JOURNAL OF BACTERIOLOGY, Apr. 2007, p. 3133–3139 Vol. 189, No. 8
0021-9193/07/$08.00+0 doi:10.1128/JB.01850-06

Characterization of PPT_{Ns}, a Cyanobacterial Phosphopantetheinyl Transferase from *Nodularia spumigena* NSOR10[▽]

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Received 10 December 2006/Accepted 2 February 2007

The phosphopantetheinyl transferases (PPTs) are a superfamily of essential enzymes required for the synthesis of a wide range of compounds, including fatty acids, polyketides, and nonribosomal peptide metabolites. These enzymes activate carrier proteins in specific biosynthetic pathways by transfer of a phosphopantetheinyl moiety. The diverse PPT superfamily can be divided into two families based on specificity and conserved sequence motifs. The first family is typified by the *Escherichia coli* acyl carrier protein synthase (AcpS), which is involved in fatty acid synthesis. The prototype of the second family is the broad-substrate-range PPT Sfp, which is required for surfactin biosynthesis in *Bacillus subtilis*. Most cyanobacteria do not encode an AcpS-like PPT, and furthermore, some of their Sfp-like PPTs belong to a unique phylogenetic subgroup defined by the PPTs involved in heterocyst differentiation. Here, we describe the first functional characterization of a cyanobacterial PPT based on a structural analysis and subsequent functional analysis of the *Nodularia spumigena* NSOR10 PPT. Southern hybridizations suggested that this enzyme may be the only PPT encoded in the *N. spumigena* NSOR10 genome. Expression and enzyme characterization showed that this PPT was capable of modifying carrier proteins resulting from both heterocyst glycoplipid synthesis and nodularin toxin synthesis. Cyanobacteria are a unique and vast source of bioactive metabolites; therefore, an understanding of cyanobacterial PPTs is important in order to harness the biotechnological potential of cyanobacterial natural products.

Phosphopantetheinyl transferases (PPTs) are enzymes that are required for the activation of carrier proteins in the pathways for synthesis of fatty acids and a wide range of diverse metabolites, including nonribosomal peptides and polyketides (24). The PPT superfamily can be separated into two families of enzymes based on sequence and substrate specificity. The first family includes the acyl carrier protein (ACP) synthase (AcpS)-type PPTs (120 amino acids), which are involved in activating carrier proteins involved in primary metabolism, including carrier proteins involved in fatty acid synthesis (FAS). The majority of microorganisms harbor an AcpS-type PPT, which typically has a limited range of specificity for carrier proteins involved in secondary metabolism. The second group of PPTs is the Sfp-like family (230 amino acids), which exhibit similarity to the *Bacillus subtilis* PPT Sfp, which is responsible for the activation of carrier proteins in the biosynthetic pathway for surfactin (24, 34). Microorganisms which require activation of carrier proteins involved in secondary metabolism pathways, such as carrier proteins involved in nonribosomal peptide synthetase (NRPS) or polyketide synthase (PKS) pathways, require the activity of an Sfp-like PPT. Some members of the Sfp-like family, which can be further divided into the W/KEA and F/KES subfamilies (11), exhibit a wide range of activity with noncognate carrier proteins. This activity has been harnessed in diverse applications, including the Sfp labeling of carrier proteins (40). In organisms such as *Pseudomonas aeruginosa* PAO1 (13), *Haemophilus influenzae*, and *Synechocystis* sp. strain PCC 6803, an Sfp-like PPT acts in both primary metabolism and secondary metabolism due to the absence of an AcpS-type PPT in their genomes. Biosynthetic gene clusters often have a colocalized Sfp-like PPT, like that present in the gene cluster responsible for the synthesis of the telomerase inhibitor griseorhodin A in *Streptomyces* sp. strain JP95 (25). However, the majority of cyanobacterial biosynthetic clusters do not have a proximally associated PPT.

Cyanobacteria have been recognized as a useful and vast source of secondary metabolites with important pharmaceutical functions (3). These metabolites include the dolastatin family of antitumor compounds, several derivatives of which are currently in phase I and II trials (26, 27), and the cancer cell toxin curacin A, which is now in preclinical cancer trials (9). However, the production of cyanobacterial compounds in sustainable, easily manipulated heterologous hosts remains elusive. The requirement for PPTs to activate carrier proteins in biosynthetic pathways is critical for successful host selection.

Despite the plethora of screening approaches and promising natural product leads for cyanobacteria, few synthesis pathways have been characterized, and the majority of the pathways that have been characterized are involved in toxin production. These pathways include microcystin pathways in *Microcystis aeruginosa* PCC 7806 (41), *Anabaena* sp. strain 90 (38), and *Planktothrix agardhii* CYA 126 (10), a nostocyclopeptide pathway (20) identified in *Nostoc* sp. strain GSV 224, and a nostopeptolide pathway identified in *Nostoc* sp. strain ATCC 53789 (1).

Bioinformatic analyses have revealed the absence of AcpS

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▽ Published ahead of print on 16 February 2007.

enzymes in almost all cyanobacterial genomes available for analysis (11). Therefore, it is likely that Sfp-like PPTs, which are typically responsible for the activation of secondary metabolite carrier proteins, are also involved in FAS. A recent phylogenetic analysis placed all cyanobacterial PPTs in the diverse W/KEA subfamily. In this subfamily a specific, heterocyst type of PPT is associated with synthesis of the heterocyst glycolipids (11). Heterocysts are specialized nitrogen fixation cells (29) with glycolipid membranes that are made up of complex lipid and carbohydrate residues. The glycolipid membrane prevents the entry of oxygen into the cell, which would otherwise inhibit the nitrogenase enzyme. Several heterocyst genetic loci have been identified, one of which is the *hetMNI* locus associated with heterocyst glycolipid synthesis (7). The *hetMNI* locus can be examined in the published genome of *Nostoc punctiforme* ATCC 29133 and the publicly available *Nostoc* sp. strain PCC 7120, *Nodularia spumigena* CCY 9414, and *Anabaena variabilis* ATCC 29413 genomes. A PPT (HetI) gene is in this locus, which also encodes an aryl carrier protein (ArCP) in an iterative polyketide synthase (HetM) (7) and an oxidoreductase (HetN) (6). *N. spumigena* NSOR10 is also able to form heterocysts; however the *hetMNI* locus in this species has not been characterized previously.

Algal blooms of *N. spumigena* occur mainly in the estuaries and coastal waters of southern Australia (18) but have also been reported in the Baltic Sea (38), New Zealand (8), and North America (28). Many blooms are highly toxic due to the nonribosomal production of the protein phosphatase-inhibiting hepatotoxin nodularin. The biosynthesis of nodularin has been characterized, and the gene cluster has been sequenced (30). The large hybrid NRPS/PKS biosynthetic gene cluster encodes four ACPs and five peptidyl carrier proteins (PCPs). The carrier proteins require a PPT for activity, and the gene encoding this PPT is not located in or adjacent to the biosynthetic cluster. A 220-bp fragment of a PPT gene from *N. spumigena* NSOR10 was recently identified by degenerate PCR-based screening (11). This PPT was designated PPT_{Ns} (*N. spumigena p*hospho*p*antetheinyl *t*ransferase) in this study.

Due to the association of PPT_{Ns} with the heterocyst cluster, it was hypothesized that this PPT phosphopantetheinylates the HetM ArCP. Furthermore, due to the general absence of genes encoding multiple PPTs in cyanobacterial genomes, it was likely that this PPT also participates in the biosynthesis of the hepatotoxin nodularin. In this study, our aims were to express and characterize the cyanobacterial PPT PPT_{Ns} of *N. spumigena* NSOR10 and to determine this enzyme's potential role in both glycolipid synthesis and nodularin synthesis.

MATERIALS AND METHODS

Media and culturing. *N. spumigena* NSOR10 was cultured at room temperature with a cycle consisting of 12 h of light and 12 h of darkness in ASM media supplemented with 1.5% NaCl (33). DNA was extracted as previously described (4).

DNA amplification, sequencing, and analysis. PCR and sequencing reactions were carried out as previously described (11). Panhandle-based gene walking with adaptor-mediated and specific primers (Table 1) (30) was utilized to amplify the unknown genomic regions flanking the *N. spumigena* PPT fragment. The outputs from BLAST (Basic Local Alignment Search Tool), Pileup from GCG, and the multiple-sequence alignment tool in CLUSTAL X (12) were utilized for analysis and alignment of sequences. Automated sequencing was performed using the PRISM Big Dye cycle sequencing system and a model 373 sequencer (Applied Biosystems) at the Automated DNA Analysis Facility, University of New South Wales. Sequence analysis was performed using the Applied Biosystems Autoassembler software.

TABLE 1. Primers used to amplify the unknown genomic region surrounding the PPT_{Ns} gene

Primer	Sequence (5′–3′)
Panhandle Roko1	GTGGTAAACCAGTATTAGCGG
Panhandle Roko2	GTCTAGTCCACAAGGCTTTAAG
Panhandle Roko3	CCATTGTTATTCCTGGTC
Panhandle Roko4	CATTAAGGCCGCATAACCGAT
Panhandle Roko5	GCATCGTGAATCACAGTGCC
Panhandle Roko6	CAATAGTAGTGGCGTGGCAA
Panhandle Roko7	CTAAATGGCCAGCATAAGCA
Panhandle Roko8	GCTTATGCTGGCCATTTAG
Panhandle Roko9	GATGAATAGCCGGGTCAAG
Panhandle Roko10	GCGTGATGCTGTAAATGC
Panhandle Roko11	CTTGACCCGGCTATTCATC
Panhandle Thorne1	GTGATAATTCCCGGCACACAGAGC
Panhandle Thorne 2	CCGTTATTGCACTTGTAAAGAGG
T7P Adapter	CCCCTATCCACCCTTACACCTATC
T7Pr2 Adapter	CCTTACACCTATCCCTCCGTAATAC

Southern hybridization. Pure genomic DNA samples (~10 μg) were digested overnight with XbaI or XmnI used according to the manufacturer's recommendations (Promega, Australia). Digests and positive controls (0.5 to 1.0 ng of linearized plasmid) were separated by electrophoresis on 0.8% agarose gels at 60 mV for approximately 2.5 h and vacuum blotted onto a nylon membrane (Amersham). A DIG-High Prime DNA labeling kit (Roche, Australia) was utilized for Southern hybridization. Specific PPT probes were created by amplification with specific primers. Primers slrup (5′-TGTTTAAACTCACCTGTG-3′) and slrdn (5′-CCCAAGGTAACGAAACGA-3′) were used to amplify slr0495 from *Synechocystis* sp. strain PCC 6803. Primers npunf (5′-GGATCCG CGATCGCCAGTCTGAGTTC-3′) and npunr (5′-GAGCTCTTTGTGTAGTA GCGAATTATC-3′) were utilized to amplify a PPT from *N. punctiforme* ATCC 29133. Primers npptf (5′-CATGAAAGATATCACGGCGCTT-3′) and npptr (5′-GAAGATAACAAGCTTGTATTGCC-3′) were used to amplify the full-length PPT_{Ns} open reading frame. Probes were labeled by PCR with digoxigenin, and their efficiency was tested by using concentrations ranging from 100 fg to 10 ng. Hybridization was performed overnight at 40°C, stringency washes were performed with 0.5% SSC–0.1% sodium dodecyl sulfate at 65°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and signals were analyzed by chemiluminescent detection with disodium 3-(4-methoxy-spiro{1,2-dioxetane-3,2′-(5′-chloro) tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CPSD) substrate by using an LAS-3000 (FUJIFILM).

Creation of expression plasmids. The *N. spumigena* NSOR10 PPT_{Ns} gene (accession no. AY836561) was amplified with primers npptf and npptr. The 720-bp PCR-amplified product was cloned into pET30 (Novagen) to obtain the expression plasmid $pPPT_{Ns}$. The *N. punctiforme* ATCC 29133 *hetM* gene was amplified from the heterocyst *hetMNI* locus encoding the ArCP/ketoreductase HetM (accession no. ZP_00107100). Primers hetmf (5′-GCCATGGCTATAAA ACAGTCTTTC-3′) and hetmr (5′-GGGATCCGAGATTCAAGAAACC-3′) were used to amplify a 1.7-kb fragment, which was cloned into pET30 to create pHetM. The *N. punctiforme* ATCC 29133 pArCP vector for expression of *N. punctiforme* ArCP ($ArCP_{Np}$) was constructed in a similar manner utilizing primers hetmf and arcpr (5′-TAGCTCGAGAACCATCTTGCAC-3′) to amplify and clone the 260-bp ArCP domain of HetM and create pArCP.

The *N. spumigena* NSOR10 PCP and ACP (designated PCP_{Ns} and ACP_{Ns}) sequences were amplified from the hybrid NRPS/PKS *ndaC* gene (accession no. AAO64404) of the *ndaS* gene cluster responsible for production of the hepatotoxin nodularin (30). Primers nspcpf (5′-CTCGAGCAGCCTCTACAACTGC A-3′) and nspcpr (5′-GGATCCGCCAGGAGAACGGCGG-3′) and primers nsacpf (5′-GGAGCTCTTTTCCAAACATTCT-3′) and nsacpr (5′-GGGATCC TCTAAGCATTCCATCAGTC-3′) were utilized. The resulting fragments were manipulated as described above to obtain pNsACP and pNsPCP, respectively.

The *M. aeruginosa* PCP (MPCP) sequence was amplified from the hybrid NRPS/PKS *mcyG* gene (accession no. AAX73195) of the *mcyS* gene cluster responsible for production of the hepatotoxin microcystin (41). PCP primers mpcpf (5′-GGATCCTGAACAGGGA-3′) and mpcpr (5′-CTCGAGATGGCG ACGGCTCC-3′) were used to construct the expression vector pMPCP, as described above.

The gene encoding the PKS *N. punctiforme* ACP (designated ACP_{Np}) was amplified from the putative nostopeptolide NosB gene cluster of *N. punctiforme* ATCC 29133. This species has been shown to produce nostopeptolide (21), and

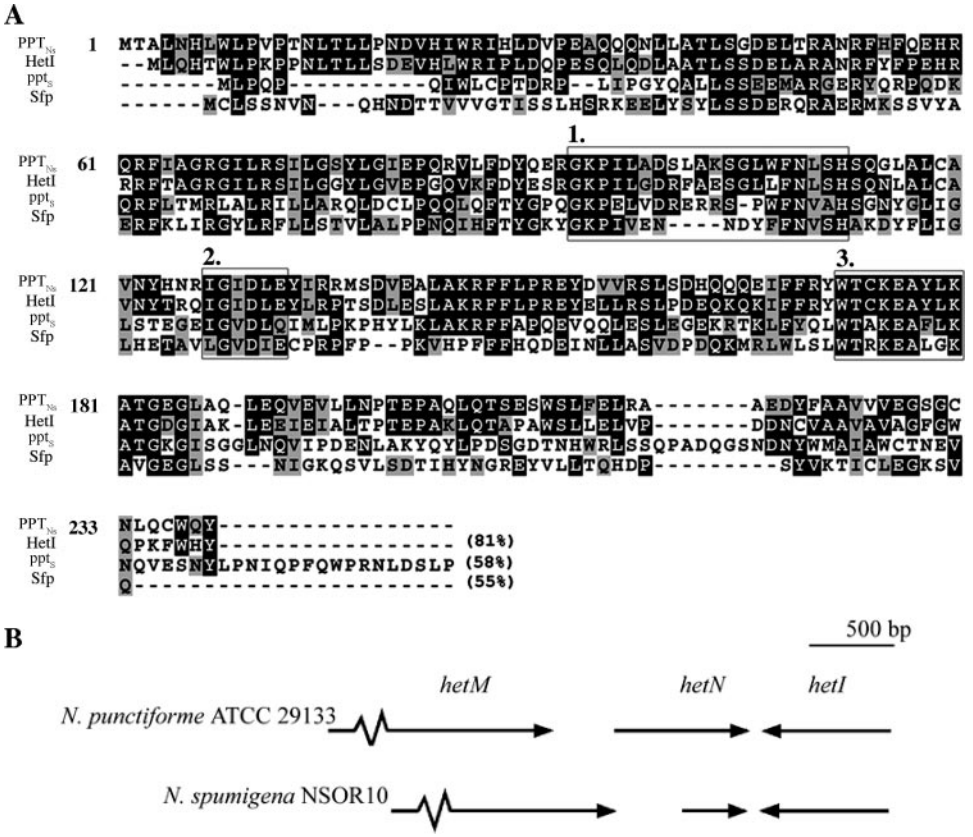


FIG. 1. (A) Alignment of PPT representatives. PPT_{Ns} from *N. spumigena* NSOR10 was aligned with HetI from *Nostoc* sp. strain PCC 7120 (accession no. P37695), ppt_S from *Synechocystis* sp. strain PCC 6803 (accession no. BAA10326), and Sfp from *B. subtilis* (accession no. P39135). PPT_{Ns} numbering is shown, and the levels of similarity to PPT_{Ns} are indicated in parentheses. Black shading indicates amino acid identity, and gray shading shows amino acid similarity. PPT motifs 1, 2, and 3 are enclosed in boxes and numbered. (B) Comparison of the *hetMNI* loci of *N. punctiforme* ATCC 29133 and *N. spumigena* NSOR10. A partial segment of the 1,520-bp *hetM* sequence is indicated by a broken arrow. The arrows indicate the direction of gene transcription.

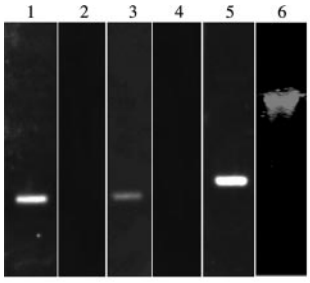
the gene cluster is similar to the characterized nostopeptolide gene cluster in *Nostoc* sp. strain GSV224 (20). NosB (accession no. ZP_00110898) is a modular type 1 PKS encoded by a gene in this cluster. The gene encoding C-terminal NosB ACP was amplified utilizing primers npacpbf (5′-GGATCCTAAAATCT AGGCTAG-3′) npacpsr (5′-GAGCTCAAATTGTTATTTCTT-3′) and was cloned as described above to obtain pNpACP.

Protein expression and purification and enzyme activity analysis. Plasmids pNsPPT, pArCP, and pMPCP were utilized for expression by incubating them at 30°C for 4 h with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for induction. ACP_{Np} was expressed at 22 to 24°C with 0.1 mM IPTG for 6 h. HetM was expressed at 18°C overnight with 0.1 mM IPTG. $ArCP_{Np}$ was expressed at 37°C for 2 h with 1 mM IPTG. After expression, cells were pelleted by centrifugation at 4,000 × *g* and frozen at −80°C overnight. Pellets were thawed on ice, resuspended in 5 ml of 50 mM HEPES (Sigma)–150 mM NaCl (pH 7.4), and either subjected to three passages through a cooled French pressure cell at 1,000 lb/in^2 or sonicated at 4°C and 30% amplitude for 25 s with 0.5-s pulses. The soluble fraction was collected after centrifugation at 20,000 × *g* for 30 min at 4°C. A HiTrap chelating column (Amersham) was utilized for purification of the recombinant proteins. Fractions containing the desired protein, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were pooled, desalted, and snap frozen in 50 mM HEPES–150 mM NaCl–8% glycerol (pH 7.4) for storage at −80°C. Protein concentrations were determined using the calculated extinction coefficient of each protein, as follows; PPT_{Ns}, 37,650 cm^{-1} M^{-1}; HetM, 67,430 cm^{-1} M^{-1}; $ArCP_{Np}$, 8,250 cm^{-1} M^{-1}; MPCP, 6,970 cm^{-1} M^{-1}; and ACP_{Np}, 13,940 cm^{-1} M^{-1}.

PPT assays were carried out as previously described (13). In brief, 100- to 400-μl reaction mixtures containing 5 mM Tris-HCl (pH 7.4), 12.5 mM $MgCl_2$, 0.5 mM coenzyme (CoA), 2 μM dithiothreitol, carrier proteins at concentrations of 10 to 100 μM, and 300 nM (final concentration) PPT were incubated at 37°C for 30 min. The reactions were terminated by addition of 1 ml of 10% trichloroacetic acid. Assay mixtures were precipitated overnight at −20°C before centrifugation at 4°C for 15 min at 16,000 × *g*. Protein pellets were analyzed by electrospray ionization mass spectrometry. Spectra were acquired using an API QStar Pulsar *i* hybrid tandem mass spectrometer (Applied Biosystems). Samples (~200 to 400 fmol) were dissolved in water-acetonitrile-formic acid (50:49:1) and loaded (1 μl) into nanospray needles (Proxeon), and the tip was positioned ~10 mm from the orifice. Nitrogen was used as the curtain gas, and a 900-V potential was applied to the needle. A time of flight mass spectrometry scan was acquired (*m*/*z* 550 to 2000, 1 s) and accumulated for ~1 min in a single file. Spectra were deconvoluted using the Bayesian reconstruction method contained in the Analyst QS software.

RESULTS

Sequencing and analysis of the *hetMNI* locus from *N. spumigena* NSOR10. Flanking regions of the partial PPT gene fragment were amplified to allow sequencing of 3,450 bp of the *N. spumigena* NSOR10 *hetMNI* locus (accession no. AY836561). The sequence analysis revealed the 240-residue PPT PPT_{Ns}, a 27,555-Da protein with an isoelectric point of 6.1. PPT_{Ns} exhibited similarity to HetI from *Nostoc* sp. strain PCC 7120 (81%) and to Sfp from *B. subtilis* (55%). PPT_{Ns} is encoded in reverse orientation compared to the *hetM* and *hetN* genes, as observed in the *hetMNI* locus of the heterocyst-forming cyanobacteria *N. punctiforme* ATCC 29133 (Fig. 1),



Probe	Positive control (plasmid)	*N. spumigena* NSOR10
ppt_S	+ (Lane 1)	– (Lane 2)
$ppt3_N$	+ (Lane 3)	– (Lane 4)
PPT_{Ns} gene	+ (Lane 5)	+ (Lane 6)

FIG. 2. Southern hybridization of PPT gene probes to digested *N. spumigena* NSOR10 genomic DNA. A plus sign indicates that a band was detected by chemiluminescence, and a minus sign indicates that no bands were visible. For positive controls we utilized linearized plasmid DNA. The lane numbers in parentheses indicated the lanes in the gel. ppt_S, PPT gene (accession no. BAA10326) from *Synechocystis* sp. strain PCC 6803; $ppt3_N$, gene (accession no. ZP_00110892) encoding a PPT in *N. punctiforme* ATCC 29133; PPT_{Ns}, gene encoding a PPT in *N. spumigena* NSOR10.

Nostoc sp. strain PCC 7120, and *A. variabilis* ATCC 29413.

The predicted protein products of the genes downstream of the PPT_{Ns} gene in *N. spumigena* NSOR10 were analyzed and compared to homologous proteins. The protein product of *hetN* is 126 amino acids long and exhibits 83% similarity to the C-terminal half of the corresponding protein in *A. variabilis* ATCC 29413. The partial *hetM* gene, encoding an iterative PKS in *N. spumigena*, exhibited 90% similarity to the gene encoding a homologous protein in *N. punctiforme* ATCC 29133, *hetM* (also designated *hglB*). No open reading frames were detected in the sequenced region extending 900 bp upstream from the PPT_{Ns} gene.

Southern hybridization. Southern analysis was utilized to ascertain the number of PPTs encoded in the *N. spumigena* NS0R10 genome. Probes were constructed from the genes encoding a diverse range of cyanobacterial PPTs, including the PPT_{Ns} gene, ppt_S in *Synechocystis* sp. strain PCC 6803 (accession no. BAA10326), and the gene encoding a PPT in *N. punctiforme* ATCC 29133 (accession no. ZP_00110892) designated $ppt3_N$. These PPTs are produced by organisms belonging to distinct cyanobacterial phylogenetic clades (11). Hybridizations performed with the PPT_{Ns} gene probe revealed a single band when *N. spumigena* NSOR10 was used (Fig. 2), and no hybridization was detected with the ppt_S- or $ppt3_N$-specific probes. Taken together, these results suggest that PPT_{Ns} may be the only genomically encoded PPT in *N. spumigena* NSOR10. However, the single band detected by Southern analysis did not exclude the slight possibility that there were two PPTs encoded by the single digested genomic fragment. The possibility that there was a more divergent PPT that was not detected by the specific Southern hybridization probes also could not be excluded.

Expression and purification of recombinant proteins. In order to confirm the PPT activity of PPT_{Ns}, the enzyme was expressed as a 32.7-kDa, His-tagged, soluble protein. Attempts to produce ACP_{Ns} and PCP_{Ns} from *N. spumigena* NSOR10 were not successful. Our attempts to resolve this problem included varying the expression time (2 to 24 h), temperature (18 to 37°C), IPTG induction concentration (0.1 to 1 mM), growth medium (Luria broth and tryptone phosphate), and helper plasmids (pRARE and pLysE; Novagen). As the partial *hetM* gene in *N. spumigena* NSOR10 does not encode an ArCP domain, the protein in *N. punctiforme* ATCC 29133 (designated $ArCP_{Np}$) that exhibited 90% similarity, encoded by *hetM*, was selected for expression and in vitro activation analysis. Although soluble HetM expression was observed, analysis of HetM by nanospray ion trap mass spectrometry could not be performed due to low yields. The expression plasmid encoding only the ArCP domain of HetM was therefore utilized, and the 15.6-kDa $ArCP_{Np}$ protein was subsequently expressed with suitable yields. MPCP and ACP_{Np} were expressed as 22.0- and 20.8-kDa proteins, respectively. No phosphopantetheinylation of the cyanobacterial carrier proteins was seen after expression in *E. coli* (Fig. 3 and Table 2).

PPT_{Ns} activity. Activity was detected by ionization mass spectrometry by addition of 340 Da. This mass was related to the incorporation of the phosphopantetheinyl arm of CoA transferred by the PPT to the carrier protein (Fig. 3). Mass spectra of phosphopantetheinylated carrier proteins were analyzed alongside controls (with no PPT) to verify phosphopantetheinylation by PPT_{Ns}.

PPT_{Ns} phosphopantetheinyl activity was confirmed by employing the HetM ArCP encoded by the *hetMNI* gene cluster in *N. punctiforme* ATCC 29133 ($ArCP_{Np}$). The conversion of apo-$ArCP_{Np}$ (15.75 kDa) to holo-$ArCP_{Np}$ (16.09 kDa) (Fig. 3) revealed that PPT_{Ns} can phosphopantetheinylate the HetM carrier protein which is involved in heterocyst glycolipid synthesis (Table 2).

PPT_{Ns} activity in PKS and NRPS secondary metabolism (ACPs and PCPs) was subsequently examined utilizing ACP_{Np} and MPCP (Table 2). Mass spectrometry analyses revealed addition of 340 Da of the phosphopantetheinyl moiety from CoA to each of these carrier proteins. Control reactions were performed without PPT_{Ns} (Fig. 3).

DISCUSSION

This study included the first enzymatic analysis of a cyanobacterial PPT. After incubation of PPT_{Ns} with apo-$ArCP_{Np}$, complete conversion to holo-$ArCP_{Np}$ was observed. No phosphopantetheinylation of the HetM $ArCP_{Np}$ was seen after heterologous expression in *E. coli*. Therefore, the PPTs of *E. coli* were not capable of phosphopantetheinylating the HetM ArCP from *N. punctiforme* ATCC 29133. The specificity of *E. coli* PPTs has been reported previously, and negligible phosphopantetheinylation of the *P. aeruginosa* PAO1 ArCP has been observed after heterologous expression in *E. coli* (13).

Although not conclusive, the Southern hybridization results suggest that only one PPT may be encoded in the *N. spumigena* NSOR10 genome. This suggestion is supported by the presence of only one PPT encoded in the recently available *N. spumigena* CCY 9414 genome. However, further phospho-

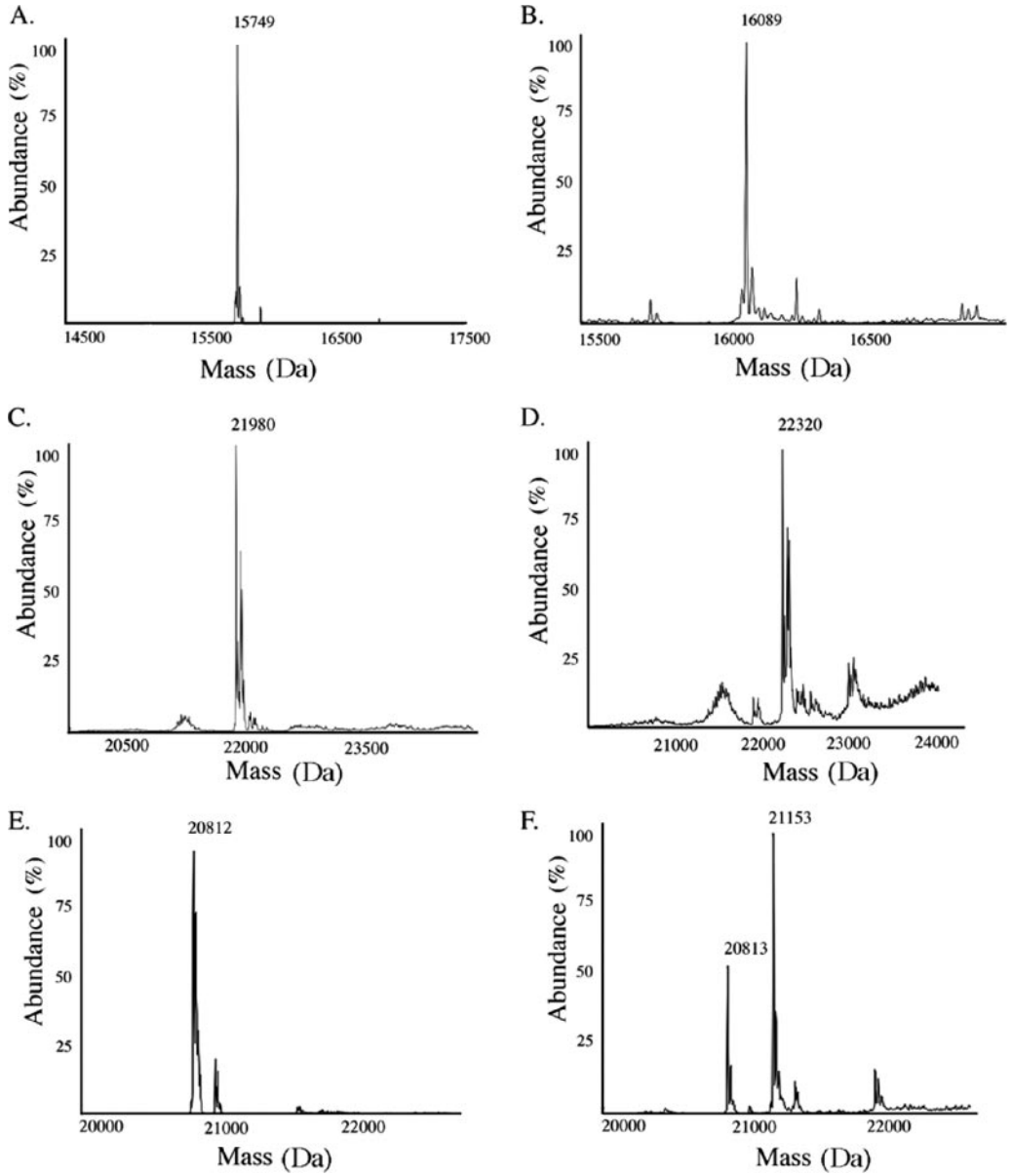


FIG. 3. Mass spectrometry of ArCP, PCP, and ACP phosphopantetheinylation. (A) Mass spectrum of $ArCP_{Np}$. (B) Mass spectrum of phosphopantetheinylated $ArCP_{Np}$ after incubation with PPT_{Ns}. (C) Mass spectrum of MPCP. (D) Mass spectrum of MPCP phosphopantetheinylated after incubation with PPT_{Ns}. (E) Mass spectrum of ACP_{Np}. (F) Mass spectrum of phosphopantetheinylated ACP_{Np} after incubation with PPT_{Ns}.

patentheinylation assays are necessary to determine whether PPT_{Ns} is able to activate the ACP involved in FAS. The possibility that PPT_{Ns} is the only PPT encoded in the genome of *N. spumigena* NSOR10 led to the hypothesis that PPT_{Ns} could also phosphopantetheinylate the PKS/NRPS carrier proteins, such as those in the hybrid PKS/NRPS multienzyme responsible for nodularin production in *N. spumigena* NSOR10 (30). However, the *N. spumigena* NSOR10 nodularin carrier proteins could not be expressed. Similar results for problematic carrier protein expression have been reported previously and have included the failure to express a *P. aeruginosa* PAO1 NRPS PCP (13) or the *Streptomyces glaucescens* NRPS PCP in *E. coli* (39). Successful expression of a PKS ACP from the *N. punctiforme* ATCC 29133 nostopeptolide A (*nosB*) biosynthetic gene cluster and an *M. aeruginosa* PCC 7806 NRPS PCP from the microcystin (*mcyG*) biosynthetic gene cluster allowed the analysis of PPT_{Ns} phosphopantetheinylation in secondary metabolism. Phosphopantetheinylation of both carrier proteins was observed in a 30-min assay, suggesting that PPT_{Ns} could also act as an efficient cofactor for the synthesis of NRPSs and PKSs in *N. spumigena* NSOR10.

Although the NRPS PCP used here is produced by a unicellular cyanobacterial species, *M. aeruginosa* PCC 7806, phosphopantetheinylation by a PPT from a filamentous, heterocyst-forming cyanobacterial species was not surprising. This PCP was from a domain in McyG, which is encoded by the *M.*

TABLE 2. Phosphopantetheinylation of carrier proteins by PPT_{Ns}, detected by addition of a phosphopantetheinyl moiety (340 Da), using mass spectrometry

Carrier protein	PPT	Expected molecular mass (Da)	Observed molecular mass (Da)	Observed molecular mass of pantetheinylated carrier protein (Da)	% Holo-carrier protein[a]
$ArCP_{Np}$ (glycolipid biosynthesis)	PPT_{Ns}	15,751	15,749	16,089	100
MPCP (microcystin biosynthesis, NRPS)	PPT_{Ns}	21,978	21,980	22,320	100
ACP_{Np} (nostopeptolide biosynthesis, PKS)	PPT_{Ns}	20,812	20,812	21,153	69

[a] The percentage of holo-carrier protein was estimated by comparing holo-carrier protein abundance and apo-carrier protein abundance in mass spectra as previously described (31).

aeruginosa PCC 7806 microcystin biosynthetic gene cluster. This 300-kDa protein is 70% identical to the corresponding NdaC protein encoded by the nodularin biosynthetic gene cluster in *N. spumigena* NSOR10. A common evolutionary pathway has been proposed for these two hepatotoxins, with transposition and NRPS module deletion thought to be responsible for the variation in the structures of the two cyclic peptides (30, 35). McyG is putatively involved in the activation of phenylpropanoids, which is the first step in the biosynthesis of the highly modified amino acid Adda (19). The unusual and complex biosynthesis of this amino acid, which occurs only in cyanobacteria, involves a hybrid NRPS/PKS.

ACP_{Np} was also phosphopantetheinylated, indicating that PPT_{Ns} can activate carrier proteins from biosynthetic pathways that are not present in *N. spumigena* NSOR10, such as those involved in nostopeptolide biosynthesis. The incomplete conversion (approximately 65% conversion to holo-ACP_{Np}) indicated that there was reduced specificity for noncognate carrier proteins. The terminal 100 amino acids (incorporating the ACP) of the putative NosB protein from *N. punctiforme* ATCC 29133 exhibit 60% similarity to the corresponding amino acids of the NdaC PKS ACP.

A similar scenario of a singular PPT participating in multiple metabolic pathways is typified by PcpS in *P. aeruginosa* PAO1 (13). This PPT was catalytically characterized to show that it is most efficient with the ACPs of FAS. It was suggested that a small reduction in the efficiency of this PPT would lead to the failure of PcpS to phosphopantetheinylate carrier proteins involved in secondary metabolism. In cyanobacteria, where the majority of species harbor a single PPT, genetic mutations could potentially prevent toxin production by inhibiting the ability of the PPT to activate carrier proteins in secondary metabolism biosynthetic pathways.

Cyanobacterial species associated with slow growth and complicated or unattainable culture requirements often include strains that harbor the gene clusters for the synthesis of pharmaceutically interesting compounds (14, 17, 37). Molecular methods may yield the genetic information (5, 30), but they have not allowed isolation and production of novel compounds without recombinant expression (2). The PPT Sfp has been used to overcome problems associated with carrier protein modification in *E. coli* heterologous hosts (23, 32); however, modification is often incomplete (15). Alternative expression hosts, including *Pseudomonas* sp. (16). and *Streptomyces* spp. (22, 36), are being investigated for heterologous expression and biochemical characterization of secondary metabolite gene clusters from cyanobacteria.

PPT_{Ns} is capable of phosphopantetheinylating a broad range of carrier protein substrates, which, based on the evidence presented here, may be a common characteristic of all cyanobacterial PPTs. If so, the occurrence of multiple PPTs in heterocyst-forming species, such as the three PPTs of *N. punctiforme* ATCC 29133, would be superfluous to the phosphopantetheinylation needs of the organism. An alternative scenario is that this broad range of activity is present only in cyanobacterial species with multiple phosphopantetheinylation pathways (FAS, NRPS, and PKS) that harbor a single Sfp-like PPT. If this is true, the multiple PPTs encoded by the genome of *N. punctiforme* ATCC 29133 would be expected to show dedication to a single phosphopantetheinylation pathway.

The ability of PPT_{Ns} to activate noncognate secondary metabolite carrier proteins has great potential for application in biotechnology. Phosphopantetheinylation of noncognate carrier proteins from alternative pathways, such as those involved in microcystin or nostopeptolide biosynthesis, has demonstrated the potential of using this PPT to harness the cyanobacterial biosynthetic pathways in heterologous hosts.

ACKNOWLEDGMENTS

The Australian Research Council and Diagnostic Technology P/L financially supported this work. J.N.C. was also supported by an Adrian Lee Travel Scholarship, University of New South Wales.

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