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Cloning and Characterization of a *Streptomyces* Single Module Type Non-ribosomal Peptide Synthetase Catalyzing a Blue Pigment Synthesis*

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In the present study, we cloned a gene, designated *bpsA*, which encodes a single module type non-ribosomal peptide synthetase (NRPS) from a D-cycloserine (DCS)-producing *Streptomyces lavendulae* ATCC11924. A putative oxidation domain is significantly integrated into the adenylation domain of the NRPS, and the condensation domain is absent from the module. When *S. lividans* was transformed with a plasmid carrying *bpsA*, the transformed cells produced a blue pigment, suggesting that *bpsA* is responsible for the blue pigment synthesis. However, to produce the blue pigment in *Escherichia coli*, the existence of the 4′-phosphopantetheinyl transferase (PPTase) gene from *Streptomyces* was necessary, in addition to *bpsA*. The chemical structure of the pigment was determined as 5,5′-diamino-4,4′-dihydroxy-3,3′-diazadiphenoquinone-(2,2′), called indigoidine. The *bpsA* gene product, designated BPSA, was overproduced in an *E. coli* host-vector system and purified to homogeneity, demonstrating that the recombinant enzyme prefers L-Gln as a substrate. The *in vitro* experiment using L-Gln also showed that the blue pigment was formed by the purified BPSA only when the enzyme was phosphopantetheinylated by adding a *Streptomyces* PPTase purified from *E. coli* cells. Each site-directed mutagenesis experiment of Lys[598], Tyr[601], Ser[603], and Tyr[608], which are seen in the oxidation domain of BPSA, suggests that these residues are essential for the binding of FMN to the protein and the synthesis of the blue pigment.

The genus *Streptomyces* is well known for its ability to produce an enormous variety of bioactive secondary metabolites, including clinically useful antibiotics. For example, D-cycloserine (D-4-amino-3-isoxazolidone: DCS),[2] which is a cyclic structural analogue of D-alanine and is produced by *Streptomyces garyphalus* and *Streptomyces lavendulae*, is a clinical medicine for the treatment of tuberculosis (1). The biosynthesis genes for antibiotics, in general, form a cluster. In some cases, the final checkpoint in the transcriptional regulation of the cluster is controlled by a family of proteins called *Streptomyces* antibiotic regulatory proteins (SARPs), which have been characterized as transcriptional activators (2).

Many peptide antibiotics are known to be synthesized by non-ribosomal peptide synthetases (NRPSs). NRPSs, which are commonly found in microorganisms, are very large proteins containing sets of modules, each of which consists of various functional domains such as adenylation (A), condensation (C), cyclization (Cy), thiolation (T), and thioesterase (TE) domains (3). The amino acid sequence of the peptide antibiotic, which is produced by each NRPS, is determined by the order of the modules.

When an NRPS, which is formed as an apoform, takes the holoform, the T-domain of apo-NRPS must be phosphopantetheinylated (4). This post-translational modification is catalyzed by a superfamily of enzymes known as 4′-phosphopantetheinyl transferases (PPTases), which transfer the phosphopantetheinyl group from CoA to a conserved serine residue of their T-domain (4). In the process of peptide synthesis catalyzed by the holoform of NRPSs, individual amino acids are activated by the respective A-domains as amino acyl adenylates and subsequently are bound to the thiol group on the T-domains of the same modules. C-domains located downstream of each T-domain catalyze the condensation between the amino acid residues of adjacent modules so that a growing peptide chain moves from one module to the next until, finally, the completed peptide chain at the last module is released by the catalysis of the TE-domain (5, 6).

In this study, during our attempt to clone DCS biosynthesis genes from a DCS-producing *S. lavendulae* ATCC11924 by the suppression subtractive hybridization method, which is a cost-effective and powerful technique for the isolation of species-specific DNA sequences from closely related microorganisms (7, 8), we unexpectedly found that a DCS producer-originated gene, designated *bpsA*, encodes a protein classified into the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB240063.

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[2] The abbreviations used are: DCS, D-cycloserine; A-domain, adenylation domain; Ap, ampicillin; C-domain, condensation domain; Cm, chloramphenicol; Cy-domain, cyclization domain; DMF, dimethylformamide; Km, kanamycin; NMP, *N*-methylpyrrolidone; NRPS, non-ribosomal peptide synthetase; ORF, open reading frame; Ox-domain, oxidation domain; PPTase, 4′-phosphopantetheinyl transferase; RBS, ribosome-binding site; SARP, *Streptomyces* antibiotic regulatory protein; T-domain, thiolation domain; TE-domain, thioesterase domain; THF, tetrahydrofuran; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; BPSA, blue pigment synthetase A.

NRPS family. Interestingly, this NRPS, designated BPSA, is a single module type enzyme and contains an oxidation (Ox)-domain. Heterologous expression of *bpsA* in *S. lividans* and *E. coli* demonstrated that BPSA functions as a synthetase for a blue pigment, which was identified as a water-insoluble blue 3,3′-bipyridyl pigment, indigoidine (9, 10). An *in vitro* study shows that the holotype of BPSA, which was activated by *in vitro* phosphopantetheinylation, catalyzes the synthesis of the blue pigment using L-Gln as a substrate. The enzymatic kinetic parameters of BPSA were also determined. Furthermore, by the mutational analysis of the Ox-domain in BPSA, amino acid residues, which may be important for the binding of cofactor FMN, were suggested. Finally, we suggest a possible mechanism whereby the blue pigment is synthesized by BPSA. To the best of our knowledge, this is the first report that characterizes the single module type NRPS catalyzing a pigment synthesis.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—*S. lavendulae* ATCC11924 and *S. lavendulae* JCM4055 are a DCS producer and a DCS-non-producer, respectively. Both *Streptomyces* strains were grown at 28 °C in a YEME medium (11) for the preparation of genomic DNA. *S. lividans* 66 was grown in a YEME medium containing 0.5% glycine at 28 °C for the preparation of protoplast. For blue pigment production, *S. lavendulae* ATCC11924 was cultured at 28 °C in medium B (12). Plasmid pIJ702 carrying the thiostrepton-resistance gene was used as a vector for *S. lividans* 66.

Escherichia coli DH5α and pUC19 were used for DNA cloning and sequencing. *E. coli* BL21(DE3), pET-21a(+), and pET-28a(+) (Novagen) were used for protein expression. Plasmid pSTV28 (TaKaRa), whose replication origin is derived from the pACYC184 vector, was used for the co-expression experiment. *E. coli* cells were grown in an LB medium (13). When necessary, appropriate antibiotics were added at the following concentrations: ampicillin (Ap), 100 μg/ml; kanamycin (Km), 30 μg/ml; and chloramphenicol (Cm), 34 μg/ml.

DNA Manipulations—The genomic and plasmid DNAs of *Streptomyces* were isolated following a standard protocol (11). The plasmid DNA in *E. coli* was isolated using the Wizard® *Plus* Minipreps DNA Purification system (Promega).

Southern Hybridization—Southern hybridization was performed using a Hybond-N+ (Amersham Biosciences) membrane. Probe labeling, hybridization, and detection were performed with the AlkPhos Direct Labeling and Detection system (Amersham Biosciences) according to the protocol supplied by the manufacturer.

Construction and Screening of Subtractive Hybridization Libraries—Subtractive hybridization between *S. lavendulae* ATCC11924 as a tester and *S. lavendulae* JCM4055 as a driver was carried out using a PCR-select bacterial genome subtraction kit (Clontech) according to the manufacturer's instruction manual, except that the hybridization temperature was set to 75 °C. The secondary PCR products, which were enriched for the *S. lavendulae* ATCC11924-specific DNA fragment, were subcloned into pGEM-T (Promega) and introduced into *E. coli* DH5α. Approximately 120 white colonies were randomly selected, and plasmid DNA was isolated from each clone. To eliminate nonspecific clones, each plasmid DNA was arrayed on Hybond-N+, and differential screening was conducted by dot-blot hybridization at 72 °C. In this case, AluI-digested genome DNAs from the tester and driver were used as probes. Ninety candidates carrying approximately a 0.5-kb DNA fragment specific to ATCC11924 were selected. The tester-specific DNA fragments obtained were analyzed to determine their nucleotide sequences followed by a BLAST search and Frame plot analysis (14). Four clones were selected by the above screening. A DNA fragment (0.5-kb) from one of four clones, designated SA49, was used for the cloning of a larger DNA fragment (see below).

Cloning of a 6-kb BamHI DNA Fragment Containing the Subtracted Region from S. lavendulae ATCC11924—To clone the DNA fragment containing the subtracted region (SA49) from *S. lavendulae* ATCC11924, Southern hybridization was performed against restriction enzyme-digested genomic DNA using the SA49 as a probe. The digestion with BamHI gave a single band having ~6-kb. The DNA fragments were eluted from agarose gels, purified, ligated to the BamHI-digested pUC19, and introduced into *E. coli* DH5α. Positive clones, which were selected by colony hybridization, were finally confirmed by Southern hybridization. One of the positive clones, designated A49, was analyzed to determine its DNA sequence.

DNA Sequencing and Homology Analysis of the Predicted Proteins—DNA sequencing was performed with ABI Prism 310 and 377 DNA sequencers (Applied Biosystems) using the Big-Dye terminator cycle sequencing ready reaction kit ver.1.1. The DNA and protein sequences were analyzed with GENETYX ver.7 for Windows (Software Development). The open reading frames (ORFs) were predicted using a FramePlot ver.2.3.2 (14). A homology search was conducted with the FASTX program at DDBJ. The domain structure of the predicted protein was analyzed using the Conserved Domain data base and Search Service, v2.05, at NCBI.

Expression of bpsA in S. lividans 66—A 4.8-kb DNA fragment containing *bpsA* (=*orfB*), which was obtained from the A49 clone by double digestion with BamHI and KpnI, was ligated to pIJ702-digested with BglII and KpnI to generate pIJA49/*bpsA*. After the chimeric plasmid was introduced into *S. lividans* 66 by the protoplast transformation, the resulting transformant was regenerated on an R5 medium (11). The regenerated candidate was inoculated into a YEME medium containing 50 μg of thiostrepton/ml and grown at 28 °C.

Co-expression of bpsA in E. coli with the Gene svp Encoding a PPTase from S. verticillus—The gene *bpsA* was amplified by PCR with KOD-Plus-polymerase (TOYOBO) using a 5′-phosphorylated sense primer, 5′-catatgactcttcaggagaccagcgtgctc-3′ (the NdeI site is underlined), and a 5′-phosphorylated antisense primer, 5′-aagcttctcgccgagcaggtagcggatgtg-3′ (the HindIII site is underlined). The amplified *bpsA* was inserted into the SmaI site of pUC19 to yield pUC/*bpsA*. The *bpsA* was cut off from pUC/*bpsA* by digestion with NdeI and HindIII and inserted into the same sites of pET-28a(+) to generate pET/*bpsA*. The resulting BPSA was obtained as a product with a His_6 tag at the N and C termini. The gene *svp*, which encodes a PPTase from

S. verticillus (15), was amplified by PCR from the *S. verticillus* genome using a 5′-phosphorylated sense primer, 5′-catatgatcgccgccctcctgccctcctg-3′ (the NdeI site is underlined), and a 5′-phosphorylated antisense primer, 5′-ctcgagcgggacggcggtccggtcgtccgc-3′ (the XhoI site is underlined). The amplified *svp* gene was inserted into the SmaI site of pUC19 to yield pUC/*svp*. The *svp* gene was removed from pUC/*svp* by digestion with NdeI and XhoI and inserted into the same sites of pET-21a(+) to give pET/*svp*. The Svp protein was obtained as a product with a His_6 tag at the C terminus. A 2.2-kb DNA fragment, which contains *svp* under the control of the T7 promoter, was cut off from pET/*svp* by digestion with SphI and PvuI and inserted into the same sites of pSTV28 to yield pSTV/*svp*. The chimeric plasmids, pET/*bpsA* and pSTV/*svp*, were introduced into *E. coli* BL21(DE3). Co-expression of *bpsA* with *svp* in *E. coli* was performed at 18 °C.

Purification and Chemical Properties of the Blue Pigment—Production of the blue pigment in *S. lavendulae* ATCC11924 was induced by γ-nonalactone.[3] A culture broth (2 liter) of the strain at 2 h after induction by γ-nonalactone was centrifuged (4,400 × *g*, 4 °C, 10 min) to remove the mycelium, and the resulting supernatant was further centrifuged (30,000 × *g*, 4 °C, 30 min) to collect the blue pigment. The pigment was washed twice with water and methanol and dried *in vacuo*. The pigment was dissolved in Me_2SO under ultrasonication and filtered through a DISMIC-13JP filter (ADVANTEC), and 5 volumes of water were added. By centrifugation (30,000 × *g*, 4 °C, 30 min), the precipitated blue pigment was collected, washed ten times with water, twice with methanol, and dried *in vacuo*, yielding 11 mg of blue pigment.

For visible spectral analysis, the purified blue pigment was dissolved in Me_2SO, *N*-methylpyrrolidone (NMP), dimethylformamide (DMF), tetrahydrofuran (THF), and pyridine with sonication. The visible spectra were recorded on a JASCO V-550 spectrophotometer (JASCO).

For the analysis of the molecular mass by electron impact (EI)-MS, the blue pigment was dissolved in THF and subjected to a JEOL JMS-SX102A spectrometer (JEOL). For the same analysis by MALDI-TOF, the pigment was dissolved in Me_2SO and subjected to Voyager™ RP-3 (Applied Biosystems).

^1H NMR was recorded on a JEOL-JNM-LA500 spectrometer (JEOL) using tetramethylsilane as an internal standard. The blue pigment was dissolved in deuterated Me_2SO.

The IR spectrum of the blue pigment, run as a potassium bromide tablet, showed the following frequencies: 3443, 3328, 3288, 3196, 3064, 2959, 2924, 2869, 2839, 2364, 1688, 1639, 1596, 1579, 1454, 1371, 1323, 1250, 1118, 1060, 1037, 956, 885, 824, 779, 752, 718, 689, 661, and 634 in wave number.

Overexpression in E. coli and Purification of BPSA and BPSAΔTE—For the production of BPSAΔTE (1–1014), which lacks the TE-domain, the expression vector for *bpsA*ΔTE was constructed using pET-28a(+) in the same way as the full-length *bpsA*, generating pET/*bpsA*ΔTE. The PCR primers used were a 5′-phosphorylated sense primer, which was used in *bpsA* amplification, and a 5′-phosphorylated antisense primer, 5′-aagcttctgggcgacctcgcgctccag-3′ (the HindIII site is underlined). The BPSAΔTE protein was produced with the His_6 tag at both the N and C termini.

E. coli BL21(DE3) cells harboring pET/*bpsA* or pET/*bpsA*ΔTE were grown in 2.5 liters of an LB medium supplemented with Km at 18 °C. Cultivation was performed without isopropyl-1-thio-β-D-galactopyranoside induction for 30 h. Cells were suspended in a binding buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 5 mM imidazole) and disrupted by sonication, and cell debris was removed by centrifugation at 27,000 × *g* for 20 min. The resulting supernatant was applied on a His-Bind resin column (1 × 10 cm, Novagen) and then washed with a wash buffer I (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 30 mM imidazole). Elution was done with a linear gradient concentration of 30–500 mM imidazole. The fractions containing the BPSA or BPSAΔTE were collected and dialyzed against a binding buffer containing 1 mM EDTA (twice) and subsequently against a binding buffer (twice). The dialysate was reapplied on a flesh His-Bind resin column. The column was washed with wash buffer II (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 60 mM imidazole). Elution was conducted with a 60–1000 mM imidazole. Each protein purified to homogeneity was stored at −20 °C in the presence of 50% glycerol until use.

Overexpression in E. coli and Purification of Svp—*E. coli* BL21(DE3) cells harboring pET/*svp* were grown in 3 liters of an LB medium supplemented with Ap at 23 °C. Cultivation was performed without isopropyl-1-thio-β-D-galactopyranoside induction for 24 h. The cells were disrupted by sonication, and cell debris was removed by centrifugation at 27,000 × *g* for 20 min. The resulting supernatant fluid was applied on a His-Bind resin column (1 × 10 cm). After the column was washed with wash buffer II, elution was conducted with 60–500 mM imidazole. The purified Svp protein was stored at −20 °C in the presence of 50% glycerol until use.

ATP/PP_i Exchange Assay—To evaluate the acyl-adenylation of NRPS, amino acid-dependent ATP/PP_i exchange assays were performed as described previously (16). The assay solution (100 μl) contained 300 nM His_6-tagged protein, 5 mM ATP, 1.72 μM [^{32}P]PP_i (1 μCi, 60 Ci/mmol; PerkinElmer), 1 mM PP_i, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM amino acid, and 20 mM Tris-HCl, pH 7.8. After 20 min of incubation at 30 °C, the reaction was terminated by addition of 0.9 ml of a charcoal suspension containing 0.25 M perchloric acid, 1.6% activated charcoal, and 0.1 M tetrasodium pyrophosphate. The charcoal was washed twice with 1 ml of distilled water and resuspended in 0.5 ml of distilled water. After addition of liquid scintillation fluid (ACSII; Amersham Biosciences), the radioactivity was measured. The reaction mixture without the amino acid substrate was used as a control. To determine the kinetic parameters, reactions were carried out in various concentrations of L-Gln for 6 min. Experiments in which ATP was varied (7.8 μM − 2 mM) at fixed L-Gln concentrations (3 mM) gave a K_m value of 192 ± 13 μM. Thus, the ATP concentration (5 mM) used in the kinetic studies for L-Gln is saturating.

PP_i Release Assay—The PP_i release rate by the A-domain was measured by a coupled continuous-spectrophotometric assay using the EnzChek Pyrophosphate Assay kit (Molecular

[3] M. Sugiyama, unpublished data.

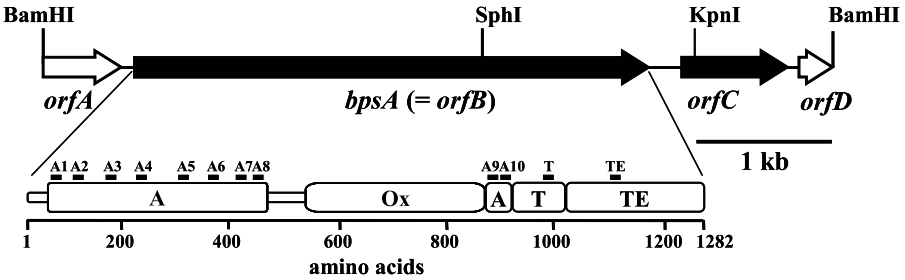

FIGURE 1. **Gene organization in a 5.9-kb DNA fragment from *S. lavendulae* ATCC11924 and the domain structure of BPSA.** *Solid* and *open arrows* indicate the complete and incomplete ORFs, respectively. The restriction enzyme sites, BamHI, SphI, and KpnI, used for the vector construction, are indicated. The location of highly conserved signature sequences within the particular domain types (A-, Ox-, T-, and TE-domains) are indicated as *bars*.

Probes). The reactions contained 20 mM Tris-HCl, pH 7.8, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM MesG, 0.2 units of purine nucleoside phosphorylase, 0.2 units of inorganic pyrophosphatase, 5 mM ATP, 3 mM L-Gln, 100 nM Svp, 0.5 mM CoA, and 100 nM BPSA. For the conversion of the apoform to the holoform, BPSA was preincubated with Svp at 30 °C for 30 min without L-Gln and ATP. The reactions were started by the addition of L-Gln and ATP and monitored every 10 s for 6 min at 360 nm. The slope of 0–100 s was correlated with a standard curve created with PP_i.

Assays for Amino Acylation of L-[^{14}C]Gln to the T-domain of BPSAΔTE—The loading of L-[^{14}C]Gln to the T-domain of BPSAΔTE was investigated by autoradiography. The reaction of 100 μl in volume contained 20 mM Tris-HCl, pH 7.8, 1 mM $MgCl_2$, 0.1 mM EDTA, 3 mM ATP, 10 μM L-[^{14}C]Gln (0.2 μCi, 210 mCi/mmol; Moravek Biochemicals), 300 nM Svp, 0.5 mM CoA, and 300 nM BPSA or BPSAΔTE and was incubated for 30 min at 30 °C to allow the phosphopantetheinylation of the T-domain prior to initiation by the addition of L-[^{14}C]Gln. At 15 min after addition of L-[^{14}C]Gln, the reaction was quenched with 0.8 ml of 10% cold trichloroacetic acid containing 2% bovine serum albumin. The precipitated protein was washed with 10% cold trichloroacetic acid and acetone and subjected to 10% SDS-PAGE. The dried gel was exposed on an imaging plate and visualized by BAS-2000.

In Vitro Synthesis of the Blue Pigment—To phosphopantetheinylate BPSA, a solution (1.4 ml) containing 660 nM BPSA, 810 nM Svp, 0.1 mM CoA, and 1 mM $MgCl_2$, which was prepared in a 50 mM sodium phosphate buffer (pH 7.8), was incubated at 30 °C for 10 min. Synthesis of the blue pigment was initiated by addition of 200 μl of 10 mM ATP (final 1 mM) and 400 μl of 5 mM L-amino acid (final 1 mM). The *in vitro* synthesis of the blue pigment was monitored by measuring the absorbance at 590 nm. The molecular mass of the synthesized blue pigment was analyzed by MALDI-TOF as described above.

MS/MS Analysis—A reaction mixture consisting of 20 mM Tris-HCl, pH 7.8, 1.5 μM BPSA, 2 μM Svp, 1 mM CoA, and 1 mM $MgCl_2$ was incubated at 30 °C for 1 h. 4′-phosphopantetheinylated BPSA was subjected to SDS-PAGE, and the corresponding band was cut off from the gel. After digestion of the protein contained in the gel by trypsin (Promega), the resulting peptide preparation was desalted with ZipTip (Millipore) and analyzed using an AXIMA-QIT (Shimadzu) mass spectrometer. A peak (m/z = 2921), which corresponds to a peptide ENASVQDDFFESGGNSLIAVGLVR (Ser^{972} is underlined) containing the carbamidemethylated 4′-phosphopantetheinyl residue, was used as a precursor ion for the MS/MS analysis.

Mutagenic Analysis—Site-directed mutagenesis was performed using the QuickChange II XL Site-directed mutagenesis kit (Stratagene) according to the supplier's instructions. The mutation in *bpsA* was confirmed by DNA sequencing. The mutants of BPSA were overproduced in *E. coli* BL21 (DE3) and purified in the same way as wild-type BPSA.

HPLC Analysis—HPLC for the analysis of the BPSA cofactor was performed using a Hydrosphere C18 column (4.6 × 200 mm, YMC). The run was conducted by a gradient elution with a 20 mM potassium phosphate buffer (pH 6.9) containing 0.1% acetonitrile (from 90 to 30%) and methanol (from 10 to 70%) for 30 min at the flow rate of 1.5 ml/min.

Nucleotide Sequence Accession Number—The sequence reported has been deposited in the DDBJ data base under the accession number AB240063.

RESULTS AND DISCUSSION

Finding an NRPS Gene in a DCS Producer S. lavendulae ATCC11924—To clone biosynthesis genes for DCS, we enriched DNA fragments specific to DCS-producing *S. lavendulae* ATCC11924 using a PCR-based subtractive hybridization method. We could not find the clone carrying the DCS biosynthesis genes. Instead, we noticed a clone harboring a 0.5-kb DNA fragment, designated SA49: a portion of the protein deduced from the nucleotide sequence had a 70% similarity to a putative regulatory protein of actinorhodin-producing *S. coelicolor* (11). We assumed that the protein encoded in SA49 may control some biosynthesis genes for secondary metabolites in *S. lavendulae* ATCC11924. Because the regulatory genes are generally clustered with the biosynthesis genes for secondary metabolites (11), a 6-kb BamHI-digested DNA fragment was newly cloned from the genome of *S. lavendulae* ATCC11924 using the SA49 DNA fragment as a probe. As shown in Fig. 1, the nucleotide sequence and FramePlot analyses (14) of the 5913-bp DNA suggest the presence of two complete ORFs, named *orfB* and *orfC*, and two incomplete ones (*orfA* and *orfD*).

The incomplete *orfA* starts out of the sequenced region. A portion of a deduced protein consisting of 193 amino acids showed high identity (60–80%) to some bacterial ribose phosphate pyrophosphokinases. The complete *orfB* consists of 3849 bp. A putative ribosome-binding site (RBS), AGGAAG, was found upstream of the start codon. The protein, encoded by *orfB*, consists of 1282 amino acids with a calculated molecular mass of 141 kDa and exhibits a significant similarity to a large number of NRPS. In particular, the protein displays a similarity with NRPSs, designated IndC (17) (GenBank™ accession number CAB87990) from *Erwinia chrysanthemi* (57% identity) and IgiD (GenBank™ accession number AAD54007) from

TABLE 1
Highly conserved core motifs of each domain in BPSA

Domain	Core	BPSA	Consensus sequence
Adenylation	A1	LTFREL (43–48)	L(S/T)YXEL
	A2	LNAGAAYLPLS (88–98)	LKAGXAY(V/L)P(L/Y)D
	A3	LAYVIYTSGSTGKPKG (166–181)	LAYXXYTSG(S/T)TGXPKG
	A4	FDAA (216–219)	FDXS
	A5	NVYGPTE (309–315)	NXYGPTE
	A6	GELYIGGIQLARGYL (365–379)	GELXIXGXG(V/L)ARGYL
	A7	YKTGDL (404–409)	Y(R/K)TGDL
	A8	GRADNQVKLRGYRVELDEIS (422–441)	GRXDXQVKIRGXRIELGEIE
	A9	LPDYMIP (892–898)	LPXYM(I/V)P
	A10	NGKIDV (912–917)	NGK(V/L)DR
Thiolation	T	DDFFESGGNSL (963–973)	DXFFXXLGG(H/D)S(L/I)
Thioesterase	TE	GYSFG (1100–1104)	G(H/Y)SXG

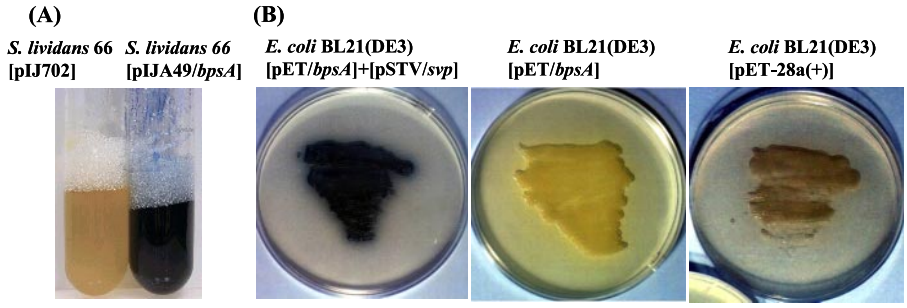

FIGURE 2. **Blue pigment production in *S. lividans* 66 and *E. coli* carrying *bpsA*.** *A*, blue pigment production in *S. lividans* 66 in a liquid medium: *S. lividans* 66 harboring pIJA49/*bpsA* was grown in a YEME medium supplemented with 50 μg/ml of thiostrepton at 28 °C for 2 days. *S. lividans* 66 harboring pIJ702 was used as the vector control. *B*, blue pigment production in *E. coli* BL21(DE3) harboring pET/*bpsA* or pET/*bpsA* together with pSTV/*svp*. Each transformant was grown on an LB medium without isopropyl-1-thio-β-D-galactopyranoside induction at 18 °C for 2 days. *E. coli* BL21 (DE3) harboring pET-28a(+) was used as the vector control.

TABLE 2
λ_{max} of the blue pigment in various solvents

Solvents	λ_{max}	
	Blue pigment	Indigoidine[a]
	nm	*nm*
Me_2SO	613	612
DMF	599	602
NMP	604	605
Pyridine	603	602
THF	583	589

[a] Data are from Kuhn *et al.* (9).

Vogesella indigofera (46% identity). Because *orfB* was found to encode a synthetase of a blue pigment, as described below, we renamed the gene as *bpsA* (blue pigment synthetase-encoding gene A). The gene *orfC*, which consists of 789 bp, encodes a protein of 262 amino acids with a calculated molecular mass of 29.5 kDa. The upstream region of *orfC* contains a potent RBS of AGGAG. The *orfC* gene product shows a significant similarity to the SARP family, which controls the secondary metabolism in *Streptomyces* sp (2). The highest similarity (59% identity) was seen with the actinorhodin operon activator protein (ActII-Orf4) from *S. coelicolor* (18), suggesting that the *orfC*-encoded protein may act as a transcriptional activator. The *orfD* gene ends out of sequence, and the deduced protein (63 amino acids), which is an N-terminal region of the protein, shows 97% identity to the *S*-adenosylmethionine synthetase from *S. spectabilis* (GenBank™ accession number Q9X4Q2).

bpsA Encodes a Single Module Type NRPS—Because the *bpsA* gene product, designated BPSA, exhibits a significant similarity to a large number of NRPS, the domain structural search of BPSA was carried out using the Conserved Domain Search Program (NCBI). We found that BPSA contains an A-domain, a T-domain, and a TE-domain at the C-terminal end; thus, the protein appears to be an NRPS of a single module type. Importantly, there is a putative Ox-domain integrated into the A-domain between the A8 and A9 signature sequences of the A-domain, and the C-domain is absent from the BPSA (Fig. 1). The motif sequences of each domain in BPSA are summarized in Table 1. The Ox-domain in BPSA exhibits similarity with those of EpoB (19) and BlmIII (16), which are involved in the biosynthesis of epothilone and bleomycin, respectively (20, 21).

The Single Module NRPS, Designated BPSA, Confers the Ability to Synthesize a Blue Pigment on S. lividans—A 4.8-kb BamHI-KpnI-DNA fragment containing *bspA* from the A49 clone was subcloned into a *Streptomyces* multicopy vector pIJ702. The resulting chimeric plasmid, designated pIJA49/*bpsA*, was introduced into *S. lividans* 66. The *Streptomyces* cells, transformed with pIJA49/*bpsA*, produced a blue pigment, but not with pIJ702 (Fig. 2*A*), suggesting that *bpsA* encodes a single module type NRPS essential for the biosynthesis of the blue pigment. In the construction of pIJA49/*bpsA*, the DNA fragment containing *bpsA* and its upstream region was inserted at the opposite direction into the melanin-synthesizing gene of pIJ702, suggesting that the cloned *bpsA* gene contains an intrinsic promoter.

BPSA Synthesizes Blue Pigment in the Presence of a Streptomyces PPTase—We tried to evaluate the blue pigment-synthesizing ability of *bpsA* in *E. coli* cells. However, *E. coli* carrying *bpsA* did not produce the blue pigment (Fig. 2*B*), suggesting that the recombinant BPSA is not phosphopantetheinylated by a PPTase produced in *E. coli* cells. Therefore, we used a gene encoding a PPTase from *S. verticillus*, designated *svp* (15), instead of the *E. coli* PPTase. As expected, the *E. coli* carrying both *bpsA* and *svp* genes produced the blue pigment clearly (Fig. 2*B*), indicating that the *svp* gene product, designated Svp, 4′-phosphopantetheinylates BPSA, and the holotype BPSA can catalyze the synthesis of the blue pigment. However, the blue pigment in *S. lividans* was successfully produced by introducing *bpsA* alone (Fig. 2*A*), showing that the PPTase derived from

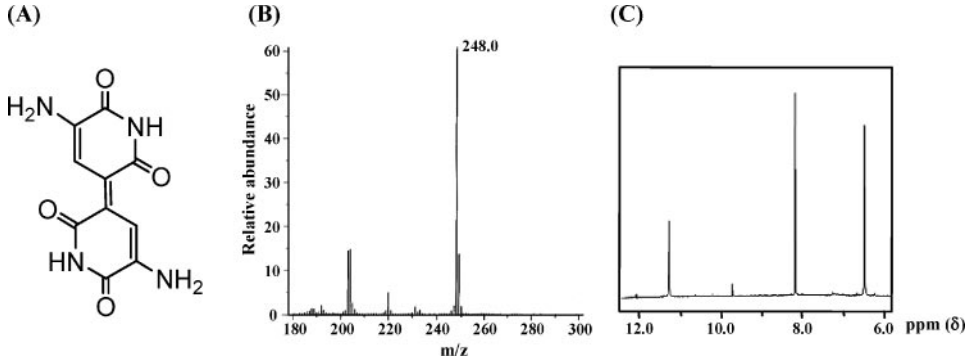

FIGURE 3. **Chemical structural analysis of the blue pigment.** *A*, structure of the blue pigment produced by *S. lavendulae* ATCC11924. *B*, mass spectrum analysis by EI-MS. The peak m/z = 248.0 was obtained and was consistent with the molecular weight of indigoidine ($C_{10}H_8N_4O_4$). *C*, ^{1}H NMR spectrum of the blue pigment. The signals obtained were 11.30 ppm (s; NH), 8.18 ppm (s; CH), and 6.46 ppm (s; NH_2).

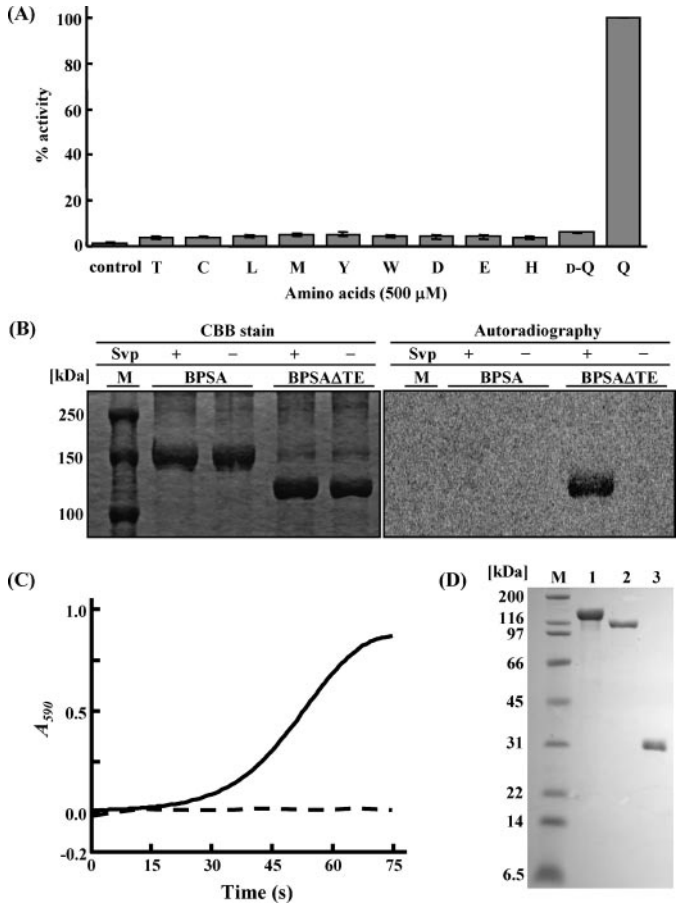

FIGURE 4. ***In vitro* analysis of BPSA and BPSAΔTE.** *A*, ATP/PP_i exchange assay of BPSA. The activity for each amino acid is expressed as relative activity to that for L-Gln. The values for 11 amino acids out of 21 amino acids tested are shown. *B*, analysis of the amino acylation of BPSA and BPSAΔTE. Proteins were stained with Coomassie Brilliant Blue (*left*), and the L-[^{14}C]Gln was visualized by autoradiography (*right*). *C*, *in vitro* blue pigment synthesis. BPSA was treated with (*straight line*) or without (*broken line*) Svp in the presence of CoA. Blue pigment synthesis was monitored by measuring the absorbance at 590 nm. *D*, SDS-PAGE profile of purified His_6-tagged proteins. The purified entire BPSA-(1–1282), BPSAΔTE-(1–1014), and Svp were analyzed by Tricine-SDS-PAGE. *Lanes M*, molecular size markers; *1*, BPSA; *2*, BPSAΔTE; *3*, Svp.

S. lividans can catalyze the 4′-phosphopantetheinylation of BPSA from *S. lavendulae*. In fact, several *Streptomyces* strains possess a PPTase that displays high similarity to Svp (15).

Chemical Structure of the Blue Pigment from S. lavendulae—We found that *S. lavendulae* ATCC11924 produces inductively the blue pigment by the addition of γ-nonalactone.[3] The *Streptomyces* blue pigment was analyzed by IR, MS, and NMR spectrometry to determine the chemical structure.

The IR spectrum of the blue pigment represented the existence of a double bond of carbon, associated amine groups, and amide. The absorption maximum of the blue pigment in various organic solvents is summarized in Table 2. These values were very similar to those of 5,5′-diamino-4,4′-dihydroxy-3,3′-diazadiphenoquinone-(2,2′), called indigoidine, as shown in Fig. 3*A* (9, 10). MALDI-TOF and EI-MS analyses yielded a peak at 248.0 m/z (Fig. 3*B*), interpreted as a radical positive ion, which corresponds to the theoretical molecular weight of indigoidine. The ^{1}H NMR spectrum of the blue pigment gave three peaks (Fig. 3*C*). The signals at 8.18 ppm, 6.46 ppm, and 11.30 ppm correspond to the protons of -C=CH-, -NH_2, and -CONH-, respectively, which can account for the structure of indigoidine. Although ^{13}C NMR analysis was hampered by the poor solubility of the pigment, we concluded that the chemical structure of the *Streptomyces* blue pigment is identical to that of indigoidine.

In Vitro Analysis of the Purified BPSA—Because BPSA is an NRPS of a single module type, the blue pigment must be synthesized from only one amino acid. In an NRPS module, amino acid recognition and activation occur by reacting with ATP bound to the A-domain. The site, which specifies an amino acid as a substrate, is located between motifs A4 and A5 in the A-domain (22, 23). The crystallographic analysis of the A-domain in gramicidin S synthetase (GrsA) in complex with L-Phe (24) revealed that eight amino acids, located between the A4 and A5 motifs, are responsible for the recognition of the amino acid utilized as a substrate. The relationship between the corresponding eight amino acids and the substrates has been investigated about many NRPSs. Now, the substrate can be easily predicted by the computer-based analysis. The result suggests that the eight amino acids seen on the A-domain of BPSA are Asp^{217}, Ala^{218}, Trp^{221}, Cys^{260}, Phe^{284}, Gly^{286}, Val^{310}, and Ile^{318}, and that the suitable substrate for the enzyme is L-Gln.

To determine the referred substrate for BPSA, we overproduced the protein and purified it to homogeneity (Fig. 4*D*). Like EpoB, which is involved in epothilone biosynthesis (20), the purified BPSA also takes on a yellow color, suggesting that FMN as a cofactor is bound to the Ox-domain of BPSA. In fact, the presence of FMN in BPSA was confirmed by measuring the characteristic absorbance of a flavin cofactor (Fig. 5*C*) and HPLC analyses (data not shown). The activation of the substrate amino acid by the purified BPSA was estimated by the ATP/PP_i exchange assay (16). Among nineteen L-amino acids, Gly, and D-Gln tested, the highest amount of radioactivity incorporated into ATP was obtained when L-Gln was used as a

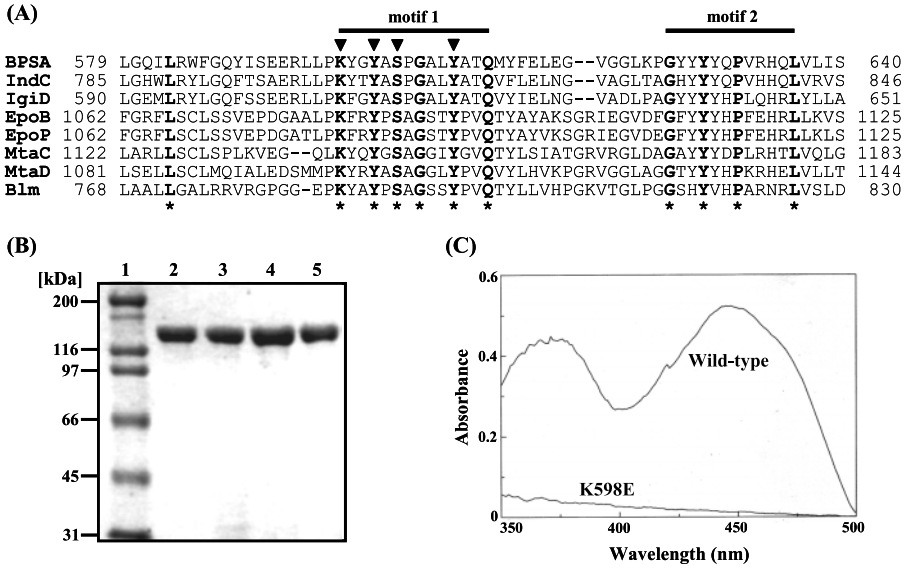

FIGURE 5. **Analysis of BPSA mutants.** *A*, alignment of the conserved region in the Ox-domains of various NRPSs. Alignment was carried out with the ClustalW program (31). The GenBank™ accession numbers are given in parentheses: IndC (CAB87990), IgiD (AAD54007), EpoB (AAF62881), EpoP (AAF26925), MtaC (AAF19811), MtaD (AAF19812), and Blm (AAG02365). The conserved residues in all sequences are shown by *bold letters* and marked with an *asterisk* below the sequences. Conserved motifs (motifs 1 and 2) are indicated by *bars* above the sequences. *Arrowheads* indicate the mutated residues. *B*, SDS-PAGE profile of the purified BPSA mutants. *Lanes 1*, molecular size markers; *2*, K598E; *3*, Y601A; *4*, S603F; *5*, Y608A. *C*, spectra of wild-type BPSA (43 μM) and K598E mutant (45 μM).

substrate (Fig. 4*A*), suggesting that the preferred substrate for BPSA is L-Gln.

The enzymatic kinetic parameter of the A-domain in BPSA for L-Gln was determined by the ATP/PP_i exchange assay. The K_m and k_{cat} values of apo-BPSA for L-Gln were 157 ± 31 μM and 324 ± 23 min^{-1}, respectively. Moreover, the PP_i release rate for the A-domain of the apoform and the holoform of BPSA was measured by a coupled-continuous spectrophotometric assay using the guanosine analogue MesG (25, 26). The conversion of the apoform to the holoform of BPSA was achieved by preincubation with the purified Svp (Fig. 4*D*). The releasing rate of the apoform was 75.8 ± 1.5 min^{-1}, whereas that of the holoform was 106.6 ± 6.6 min^{-1}. A drastic increase of the PP_i release rate by the conversion from the apoform to the holoform, which has been reported in EntE (25), was not observed in BPSA.

Amino Acylation Assay—In the blue pigment synthesis by BPSA, first, L-Gln is acyl-adenylated by the A-domain to generate L-glutaminyl-AMP. The unstable intermediate, L-Gln-AMP, is subsequently transferred to the T-domain, where it is bound as a thioester to the cysteamine group of a covalently enzyme-bound 4′-phosphopantetheine cofactor. To confirm that the acyl-adenylated L-Gln could be loaded to the 4′-phosphopantetheinylated T-domain in BPSA, we carried out an *in vitro* amino acylation assay. In this assay, BPSAΔTE, which lacks the TE-domain in BPSA, was prepared and used in addition to BPSA, because the covalently bound variant of L-Gln on the T-domain in the 4′-phosphopantetheinylated BPSA is expected to be released immediately by the TE-domain. The enzyme kinetic parameters of BPSAΔTE (Fig. 4*D*) were determined by the ATP/PP_i exchange assay. The values of K_m and k_{cat} (104 ± 15 μM and 209 ± 20 min^{-1}, respectively) were similar to those of BPSA.

The L-[^{14}C]Gln, which was tightly bound to BPSAΔTE, was detected by autoradiogram after SDS gel electrophoresis. As shown in Fig. 4*B*, the autoradiogram indicated the highly radioactive band corresponding to BPSAΔTE reacted with Svp, whereas no radioactive bands were seen in the BPSA reacted with Svp. These results suggest that L-Gln is covalently loaded on the activated T-domain of BPSAΔTE and that the TE-domain of BPSA functions to release the variant of L-Gln covalently attached on the T-domain. By MS/MS analysis, we confirmed that Ser^{972} in the T-domain of BPSA is 4′-phosphopantetheinylated with Svp (data not shown).

In Vitro Formation of the Blue Pigment by the Purified BPSA and Svp—Using both purified BPSA and Svp, we examined whether the blue pigment is synthesized *in vitro* using nineteen L-amino acids, Gly, and D-Gln as substrates. As shown in Fig. 4*C*, the pigment was clearly synthesized when L-Gln was used together with Svp, but not without Svp. The other eighteen L-amino acids, Gly, and D-Gln were not utilized even in the presence of Svp (data not shown). Furthermore, MALDI-TOF analysis of the blue pigment, which was generated *in vitro*, exhibited the same 248.0 *m*/*z* value as that produced by *S. lavendulae* ATCC11924. These results demonstrate that the blue pigment is synthesized from L-Gln by the 4′-phosphopantetheinylated BPSA.

Site-directed Mutagenesis of BPSA—BPSA has an Ox-domain integrated into the A-domain. To evaluate the function of the intricate domain, we introduced mutations into the Ox-domain of BPSA. Alignment of the putative Ox-domains of various NRPSs, which is shown in Fig. 5*A*, reveals several residues invariant in the conserved region. Among these residues, we replaced Lys^{598}, Tyr^{601}, Ser^{603}, and Tyr^{608} by Glu, Ala, Phe, and Ala to create K598E, Y601A, S603F, and Y608A, respectively.

The solution dissolving the purified wild-type BPSA displayed a yellow color, whereas that dissolving each BPSA mutant (Fig. 5*B*) did not, suggesting that these mutants may lose FMN as a cofactor. The spectrometric analysis clearly shows the absence of FMN in K598E, while the wild type contains one molar equivalent of FMN (Fig. 5*C*). The absence of FMN in three other mutants was also confirmed spectrometrically, suggesting that invariant Lys^{598}, Tyr^{601}, Ser^{603}, and Tyr^{608} residues may be essential for FMN binding. A crystallographic analysis of an oxidoreductase from *Vibrio fischeri* has shown that Lys and Arg residues stabilize a phosphate group of FMN by the formation of hydrogen bonding (27). Therefore, Lys^{598} in the Ox-domain of BPSA may form a hydrogen bond with the phosphate group of FMN.

The *in vitro* experiment showed that each of four mutants maintains an ATP/PP_i exchange activity essential for adenylation of L-Gln but not the blue pigment-synthesizing ability (data not shown). This may be a result of the absence of FMN bound in the Ox-domain. In this study, we observed that the ATP/PP_i exchange activity on the A-domain in the BPSA mutant is significantly lower (50–70%) than that in the wild-type BPSA. Because BPSA has the Ox-domain integrated into the A-domain, the adenylation activity of A-domain might be reduced if FMN is lost from the Ox-domain.

Possible Mechanism of Blue Pigment Formation Catalyzed by BPSA—Although we must clarify the mechanism whereby the blue pigment is synthesized, after the binding of the acyl-adenylated L-Gln to a thiol-group seen on the T-domain of BPSA, we propose a hypothesis at the present time: after transfer of L-Gln to the T-domain, the molecule is released from the T-domain by cyclization with forming an intramolecular amide bond. The reaction might be catalyzed by the TE-domain, as suggested by the amino acylation analysis using BPSAΔTE. We will investigate hereafter when the oxidation of the substrate by the Ox-domain occurs. Judging from a report that the oxidation by the Ox-domain of EpoB occurs when the substrate is bound to the T-domain (19), the oxidation of L-Gln by the Ox-domain of BPSA may take place when bound to the T-domain. However, we cannot rule out a possibility that the oxidation occurs after L-Gln is cyclized and released from the T-domain. We have observed an experimental phenomenon that when the Ox-domain purified from the cell-free extract of *E. coli* harboring the Ox-domain region of *bpsA* is added to the reaction mixture containing the holo-K598E mutant protein and L-Gln, the blue pigment synthesis is restored.[3] This result may support a hypothesis that after L-Gln is cyclized and released from the T-domain, the oxidation of the released intermediate will occur. The resulting oxidative intermediate may be combined between the two molecules and followed by auto-oxidation to generate the blue pigment, like the formation of indigo from indoxyl.

Genetic loci, involved in the biosynthesis of indigoidine, have been found in *E. chrysanthemi* (*indA*~*indC*) (17), *V. indigofera* (*igiA*~*igiE*) (GenBank™ accession number AF088856), and *Photorhabdus luminescens* (*plu2182*, *plu2186*, and *plu2187*) (28). It has been suggested that the *indC*, *igiD*, and *plu2186* genes in these loci encode NRPS. The present study shows that only NRPS is responsible for the biosynthesis of indigoidine in these four bacteria containing *Streptomyces* strain. The *indA*-like gene is widely distributed in bacteria (29). Interestingly, the crystal structure of an IndA-like protein (TM1464) from *Thermodoga maritima* has been determined (30). In the crystallographic analysis; however, it has been suggested that the ligand assigned for TM1464 may be a glycerol 3-phosphate analogue. Thus, the *indA*-like gene product is not involved in the biosynthesis of indigoidine, which requires L-Gln as a substrate.

The present study is the first report with respect to the characterization of a single module type NRPS, which functions as a synthetase without entering into combination with multi-NRPS module or polyketide synthase. Furthermore, using the technique of site-directed mutagenesis, we first estimated the amino acid residues essential for the oxidase activity on the NRPS. Judging from the fact that BPSA can synthesize the blue pigment from only one amino acid (L-Gln) as a substrate, the enzyme is an unusual one. Because BPSA is a single module type NRPS, its x-ray crystallographic analysis will provide new insights into the catalytic mechanism of NRPS. The crystallization of BPSA is in progress.

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Cloning and Characterization of a *Streptomyces* Single Module Type Non-ribosomal Peptide Synthetase Catalyzing a Blue Pigment Synthesis

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