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 indigoideine. 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 Lys⁵⁹⁸, Tyr⁶⁰¹, Ser⁶⁰³, and Tyr⁶⁰⁸, 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), 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 holof orm, 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 holof orm 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 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.

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‡§ 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; Nm, N-methylpyrrolidone; NRPSs, non-ribosomal peptide synthetases; ORF, open reading frame; Ox-domain, oxidation domain; PPTase, 4’-phosphopantetheinyl transferase; RBS, ribosome-binding site; SARPs, antibiotic regulatory proteins; 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® 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 BigDye 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 pIJA49bpsA. 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′-catagcactcttcgagcagccggtc-3′ (the Ndel site is underlined), and a 5′-phosphorylated antisense primer, 5′-agagtgtcctcgagcagccggttcggtc-3′ (the HindIII site is underlined). The amplified bpsA was inserted into the Smal site of pUC19 to yield pUC/bpsA. The bpsA was cut off from pUC/bpsA by digestion with Ndel 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 His6 tag at the N and C termini. The gene svp, which encodes a PPTase from...
A Single Module Type NRPS from S. lavendulae

S. verticillus (15), was amplified by PCR from the S. verticillus genome using a 5′-phosphorylated sense primer, 5′-catatgatagctgcggctctcccccgtc-3′ (the NdeI site is underlined), and a 5′-phosphorylated antisense primer, 5′-ctgacgccgagcgctgccgctgcgctgccg-3′ (the XhoI site is underlined). The amplified sgp gene was inserted into the SmaI site of pUC19 to yield pUC/sgp. The sgp gene was removed from pUC/sgp by digestion with NdeI and XhoI and inserted into the same sites of pET-21a(+) to give pET/sgp. The Sgp protein was obtained as a product with a His<sub>6</sub> tag at the C terminus. A 2.2-kb DNA fragment, which contains sgp under the control of the T7 promoter, was cut off from pET/sgp by digestion with SphI and PvuI and inserted into the same sites of pSTV28 to yield pSTV/sgp. The Svp protein was obtained as a product with the expression vector for bpsA and BPSAΔTE 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 60 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 dialedyzed against a binding buffer containing 1 mM EDTA (twice) and subsequently against a binding buffer (twice). The dialysate was reapplied on a Hi-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/sgp 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 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<sub>i</sub> Exchange Assay—To evaluate the acyl-adenylation of NRPS, amino acid-dependent ATP/PP<sub>i</sub> exchange assays were performed as described previously (16). The assay solution (100 μl) contained 300 mM His<sub>6</sub>-tagged protein, 5 mM ATP, 1.72 μM [32P]PP<sub>i</sub> (1 μCi, 60 Ci/mm; PerkinElmer), 1 mM PP<sub>i</sub>, 1 mM MgCl<sub>2</sub>, 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% 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<sub>m</sub> value of 192 ± 13 μM. Thus, the ATP concentration (5 mM) used in the kinetic studies for L-Gln is saturating.

PP<sub>i</sub> Release Assay—The PP<sub>i</sub> 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.
A Single Module Type NRPS from S. lavendulae

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 MgCl₂, 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 mM Svp, 0.5 mM CoA, and 100 mM 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 molecular mass of the synthesized blue pigment was measured by MALDI-TOF as described above.

10% SDS-PAGE. The dried gel was exposed on an imaging plate with 10% cold trichloroacetic acid and acetone and subjected to bovine serum albumin. The precipitated protein was washed with 0.8 ml of 10% cold trichloroacetic acid containing 2% form, 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 PP₆.

Assays for Amino Acylation of [14C]Gln to the T-domain of BPSAΔTE—The loading of [14C]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 MgCl₂, 0.1 mM EDTA, 3 mM ATP, 10 μM [14C]Gln (0.2 μCi, 210 Ci/mmol; Moravek Biochemicals), 300 mM Svp, 0.5 mM CoA, and 300 mM 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-Gln. At 15 min after addition of L-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 mM Svp, 0.1 mM CoA, and 1 mM MgCl₂, 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 mM BPSA, 2 μM Svp, 1 mM CoA, and 1 mM MgCl₂ 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 ENASVQD-

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 CA87990) from Erwinia chrysanthemi (57% identity) and IgiD (GenBank™ accession number AAD54007) from DFFESGGNSLIAVGLVR (Ser⁷⁷² 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 XI 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
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 EpO (19) and BmIII (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 bpsA from the A49 clone was subcloned into a Streptomyces multicopy vector pIJ702. The resulting chimeric plasmid, designated pJA49/bpsA, was introduced into S. lividans 66. The Streptomyces cells, transformed with pJA49/bpsA, produced a blue pigment, but not with pIJ702 (Fig. 2A), suggesting that bpsA encodes a single module type NRPS essential for the biosynthesis of the blue pigment. In the construction of pJA49/bpsA, the DNA fragment containing bpsA and its upstream region was inserted at the opposite direction into the melamin-synthesizing gene of pIJ702, suggesting that the cloned bpsA gene contains an intrinsic promoter.

BPSP Synthesizes Blue Pigment in the Presence of a Streptomycetes 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. 2B), 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. 2B), 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. 2A), showing that the PPTase derived from

TABLE 2

| Solvents | λ<sub>max</sub> in Blue pigment | λ<sub>max</sub> in Indigiodine |
|----------|-------------------------------|-----------------------------|
| Me<sub>2</sub>SO | 613  | 612  |  
| DMF | 599  | 602  |  
| NMP | 604  | 605  |  
| Pyridine | 603  | 602  |  
| THF | 583  | 589  |  

* 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 orfC—orfD encoded. orfD 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

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 pJA49/bpsA was grown in a YEME medium supplemented with 50 μg/ml of thioistrepton at 28 °C for 2 days. S. lividans 66 harboring pIJA49/E. coli 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.
A Single Module Type NRPS from *S. lavendulae*

![Chemical structural analysis of the blue pigment](image)

**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_{8}N_{4}O_{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$_2$).

*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. 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'-diazadiaphenoquinone-(2,2'), called indigoidine, as shown in Fig. 3A (9, 10). MALDI-TOF and EI-MS analyses yielded a peak at 248.0 *m/z* (Fig. 3B), 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. 3C). 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$^{221}$, Tyr$^{260}$, Phe$^{284}$, Gly$^{286}$, Val$^{310}$, and Ile$^{310}$, 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. 4D). 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. 5C) 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
substrate (Fig. 4A), 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/PPi exchange assay. The $K_m$ and $k_{cat}$ values of apo-BPSA for L-Gln were 157 ± 31 nM and 324 ± 23 min$^{-1}$, respectively. Moreover, the PPi 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 A-domain of the apoform and the holoform of BPSA was shown 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 (45 µM) and K598E mutant (45 µM).

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 (CA887990), IgiD (AAD54007), EpoP (AAF62881), EpoB (AAF26925), MtaC (AAF19811), MtaD (AAF19812), and Blm (AAO2365). 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 arrowheads below the sequences.

Site-directed Mutagenesis of BPSA—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. 4C, 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. 5A, 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 with the k598e, y601a, s603f, and y608a mutants 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. 5C). 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.
A Single Module Type NRPS from S. lavendulae

The *in vitro* experiment showed that each of four mutants maintains an ATP/PPi 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/PPi 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 aminoaacylation 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 gate hereafter when the oxidation of the substrate by the Ox-domain occurs. The resulting oxidative intermediate may be combined (in the Ox-domain). In this study, we observed that the ATP/PPi exchange activity on the A-domain in the BPSA mutant is significantly lower (50–70%) than that in the wild-type BPSA.

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*A Single Module Type NRPS from S. lavendulae*