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Functional Analysis of A Pyoverdine Synthetase from *Pseudomonas* sp. MIS38

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Fluorescent *Pseudomonas* sp. MIS38 produces a cyclic lipopeptide, arthrofactin. Arthrofactin is synthesized by a unique nonribosomal peptide synthetase (NRPS) with dual C/E-domains. In this study, another class of cyclic peptide, pyoverdine, was isolated from MIS38, *viz.*, Pvd38. The main fraction of Pvd38 had an *m/z* value of 1,064.57 and contained Ala, Glu, Gly, (OHOrn), Ser, and Thr at a ratio of 2:1:1:(1):1:1 in the peptide part, suggesting a new structure compound. A gene encoding NRPS for the chromophore part of Pvd38 was identified, and we found that it contained a conventional E-domain. Gene disruption completely impaired the production of Pvd38, demonstrating that the synthetase is functional. This observation allows us to conclude that different NRPS systems with dual C/E-domains (in arthrofactin synthetase) and a conventional E-domain (in pyoverdine synthetase) are both functional in MIS38.

Key words: fluorescent *Pseudomonas*; pyoverdine synthetase; nonribosomal peptide; gene disruption; arthrofactin

In bacteria and fungi, a class of huge multicomponent enzyme systems, called nonribosomal peptide synthetases (NRPSs), play important roles in producing a variety of complex peptides. NRPS assembly lines are consist of multiple unit modules. Each module contains a set of core and accessory domains with specific catalytic functions.[1] The first core domain is the adenylation (A) domain, which selects and activates amino acids carrying ATP consumption. The A-domain is about 550 amino acids (aa) in size and shares significant homology with the family of acyl-CoA synthetases and luciferases.[2] The second core domain is the thiolation (T) domain, which is also called peptidyl carrier protein (PCP), about 100 aa in size. The T-domain is located downstream of the A-domain and represents a transport unit that accepts an adenylated amino acid and is covalently bound to its 4′-phosphopantetheinyl (4′-PP) cofactor by a thioester bond. The third core domain is the condensation (C) domain, about 450 aa in size. The C-domain catalyzes elongation of peptide chain and is responsible for the peptide bond formation between the substrates. A set of C/A/T-domains, called a module, is repeated in the same number and order as the substrate amino acid residues in the product peptide. A C-terminally located accessory domain, about 250 aa in size, is the thioesterase (Te) domain, responsible for the release of a product peptide. Another accessory domain, the epimerization (E) domain, about 450 aa in size, is responsible for the conversion of substrate amino acid from the L- to the D-form. The reaction of epimerase involves abstraction and re-addition of the proton bound to C_α of the aminoacyl or peptidyl moiety, which results in the conversion of the chirality of amino acid residues.[3] The E-domain is usually contained in every module that incorporates the D-form amino acid in the product peptide. D-form amino acid residues play important roles in the product peptide, expressing specific biological activity and tolerance to proteolytic cleavage.[4]

Pseudomonas sp. MIS38 produces an effective lipopeptide biosurfactant, arthrofactin.[5] Arthrofactin contains 11 amino acid residues in the peptide part, where seven of them are of the D-form. Several NRPSs in *Pseudomonas* spp. lack a conventional E-domain, but the product peptide contains D-form amino acid residues.[6,7] Such is the case for arthrofactin synthetase (ArfA/B/C).[5] When the primary structures of the C-domains of L-peptidyl donors and D-peptidyl donors were compared, it was found that they belonged to different lineage.[8] Recently, Balibar *et al.* have shown that D-peptidyl donors ($^{D}C_{L}$ and $^{D}C_{D}$-domains) in ArfA and ArfB are unique dual-functional C/E-domains that catalyze both epimerization and condensation reactions,[9] but it remains unclear whether NRPSs with conventional E-domains also function in MIS38.

We have noticed that MIS38 culture showed yellow-green fluorescence under an iron-limiting condition. This observation suggests that MIS38 produced a pyoverdine (Pvd). Pvd is a yellow-green fluorescent

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water-soluble pigment and high affinity iron chelating siderophore that is synthesized by a NRPS, Pvd synthetase.[10] Pvd is excreted by all fluorescent *Pseudomonas* species under iron limiting conditions.[11] It is consists of an invariable dihydroxyquinoline chromophore part to which attach a small dicarboxylic acid (or its monoamide) and a peptide part of variable composition and length, usually six to 12 amino acids. Because Pvd synthetase generally contains a conventional E-domain, we decided to identify the encoding gene and to examine the functionality of the enzyme with conventional E-domains in MIS38.

In this research, a pyoverdine from MIS38 (Pvd38) was purified and the structure was partially analyzed by LC/MS and amino acid composition analysis. Next, identification and disruption of the gene encoding a conventional E-domain of NRPSs for the Pvd38 chromophore part were carried out.

Materials and Methods

Bacterial strains and plasmids. Pseudomonas sp. MIS38 was previously isolated from the soil at an oil spill in Shizuoka prefecture, Japan.[12] *Pseudomonas* sp. NC1 was an arthrofactin non-producing mutant.[5] *Pseudomonas* sp. MT21, a *pvd38* gene disrupting mutant, was constructed in this study. *Escherichia coli* DH5α was used as a host strain for gene cloning and the construction of plasmids. *E. coli* SM10λpir was used as a donor strain of the suicide plasmid pCVD442-EpiPvd38:kan (Km^r) in the gene disruption experiment. *E. coli* LE392 was used as a host strain to construct and maintain a λEMBL3 genomic library of *Pseudomonas* sp. MIS38.[5] Cloning vectors pGEM-T Easy and pBlueScript SK$^+$ were purchased from Promega (Madison, WI) and Stratagene (La Jolla, CA) respectively. A kanamycin resistant gene (*kan*) was prepared from pSMC32 (Km^r), a derivative of pSU36.[13]

Analysis of pyoverdine production. Wild type MIS38 and mutant MT21 were grown at 30 °C for 24 h in an iron limiting casamino acid (CAA) medium. CAA medium contains (per liter): 5 g casamino acids, 1.18 g K_2HPO_4–$3H_2O$, and 0.25 g $MgSO_4$–$7H_2O$, pH 7.0. All culture flasks were washed beforehand with 6 N HCl to remove any iron traces. Culture supernatant was collected and analyzed using either HPLC or a scanning UV spectrophotometer (UV Mini 1240, Shimadzu, Kyoto, Japan). Pyoverdine was detectable by its specific absorbance at 403 nm.[14] Production of pyoverdine was also observed under UV light (325 nm) by the formation of a fluorescent zone around the colony.

Analysis of arthrofactin production. Wild type MIS38 and mutant MT21 were grown at 30 °C in L-broth for 40 h. An arthrofactin deficient mutant, NC1,[5] was used as a negative control. Extraction and analysis of arthrofactin were performed as previously described.[15]

Purification of a pyoverdine from Pseudomonas sp. *MIS38.* Purification of pyoverdine was performed using a reverse-phase HPLC system (HP1100, Hewlett Packard, Palo Alto, CA) equipped with a 5C18-ARII column (4.6 × 150 mm, Nacalai Tesque, Kyoto, Japan). Pyoverdines were eluted at 10% acetonitrile in 0.1% trifluoroacetic acid. A main peak fraction (m/z 1064.57) was collected and the amino acid composition was analyzed. Other impurities bound to the column were eluted in a linear gradient of acetonitrile from 10 to 100%.[5] The m/z ($[M + H]^+/z$) value was determined with a benchtop mass spectrometer LCQ in positive mode (Thermo Fisher Scientific, Waltham, MA). Before injection into HPLC, culture supernatant containing pyoverdine was roughly purified using a Sep-Pak RP-C_{18} cartridge (Waters, Milford, MA).[16] After the cartridge was washed with water, the fraction containing pyoverdine was recovered at 70% methanol and then diluted with water to 5% methanol solution. Insoluble compounds were removed by centrifuge (8,000 g for 10 min), and the resulting clear solution was applied to HPLC.

Amino acid composition analysis. Purified pyoverdine (0.3 mg) was dissolved in 0.3 ml of 200 mM sodium citrate. The sample was analyzed at Hokkaido University Analytical Center using a Hitachi L-8500 amino acid analyzer (Hitachi, Tokyo) to determine the amino acid composition.

General DNA manipulation and analysis. Genomic DNA of MIS38 was extracted by a modification of the Marmur's method.[17] DNA preparation from λEMBL3 genomic library was performed using a Qiagen Lambda Mini Kit (Qiagen, Hilden, Germany). Plasmid DNA was purified using a QIAprep Spin Miniprep Kit (Qiagen). DNA fragments were recovered from agarose gel using a QIAquick Gel Extraction Kit (Qiagen). DNA ligation was performed using a Takara Ligation Kit (ver 2.1) (Takara Bio, Ohtsu, Japan). All other DNA manipulations were performed according to standard protocols.[18]

Nucleotide sequences of the gene fragment were determined using a BigDye Terminator v3.1 Cycle Sequencing Kit and an autosequencer ABI Prism 3100 (Applied Biosystems, Foster City, CA). The DNA sequences were analyzed with GENETYX software (Genetyx Corporation, Tokyo) and the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Deduced amino acid sequence was aligned with other related sequences by the CLUSTALW programs (http://clustalw.genome.jp/).

Cloning of pvd38-chr. The DNA region containing *pvd38-chr* was obtained as follows: PCR was performed by standard method[18] using a thermal cycler, PTC-100 (MJ Research, Waltham, MA) or Takara Dice standard (Takara Bio) and KOD plus DNA polymerase (Toyobo, Osaka, Japan). Oligodeoxyribonucleotides for PCR primers were synthesized at Hokkaido System Science

(Sapporo, Japan). For initial cloning of part of the pyoverdine synthetase gene from MIS38, a set of primers PA346f, 5′-GTCAACGAYCTKCTGCTGAC-3′ and PA472r, 5′-RCTGGTGAACCARCCSACSG-3′ was constructed at highly conserved regions in the pyoverdine synthetase among *Pseudomonas* strains. Y, R, and S denote C/T, A/G, and C/G respectively. A 146-bp DNA fragment was obtained, and the nucleotide sequence was determined to confirm that it encoded part of *pvd38-chr*. Based on the sequence of the DNA fragment, a set of primers for inverse PCR, 103f 5′-CGGGAATTCGCTCCAGCGGCACAGCAC-3′ and 177r 5′-GCTGAATTCCGATCGACCTGACCCGC-3′ was constructed in the opposite direction, where underlines indicate *Eco*RI recognition sites. Inverse PCR[18] was performed using *Ex-Taq* DNA polymerase (Takara Bio). Genomic DNA which had been digested with *Sal*I or *Pst*I was self-ligated using a Takara Ligation Kit and used as template. Temperature conditions were 94 °C 3 min followed by 35 cycles of (94 °C 1 min, 64 °C 2 min, 72 °C 3 min), and then the reaction mixture was held at 4 °C. A 2-kb fragment was amplified from *SalI* digestion/ligation of MIS38 genomic DNA, and then a further flanking 3-kb region was obtained from *PstI* digestion/ligation in the second inverse PCR. The primers for the second inverse PCR were Sal58f 5′-GCAGAATTCCCGACTGTTCGGCTTCGAG-3′ and Sal2216r 5′-CTGGAATTCGACCAGCGTTACTTCG-ACG-3′. It was found that the sequence of a terminal region of the 3-kb fragment overlapped with an end of a λEMBL3 genome clone, λ-S8. The nucleotide sequence of the insert DNA of λ-S8 was determined by the primer walking method, and then the entire gene, *pvd38-chr*, was obtained. The *pvd38-chr* gene sequence has been submitted to DDBJ/ EMBL/ GenBank under accession no. AB244732.

Southern hybridization analysis. Southern hybridization was carried out to identify the DNA fragments containing the target gene and verify successful gene disruption in MIS38. Vacuum blot cell and Hybond N^+ membrane (Amersham Biosciences, Piscataway, NJ) were used to transfer restriction enzyme digests of MIS38 genomic DNA or λEMBL3 library DNA from an agarose gel. Labeling the probe DNA fragment and detecting the signals were performed by the Gene Images AlkPhos Direct Labeling and the Detection System (Amersham Biosciences) respectively.

Construction of a gene disruption mutant in the E-domain of Pvd38-chr synthetase. Oligonucleotide primers 5′-CTAGTCTAGACAGCATCAGACCGTGCA-GAC-3′ and 5′-CTAGTCTAGAGTGGTGCGTTCGA-AACCTTG-3′ supplemented with the *Xba*I restriction site (underlined) were constructed. The target gene (2,068 bp), containing all seven conserved motifs of an E-domain, was amplified by PCR using the above set of primers and genomic DNA of *Pseudomonas* sp. MIS38 as a template. The resulting DNA fragments were cloned into pBlueScript SK^+ vector to construct pBlue-EpiPvd38. The kanamycin resistant gene (*kan*), 1,210 bp, was amplified by PCR from pSMC32 using oligonucleotide primers 5′-CATGGGTACCGTTTTATGG-ACAGCAAGCGA-3′ and 5′-CATGGGTACCCCGTC-AGTAGCTGAACAGGA-3′ with the restriction site of *Kpn*I (underlined). The DNA fragment was digested with *Kpn*I and inserted into the *Kpn*I gap of pBlue-EpiPvd38. The EpiPvd38:kan fragment was then prepared from the plasmid by *Xba*I digestion, subcloned into the *Xba*I gap of pCVD442, and transferred into *E. coli* SM10λpir by electroporation (Shimadzu GTE-10, Kyoto, Japan). The resulting pCVD442-EpiPvd38:kan plasmid was transferred into *Pseudomonas* sp. MIS38 using the conjugation method, as described below.

Conjugation of donor strain E. coli *SM10λpir and recipient strain* Pseudomonas sp. *MIS38.* Conjugation was performed by mixing 0.5 ml each of MIS38 (Cm^r) and SM10λpir (harboring the gene disruption plasmid, pCVD442-EpiPvd38:kan) culture at OD_{600} 0.4–0.5, where heat shock was initially applied to MIS38 at 42 °C for 15 min. Cells were centrifuged, and the supernatant discarded, leaving a 50 µl cell suspension, and spotted on L-agar (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar, pH 7.3) plates without antibiotics. The plates were incubated at 30 °C for 18 h. Then cells were recovered and spread onto new L-agar plates supplemented with chloramphenicol (Cm) (34 mg/l) and kanamycin (Km) (35 mg/l). Plates were incubated at 30 °C for 30 h. Colonies were transferred to a new plate. Gene disruption at the expected position by a double-crossing over event was confirmed by the size of DNA fragments obtained from PCR and Southern hybridization. One of the positive mutant strains was named MT21 and was used in further experiments.

Results and Discussion

Purification and molecular weight analysis of Pvd38

When MIS38 was grown in CAA medium, the culture showed fluorescence (yellow-green color, λ_{max} at 403 nm), which is a characteristic of pyoverdine production. The pyoverdine from MIS38, Pvd38, was purified as described in "Materials and Methods." The fraction of culture supernatant eluted under the 70% methanol condition from the Sep-Pak RP-C_{18} cartridge was separated by reverse phase HPLC. It showed a series of peaks corresponding to the fractions of Pvd38 (Fig. 1A). Molecular mass analysis revealed that Pvd38 had m/z values of 1,064.57, 1,063.70, 1,062.74, and 1,047.91 (major), and 1,036.04, 1,035.80, and 1,019.67, (minor). It is known that bacterial lipopeptides and cyclic peptides, including pyoverdines, are generally produced as a mixture with different modifications such as hydroxylation, amidation of carboxylic acid, and differ-

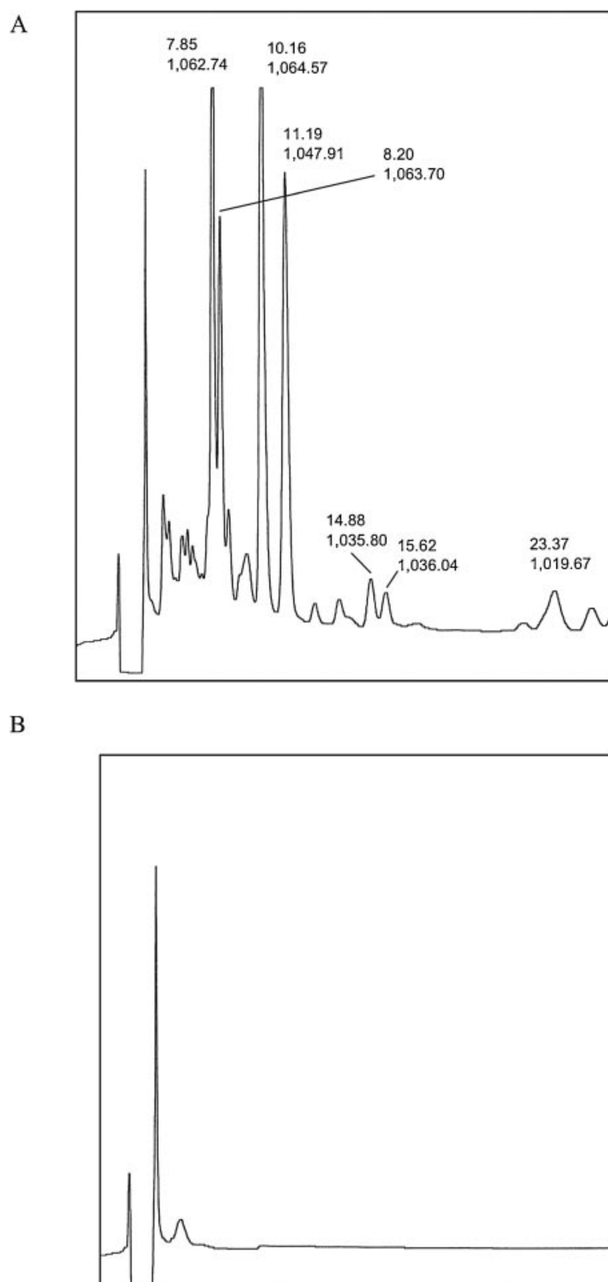

Fig. 1. LC/MS Analysis of Pyoverdines from MIS38 (A) and MT21 (B).

Upper and lower case numbers of the major peaks indicate retention times and m/z values respectively. Elution condition was 10% acetonitrile containing 0.1% TFA.

ent alkyl chain lengths in the fatty acid part.[16,19) The fact that all the peaks were eluted under the 10% acetonitrile condition on the HPLC suggested that Pvd38 contains several hydrophilic amino acids in the peptide part.

Amino acid composition analysis

It has been found that the structure of the chromophore part of pyoverdines is highly conserved among molecular species, while the peptide chains and dicarboxylic acid bound to the NH_2 group of the chromophore part are various.[20] We analyzed the peptide structure of a Pvd38 (m/z 1,064.47) by amino acid composition analysis. We found alanine (Ala), glutamic acid (Glu), glycine (Gly), serine (Ser), threonine (Thr), and an unknown amino acid X at a ratio of 2:1:1:1:1:1. In the vast majority of known Pvd obtained from *P. fluorescens* strains, there is always a hydroxyornithine (OHOrn). Based on this knowledge, it is highly probable that X is OHOrn. We could not test this possibility because standard OHOrn for amino acid analysis was not commercially available. Pvd38 was indeed a hydrophilic compound, since four of amino acids out of the seven identified were hydrophilic amino acids Glu, Ser, Thr, and putative OHOrn, and there were no hydrophobic amino acids in the molecule, such as Ile, Leu, Val, or Phe.

To the best of our knowledge Pvd38 is a new compound whose structure is different from any other known pyoverdine.[16,20]

Structure of pyoverdine chromophore part synthetase from MIS38, Pvd38-chr

Pseudomonas sp. MIS38 is known to produce arthrofactin, and the structure of arthrofactin synthetase (ArfA/B/C) is unique. ArfA/B/C does not have an E-domain to convert the L-form amino acid to the D-form, but has dual functional C/E-domains instead. We were interested whether the synthetase for the Pvd38 chromophore part has dual functional C/E-domains or a conventional E-domain like other *Pseudomonas*. Hence, *pvd38-chr* was obtained and the structure of the enzyme encoded was analyzed.

There was an open reading frame composed of 13,137 bp in *pvd38-chr* that encoded four NRPS modules (Mod1, 2, 3, 4) (Fig. 2). A BLAST homology search of the deduced amino acid sequence, 4,379 amino acids, showed high identity with pyoverdine chromophore part synthetases from plant pathogenic *P. fluorescens* Pfo-1 (93%, YP_349668), *P. syringae* pv. *Tomato str*. DC3000 (78%, AA055652), fungal antagonistic *P. fluorescens* ATCC 17400 (80%, AAF40219), opportunistic human pathogenic *P. aeruginosa* PAO1 (75%, AAG05812), and *P. putida* KT2440 (72%, NP_746359). Although the complete genome sequence of Pfo-1 has been determined, there are no experimental data convincing as to the function of this gene product. The 16 S rRNA gene sequence of MIS38 (AB028924) was 99.4% [504/507] identical to Pfo-1 (NC_007492). Moreover, we found that the genes encoding putative ArfA/B/C existed in the genome sequence of Pfo-1 (YP_347943/YP_347944/YP_347945). These facts strongly suggest that MIS38 is a close relative of *P. fluorescence* Pfo-1.

As is characteristic of Pvd-chr synthetase, the first module, Mod1, represented high homology with acyl-CoA ligases, which are involved in the fatty acid synthesis pathway.[21] Mod1 catalyzes incorporation of the dicarboxyl group to the chromophore part.[22] Mod2,

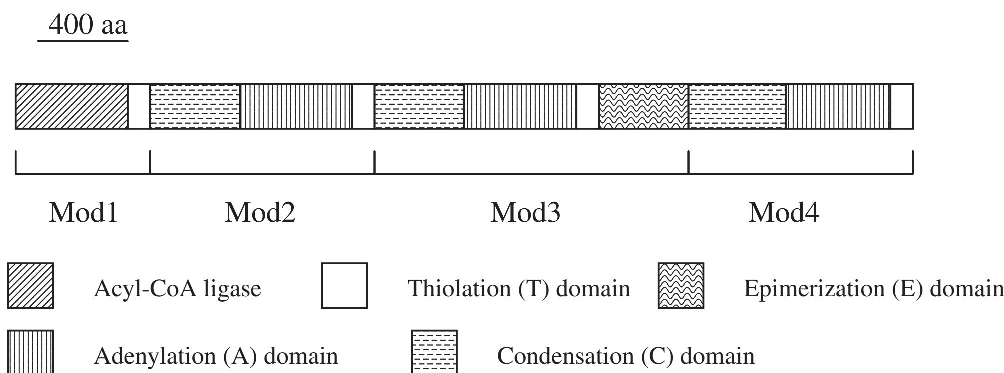

Fig. 2. Schematic Diagram Showing the Structure of the Pvd38-chr.

The substrate for each module was predicted as follows; Mod1, acyl-CoA; Mod2, L-glutamic acid; Mod3, L-tyrosine; Mod4, L-diaminobutyric acid (Table 1).

Table 1. Amino Acid Alignment of Pvd-chr Synthetase A-Domains from Several Fluorescent *Pseudomonas* sp. with Those of GrsA (P0C061).[24] FenA (AAB80955), Pvd38-Mod2 (AB244732), Pvd-Pfo-1 (YP_349668), PvsA (AA40219). Bold italics correspond to signature amino acid residues and the substrate.

A-domain	235	236	239	278	299	301	322	330	331	Substrate
GrsA	***D***	***A***	***W***	***T***	***I***	***A***	***A***	***I***	***C***	***L-Phe***
FenA-M2	***D***	***A***	***W***	***H***	***F***	***G***	***S***	***V***	***E***	***L-Glu***
Pvd38-Mod2	D	V	W	H	F	G	R	I	N	
Pvd-Pfo-1-M2	D	V	W	H	F	G	R	I	N	
PvsA-M2	D	V	W	H	F	G	R	I	N	
TycC-M3	***D***	***A***	***L***	***G***	***T***	***G***	***E***	***V***	***V***	***L-Tyr/L-Trp***
Pvd38-Mod3	D	A	E	G	I	G	A	V	M	
Pvd-Pfo-1-M3	D	A	E	G	I	G	A	V	M	
PvsA-M3	D	A	E	F	I	G	A	V	M	
SyrE-M3	***D***	***L***	***E***	***W***	***N***	***T***	***T***	***V***	***S***	***L-Dab***
Pvd38-Mod4	D	I	W	E	L	T	A	D	D	
Pvd-Pfo-1-M4	D	I	W	E	L	T	A	D	D	
PvsA-M4	D	I	W	E	L	T	A	D	D	

3, and 4 represented the typical module structure of NRPSs, in which all of the core domains (the C-, A-, and T-domains) were found.[1] It was found that Mod3 contained a conventional E-domain.

A method of predicting amino acid recognition by the A-domain in a NRPS module has been proposed.[23,24] It is based on amino acid alignments with gramicidin S synthetase (GrsA) at positions 235, 236, 239, 278, 299, 301, 322, 330, and 331, whose structures represent a substrate binding pocket that most NRPSs can fit. The respective amino acid residues at these positions of Pvd38 were D/V/W/H/F/G/R/I/N [Mod2], D/A/E/G/I/G/A/V/M [Mod3], and D/I/W/E/L/T/A/D/D [Mod 4]. These are the same constituents in the same order as those of known Pvd-chr synthetase from other represented *Pseudomonas* spp. (Table 1). The structure of the substrate binding pocket of Mod2 was highly similar to the L-glutamate incorporating modules in fengycin synthetase.[25] Mossialos *et al.* analyzed the Mod3 structure of Pvd by constructing a three-dimensional model based on the co-ordinate of the GrsA A-domain, and suggested the possibility of activating L-Tyr or L-TOPA (L-tri-hydroxyphenylalanine).[22] Structure prediction of Mod4 indicated the presence of a glutamate and a tryptophan residue at the bottom of the pocket (positions 239 and 278), allowing the hydrogen bond with the terminal amino group of substrate Dab (diaminobutyric acid). In summary, Mod2, Mod3 and Mod4 represented units of enzyme that accept, activate, and bind L-Glu, L-Tyr or L-TOPA, and L-Dab respectively. Since a conventional E-domain was found in Mod3, L-Tyr should be transformed to the D-form in the synthetic pathway. It has been proposed that the precursor of Pvd-chr is gamma-L-Glu-D-Tyr-L-Dab, and D-Tyr and that L-Dab are then condensed to a tetrahydropyrimidine ring.[22,26]

In most NRPSs, release of the product peptide is catalyzed out by a Te-domain located at the C-terminal end of the synthetase. Because no Te-domain was found in Pvd38-chr, it should be contained in another part of Pvd38 synthetases that is cooperatively functional for the synthesis of the peptide part. When we analyzed the

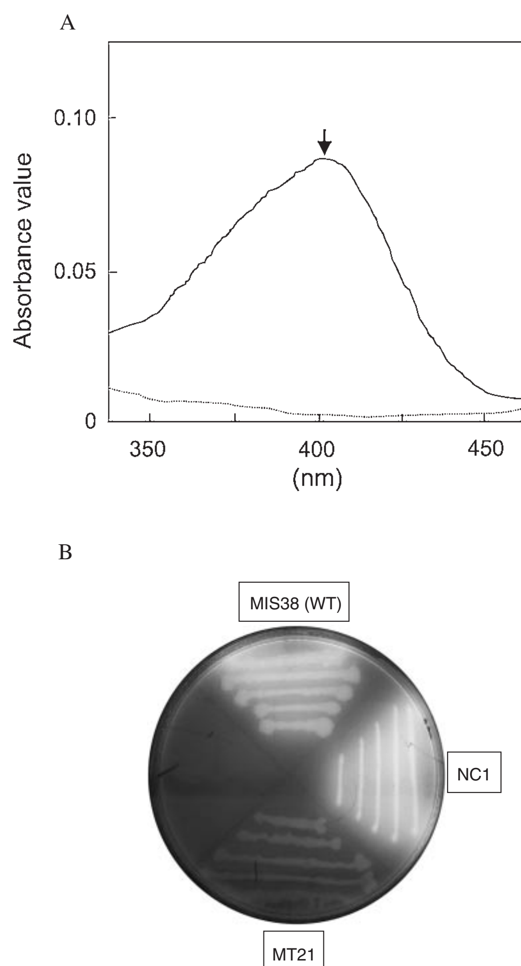

Fig. 3. Production of Pyoverdine by MIS38 and Mutant MT21.
A, Cells were grown on CAA for 24 h. MIS38 culture showed maximum absorbance at 403 nm (solid line) while this peak was absent for MT21 (dotted line). An arrow indicates the position of 403 nm. B, Pyoverdine producing MIS38 and NC1 showed fluorescent zones around the colonies under UV light (325 nm), while non-producing MT21 did not.

genome sequence of *P. fluorescens* Pfo-1, there was a putative Pvd peptide part synthetase gene 2.3 Mbp away from *pvd-chr*. This enzyme indeed carried a Te-domain at its C-terminal end.

Characterization of pvd38-chr *gene disruption mutant MT21*

Mutant strain MT21, which contains the *kan* gene in the E-domain of *pvd38-chr*, was obtained as described in "Materials and Methods." The CAA culture supernatant of wild type MIS38 showed signature absorbance at 403 nm, verifying production of Pvd38. When mutant MT21 was similarly grown in CAA medium, there was no absorbance peak at 403 nm in the culture (Fig. 3A). When the CAA agar plate was illuminated under UV light at 325 nm, a fluorescent zone was observed around the colony of MIS38 and NC1, and no zone was not observed for MT21 (Fig. 3B). Moreover, when the culture supernatant of MT21 was analyzed by reverse-phase HPLC, we found that all peaks corresponding to Pvd38 were entirely missing (Fig. 1B). These results clearly indicate that the *pvd38-chr* gene encodes an active NRPS responsible for pyoverdine synthesis in MIS38.

Pyoverdine is a siderophore responsible for uptake of essential iron for cell growth, but MT21 grew normally in CAA, probably because it might have another iron acquisition system. It is known that *P. aeruginosa* utilizes both a high-affinity pyoverdine system and a low-affinity pyochelin system for iron uptake when the cells are grown under an iron-limiting condition.[27] Even though *P. aeruginosa* has the ability to produce both of these two types of siderophores, pyoverdine-producing strains tend to produce little pyochelin. This is probably because that superior iron chelator, pyoverdine, first binds to Fe(III) with high affinity and then interacts with its cognate receptor. Mossialos *et al.* have reported that another siderophore, known as quinolobactin, was produced by *P. fluorescens* ATCC17400 when pyoverdine synthesis was suppressed.[22] In their research, it was found that the receptor for ferric quinolobactin was absent from pyoverdine-producing wild strains, while a pyoverdine deficient mutant produced substantial amounts of quinolobactin and its receptor. These multiple uptake systems reflect the fact that iron plays an essential role in a growth and survival of bacteria in a competitive environment.

When we compared the productivity of arthrofactin with MIS38 and MT21, they produced similar amounts of arthrofactin (data not shown). Moreover, arthrofactin-deficient NC1 formed a fluorescent zone around the colony, like MIS38, suggesting normal production of Pvd38 (Fig. 3B). These results indicate that the production pathways of arthrofactin and Pvd38 are entirely or largely independent.

Two different NRPS systems in Pseudomonas

It is interesting that a *Pseudomonas* strain holds NRPSs of clearly different lineages, such as Arf and Pvd38-chr, which adopt different conversion mechanisms of the L-form to the D-form amino acid in peptide synthesis. Most NRPSs in bacteria, especially Gram-positives, utilize conventional C- and E-domains to incorporate D-amino acids into the product peptide,[28] while other types of NRPSs such as Arf, harbor dual function C/E-domains in which the activities of both C- and E-domains are embedded in a single domain. These are found in Gram-negative bacteria, including *Pseudomonas*.[8] Although the mol%GC contents of *arf* and *pvd38-chr* were similar at about 64%, horizontal gene transfer of *pvd38* from Gram-positive bacteria may be possible. We have analyzed the phylogenetic tree of C-domains from various bacterial NRPSs. The results show that D-peptidyl donors, C-domains just downstream of an E-domain, of pyoverdines from *Pseudomonas* are exceptionally close in lineage to those of Gram-positive filamentous actinomycetes.[8] Recently,

enduracidin synthetase from a Gram-positive filamentous actinomycete, *Streptomyces fungicidicus*, has been reported to maintain a dual C/E-domain.[29] Based on the fact that both fluorescent *Pseudomonas* and *Streptomyces* constitute major soil bacterial groups, especially in the rhizospheres of plant roots, there should have been many chances to exchange genetic information in their evolution.[30]

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