzyme by a thioester bond between the carboxyl group of the amino acid and a thiol group, which is probably supplied by a 4′-phosphopantetheine cofactor. The amino acid is then incorporated into the peptide by the enzymatic formation of a peptide bond which joins it to the amino acid bound to the adjacent domain. This is coupled with scission of the thioester bond between the cofactor and the amino acid, regenerating the enzyme (26).

Several consensus motifs associated with these activities are present in different peptide synthetases (19), and all of these are present in PvdD (Fig. 3). From the extensive similarities found with peptide synthetases, including the presence of consensus sequence motifs (Fig. 3), it is very likely that each domain of PvdD recognizes, activates, and incorporates a specific amino acid into the peptide moiety of pyoverdine.

PvdD also contains a thioesterase active-site motif. The C-terminal segment of PvdD (336 amino acids), which is not part of either repeat sequence, was independently compared with sequences in the translated GenBank database. The only significant similarities were to EntF from *E. coli* (37) and the AcvA protein from *Penicillium chrysogenum* (40), both of which are peptide synthetases. In each case, similarity was centered around a thioesterase active-site motif (GXSXG [20], where X is any amino acid) which is present at residues 2240 to 2244 in PvdD. Similar sequence motifs are also present downstream from the domains of gramicidin S synthetase and surfactin synthetase which incorporate the C-terminal amino acids into the peptides (10, 53). Motifs of this sort are thought to be involved in termination of peptide synthesis by cleavage of the thioester bond between the 4′-phosphopantetheine cofactor of the final amino acid-activating domain and the last amino acid incorporated into the peptide (reference 10 and references therein). The presence of this motif indicates that PvdD is likely to have a thioesterase activity associated with release of the completed pyoverdine peptide, and this implies that the protein incorporates the two C-terminal threonines into the peptide. Further experiments will be required to investigate the possible thioesterase activity and to determine the amino acid substrates of PvdD.

Similarity of PvdD to PbsC. The protein found during database searching which was most similar to the first repeat of PvdD was the product of the *pbsC* gene of *Pseudomonas* sp. strain M114. This protein is predicted to contain 803 amino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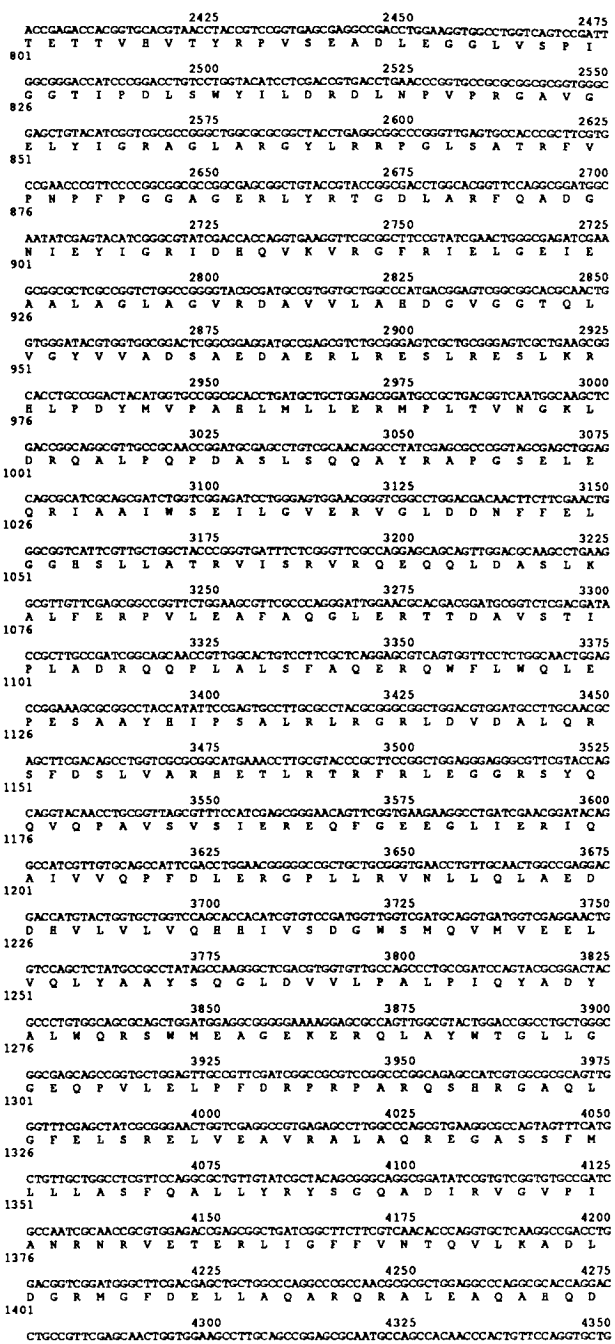
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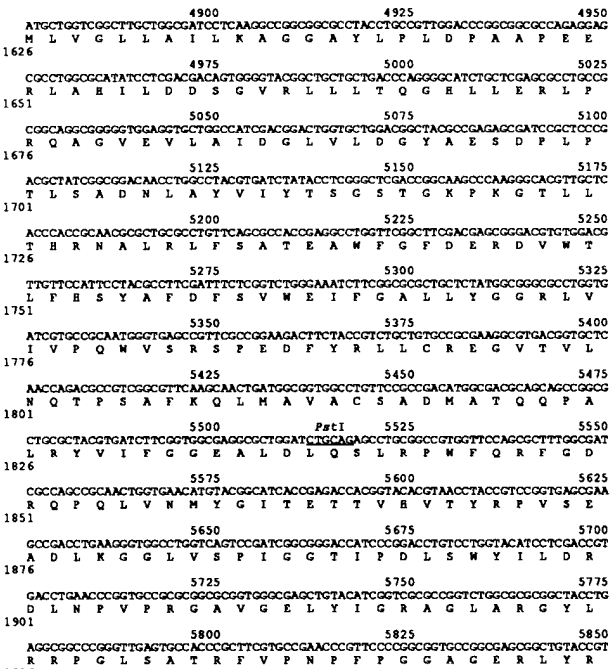
TGGGACGGCCAGACTGCGCAGTTCGACCTGACGCTGGATATTCAGGAAGACGAAAACGGCATCTGGGCCTCCTTC
W D G Q T A Q F D L T L D I Q E D E N G I W A S F
1476
4525 4550 4575
GACTATGCCACCGATCTGTTCGACGCCTCCACCGTGGAAGCCCTGGCCGGCCATTGGCGCAACCTGTTGCGGGGC
D Y A T D L F D A S T V E R L A G H W R N L L R G
1501
4600 4625 4650
ATCGTCGCCAACCCACGACAGCGGCTCGGCGAGTTGCCGCTGCTGGATGCGCCGGAGCGCCGGCAGACCCTCTCC
I V A N P R Q R L G E L P L L D A P E R R Q T L S
1526
4675 4700 4725
GAATGGAACCCGGCCCAGCGCGAGTGCGCGGTGCAGGGCACCTTGCAGCAGCGTTTCGAGGAGCAGGCGCGGCAA
E W N P A Q R E C A V Q G T L Q Q R F E E Q A R Q
1551
4750 4775 4800
CGGCCACAGGCGGTTGCGCTGATCCTCGACGAACAACGGTTGAGCTACGGCGAACTGAATGCGCGGGCCAATCGC
R P Q A V A L I L D E Q R L S Y G E L N A R A N R
1576
4825 4850 4875
CTGGCGCACTGCCTGATCGCTCGCGGCGTTGGCGCGGACGTGCCGGTCGGGCTGGCGCTGGAGCGTTCGCTGGAC
L A H C L I A R G V G A D V P V G L A L E R S L D
1601



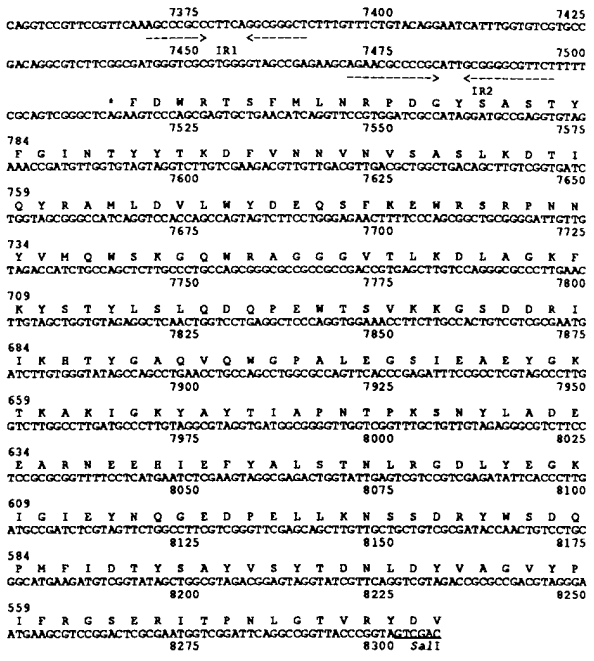


FIG. 2. Nucleotide sequences of the *pvdD* gene and part of the *fpvA* gene. The predicted amino acid sequences of the proteins are also shown. Nucleotide numbering is relative to the likely translational start site of *pvdD*. Transcription start sites for *pvdD* ($|^{-->}$), a Shine-Dalgarno sequence (SD), and inverted repeats downstream from *pvdD* (IR1 and IR2) are shown. *Bam*HI, *Pst*I, and *Sal*I restriction sites are indicated. Part of the sequence (nucleotides −75 to 390) has been published elsewhere (36) and is included here for completeness.

acids and to have a molecular mass of 89 kDa (2). However, when the translated *pbsC* sequence was aligned with that of PvdD by using the GAP program (12), similarity extended over the first 988 amino acids of PvdD, with the start of PbsC predicted by Adams et al. (2) corresponding to amino acid 185 of PvdD. It seems likely that the GTG codon in the *pbsC* sequence which corresponds to the proposed GTG start site in *pvdD* is the start of the *pbsC* gene.

Mutations of *pbsC* in *Pseudomonas* sp. strain M114 prevent the synthesis of pseudobactin, and it has been suggested that PbsC may be a member of the peptide synthetase family which is involved in the biosynthesis of M114 pseudobactin (2). PbsC contains most of the sequence motifs present in peptide synthetases, but motifs VI and X (Fig. 3) are missing. The exact relationship of PbsC with members of the peptide synthetase family remains to be determined, but its striking similarity to PvdD indicates that the two proteins are likely to have some functions in common in siderophore synthesis. DNA hybridization experiments have shown that genes similar to *pvdD* are also present in siderophore synthesis DNA from other fluorescent pseudomonads (34, 35), indicating that proteins similar to PvdD are also present in these species.

***pvdD* is adjacent to a gene encoding a ferripyoverdine uptake protein.** DNA sequencing also revealed a partial ORF of 792 bp downstream from *pvdD* and orientated in the opposite direction (Fig. 2). The 264-residue predicted protein product of the partial ORF downstream from *pvdD* was 99.2% identical to amino acids 552 to 814 of FpvA, the outer membrane receptor for ferripyoverdine (33). There was a two-amino-acid insertion and one amino acid difference when the translated sequence of this ORF was compared with that of Poole et al. (33). Further sequencing and genetic analysis has shown that this ORF does indeed encode the ferripyoverdine outer membrane receptor (51). The differences between our *fpvA* se-

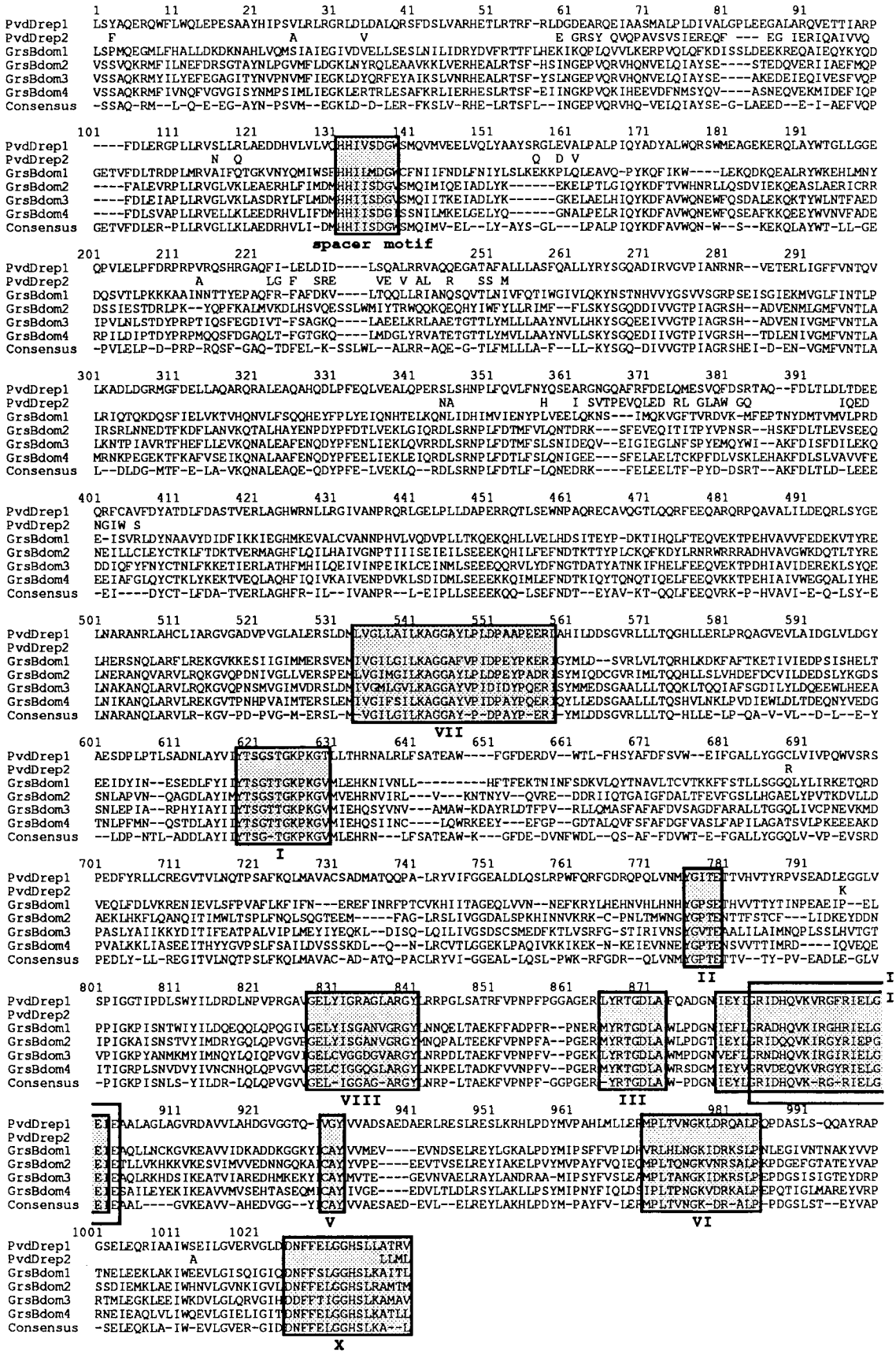

spacer motif
I
II
III
IV
V
VI
VII
VIII
IX
X

FIG. 3. Alignment of the predicted peptide synthetase domains of PvdD with those of GrsB. The two repeats of PvdD (PvdDrep1 and PvdDrep2) were aligned with the four domains of gramicidin S synthetase B (GrsBdom1 to GrsBdom4) (53), using Homed (49) and CLUSTAL V (23). The two repeats of PvdD were identical except at sites where a residue is shown for PvdDrep2. Boxes denote consensus sequence motifs which have been identified for peptide synthetase-type enzymes; the consensus sequences are listed in reference 19. Motifs I to VI are conserved among the superfamily of adenylate-forming enzymes, including nonpeptide synthetases. Motifs VII to X are conserved among a subfamily of the adenylate-forming superfamily which includes all known peptide synthetases and also EntF and AngR. Motifs I and III have been associated with ATP-binding and hydrolysis activities, respectively (15, 17, 38). Motif X contains a conserved serine (position 1034 in the alignment) to which a 4′-phosphopantetheine cofactor is very probably attached (13, 39). A so-called spacer motif present at the N-terminal end of peptide synthetase domains involved in elongation, but not initiation, of peptide synthesis (15) is also indicated. It has been suggested that amino acids between 627 and 829 in the alignment are involved in substrate recognition by peptide synthetase domains (10).

quence and that of Poole et al. (33) probably reflect minor differences between the *P. aeruginosa* strains used in the respective studies. The presence of *fpvA* downstream from *pvdD* indicates that ferripyoverdine synthesis and uptake genes are clustered, as is the case in other fluorescent pseudomonads (6, 28, 31). In addition, this allows the *fpvA* gene to be located at 47 min on the genetic map of *P. aeruginosa*, as we have previously shown that clone pSOT1, which includes this DNA, is derived from that part of the chromosome (35).

Nucleotide sequence accession number. The sequence shown in Fig. 2 has been assigned accession number U07359 in the GenBank and EMBL libraries.

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