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The *Synechocystis* sp. PCC6803 Sfp-Type Phosphopantetheinyl Transferase Does Not Possess Characteristic Broad-Range Activity

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The cyanobacterium *Synechocystis* sp. PCC6803 harbours one phosphopantetheinyl transferase (PPTase), Sppt. Protein modelling supported previous bioinformatics analyses, which suggested that Sppt is a Sfp-type PPTase with the potential to phosphopantetheinylate a broad range of carrier proteins from both primary and secondary metabolism. However, no natural products are synthesised by this species, which raises interesting evolutionary and functional questions. Phosphopantetheinylation assays and kinetic data demonstrate that Sppt was able to activate its cognate fatty acid synthesis carrier protein, SACP, but was unable to effectively activate various cyanobacterial carrier proteins from secondary metabolism or glycolipid biosynthesis pathways. To our knowledge, this is the first example of a PPTase with a Sfp-type structure, but with activity more closely resembling AcpS-type enzymes. The broad-range PPTase from *Nodularia spumigena* NSOR10 was introduced into *Synechocystis* sp. PCC6803 and was shown to activate a non-cognate carrier protein, in vivo. This engineered strain could provide a future biotechnological platform for the heterologous expression of cyanobacterial biosynthetic gene clusters.

Introduction

Cyanobacteria produce a vast array of structurally unique secondary metabolites with useful bioactive properties, including antimicrobial, immunomodulatory and antiproliferative activities.[1–3] The filamentous species, *Lyngbya majuscula*, which produces over 100 natural products including curacins, lyngbyatoxins and microcolins, is a striking example of the diversity of natural compounds produced by cyanobacteria.[4–7] Several derivatives of cyanobacterial natural products are currently in clinical trials as cancer therapeutics, including the potent anticancer dolastatins and cryptophycins produced by *Symploca* sp. and *Nostoc* spp.[8–11]

Most cyanobacterial bioactive products are synthesised by large multimodular enzymes known as nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS). Traditional NRPS/PKSs are repeated modules made up of a minimum of three domains, which activate, tether and form bonds between the cognate amino acid or organic acid precursors.[12–14] The peptidyl- or acyl-carrier protein domain (PCP or ACP) is responsible for the transport of the nascent product along the assembly line complex, and first requires activation by a phosphopantetheinyl transferase (PPTase).[15] Carrier proteins are activated by PPTases by the addition of a 340 Da 4′-phosphopantetheinyl arm, which is covalently bound from the coenzyme A (CoA) substrate. PPTases are also essential for the activation of carrier proteins in fatty acid and heterocyst synthesis.[16–18] While all organisms require a PPTase for primary metabolism, some also encode additional PPTases that are capable of activating carrier proteins from secondary metabolite pathways.[19,20] PPTases can be classified into two main groups: the acyl carrier protein synthase (AcpS)-type PPTases, which are capable of activating ACPs from PKSs and fatty acid synthesis (FAS),[21,22] and the Sfp-type PPTases, which are named for the broad range activity PPTase from surfactin synthesis in *Bacillus subtilis* and typically activate NRPS/PKS multienzymes.[20,23] Harnessing the diverse pharmaceutical potential of NRPS/PKS compounds through heterologous expression and combinatorial biosynthesis requires the use or development of hosts that possess broad range PPTase activity.[24–27]

Within cyanobacterial species, NRPS/PKS gene clusters are more abundant among the filamentous and heterocystous strains.[28] Recently, the filamentous cyanobacteria *Nodularia spumigena* NSOR10 has been shown to harbour a broad range specificity PPTase, NsPPT, which is able to phosphopantetheinylate carrier proteins from a variety of pathways and species.[29] However, genetic techniques for the manipulation of this species have not yet been developed. Therefore, a host

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with a broad range PPTase is necessary to harness the diversity of cyanobacterial natural products.

The majority of unicellular cyanobacteria, including *Prochlorococcus*, *Synechococcus* and *Synechocystis* species do not produce secondary metabolites,[30] yet they have the potential to be efficient heterologous hosts for the expression of novel cyanobacterial derived compounds due to their fast doubling times and well characterised genetic manipulation methods.[31] Previous bioinformatics studies have suggested that *Synechocystis* sp. PCC6803 harbours a broad-range specificity PPTase capable of activating carrier proteins from secondary metabolite pathways,[19] however, this has not been confirmed experimentally. Sequencing of the *Synechocystis* sp. PCC6803 genome has revealed that this species does not contain any NRPS/PKS gene clusters, which raises interesting functional and evolutionary questions in regards to its Sfp-type PPTase. Furthermore, the lack of biosynthetic gene clusters in this strain provides a clean expression background, which would make *Synechocystis* sp. PCC6803 an ideal host for heterologous natural product biosynthesis. In this study, the functional, evolutionary and biotechnological implications of this cyanobacterial Sfp-type PPTase were explored through in silico, molecular and biochemical characterisation of Sppt.

Results

Protein modelling of Sppt

Modelling of Sppt on the crystal structure of Sfp from *B. subtilis* revealed a similar protein structure between the two enzymes (Figure 1). Sppt possesses 28% amino acid sequence identity and 52% similarity to Sfp. In contrast, Sppt could not be threaded on the *B. subtilis* AcpS crystal structure[32] (PDB ID: 1f80) by using SWISS-MODEL (data not shown); this suggested that Sppt is more similar in structure to the broad-range Sfp-type PPTases than the fatty acid specific AcpS-type. Alignment of the Sppt and Sfp amino acid sequences indicated six residues in the Sppt CoA/Mg^{2+} binding pocket that differed from that of Sfp.[33,34] E28 and Q109 (K28 and E109 in Sfp), which are involved in hydrogen bonding to CoA and Mg^{2+}, have oppositely charged side chains when compared to the Sfp residues (Figure 2). A89 and F44 (S89 and T44 in Sfp) have neutral side chains in contrast to the charged hydroxyl groups of the residues in Sfp; therefore, there are no interactions between these amino acids and CoA in Sppt. The electrostatic interactions with CoA and Mg^{2+} are not likely to be altered from those of K31 and Y73 in Sfp with the residues Q73 and R31 in Sppt.

Figure 1. Theoretical model of the Sppt enzyme (left) from *Synechocystis* sp. PCC6803 by using SWISS-MODEL, threaded on the *B. subtilis* Sfp crystal structure (right). The images were generated with PyMOL (Delano Scientific LLC).

Phosphopantetheinylation of SACP by Sppt in vitro

The genome of *Synechocystis* sp. PCC6803 only encodes one carrier protein, SACP, which is involved in FAS. The activity of Sppt towards its cognate SACP was determined by phosphopantetheinylation assays. Although SDS-PAGE analysis shows the purified SACP at an observed molecular weight of 17 kDa (Figure 3), mass spectrometry revealed the purified protein to be 14.594 kDa (Figure 4A); this corresponds to a loss of the N-terminal methionine residue when compared to the theoretical mass of 14.73 kDa. The ratio of holo- to apo-carrier protein after heterologous expression in *E. coli* was analysed by mass spectrometry. SACP was partially phosphopantetheinylated after heterologous expression in *E. coli*, with a holo:apo ratio of 30:70. After in vitro incubation with Sppt, SACP showed an increased abundance of phosphopantetheinylated carrier protein; this indicates that Sppt can activate the cognate SACP (Figure 4B).

Enzymatic analysis of Sppt activity

Phosphopantetheinylation assays and subsequent HPLC analyses were carried out to determine the pH and CoA activity range of Sppt towards its cognate SACP3 carrier protein (Figure 5). SACP3, a truncated form of SACP, was used in kinetic assays as the full-length apo protein could not be separated from the holo protein despite various experimental optimisations. SACP3 was partially phosphopantetheinylated after heterologous expresssion in *E. coli*, with a holo:apo ratio of 45:55. The optimum activity for Sppt was approximately pH 7.7, with a relatively broad pH activity range where approximately 25% of the activity was retained at pH 5.5 and 9.8. Sppt activity increased with increasing CoA concentrations and reached a maximum activity with concentrations greater than 1500 μM. The Michaelis–Menten fit of the experimental data yielded a K_m value of (169.17 ± 37.48) μM for Sppt with respect to CoA, and a k_{cat} value of (28.41 ± 2.12) min^{-1}. The catalytic efficiency of Sppt for CoA was 0.168 $\mu M^{-1} min^{-1}$.

Phosphopantetheinylation of noncognate carrier proteins by Sppt in vitro

The ability of Sppt to activate carrier proteins from a range of secondary metabolism pathways was tested, including those from unicellular and filamentous, heterocyst-forming cyano-

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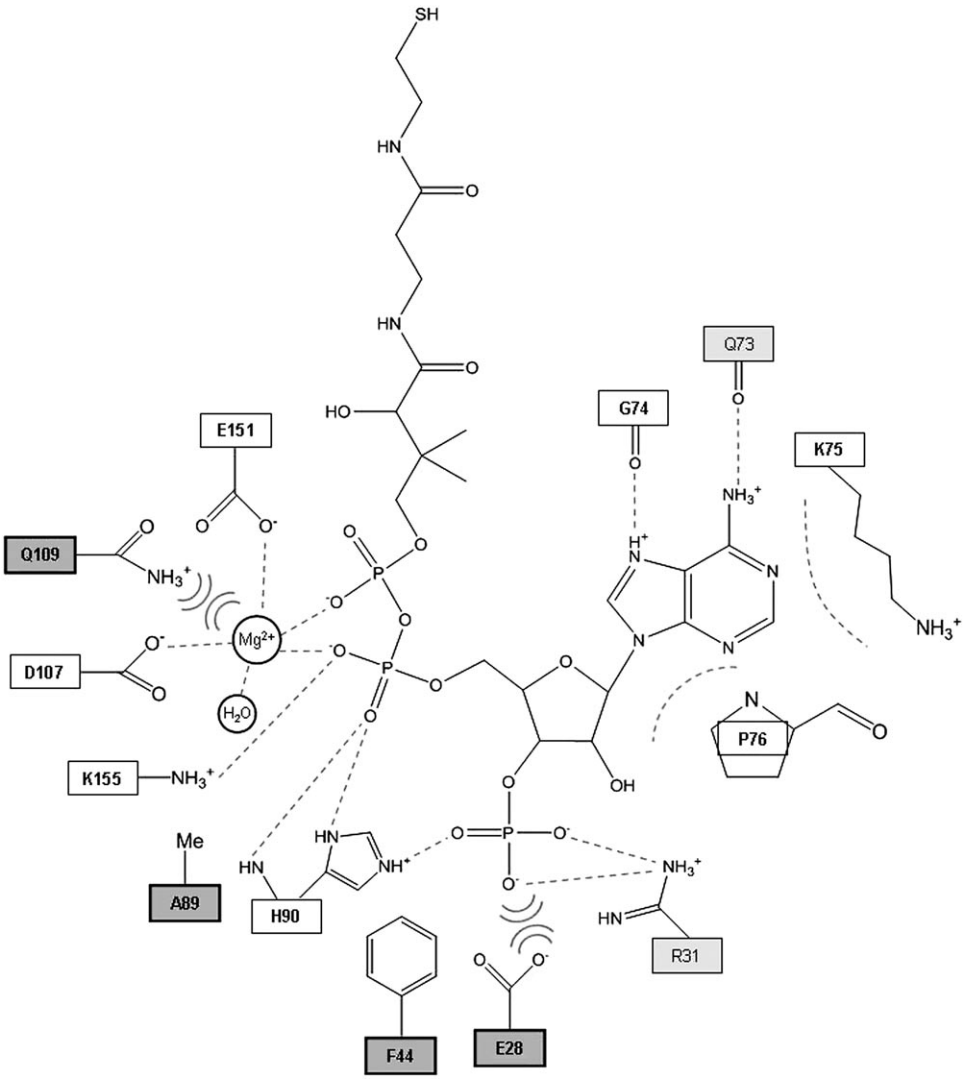

Figure 2. Amino acid residues in the CoA/Mg^{2+} binding site of Sppt as compared to those of Sfp.[33,34] Grey boxes indicate variations in the sequence of Sppt when aligned with Sfp. Bold grey boxes correspond to amino acid differences that could affect the electrostatic interactions (-----) between the residues and CoA or Mg^{2+}. Repulsion forces are indicated by unbroken, grey, short-curved lines. The corresponding key Sfp residues are: Y73, K31, K28, T44, S89, E109.

bacteria. The carrier proteins tested included the *Nostoc punctiforme* ATCC 29133 PKS aryl carrier protein (ArCP) from HetM of glycolipid synthesis (NpArCP), the PKS ACP of nostopeptolide biosynthesis (NpACP), and the *Microcystis aeruginosa* PCC7806 NRPS PCP from the loading module McyG of microcystin synthesis (MPCP). No phosphopantetheinylation of NpACP, MPCP or NpArCP by the *E. coli* PPTases was detected after heterologous expression.

Sppt was unable to phosphopantetheinylate the *N. punctiforme* ATCC 29133 NpArCP as shown by mass spectrometry (Figure 6B). The spectrum of *M. aeruginosa* PCC7806 MPCP, after incubation with Sppt, showed a very low intensity mass peak corresponding to the poor efficiency addition of the 340 Da phosphopantetheinyl moiety (22319 Da; Figure 6D). Phosphopantetheinylated NpACP was not reproducibly detected after incubation with Sppt (Figure 6F) and the mass peak of 20153 Da was only slightly above that of the spectrum background. In contrast, the broad-range cyanobacterial PPTase, NsPPT, was able to phosphopantetheinylate all three carrier proteins in vitro,[29] and was used as a positive control (Table 1).

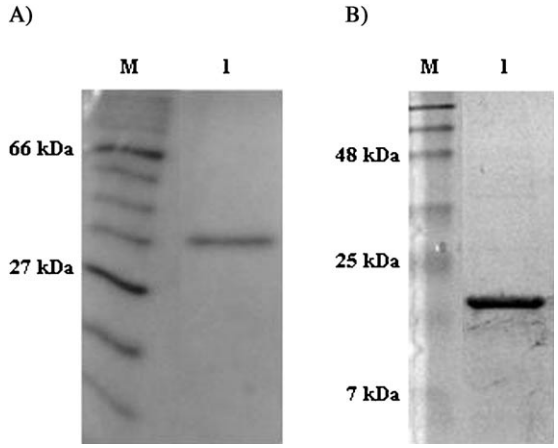

Figure 3. SDS-PAGE gel of purified A) Sppt and B) SACP; M: broad-range and prestained markers (New England Biolabs).

Engineering of a broad-range PPTase activity *Synechocystis* sp. PCC6803 heterologous host

In order to utilise *Synechocystis* sp. PCC6803 as a heterologous host for the biosynthesis of cyanobacterial natural products, the gene for the broad-range PPTase from *N. spumigena* NSOR10, *NsPPT*, was introduced into the wild-type (WT) strain. PCR screening confirmed the presence of the introduced *NsPPT* gene (data not shown). In order to verify the activity of NsPPT in the engineered host, the native PPTase, *Sppt*, was insertionally inactivated. Initial transformants were visible on chloramphenicol plates and were subcultured repeatedly on selective medium (Figure 7A). PCR screening of these transformants revealed that complete segregation of the *Sppt* gene disruption was not possible in the WT strain (Figure 8, lane 4) and could only be achieved when complemented with NsPPT

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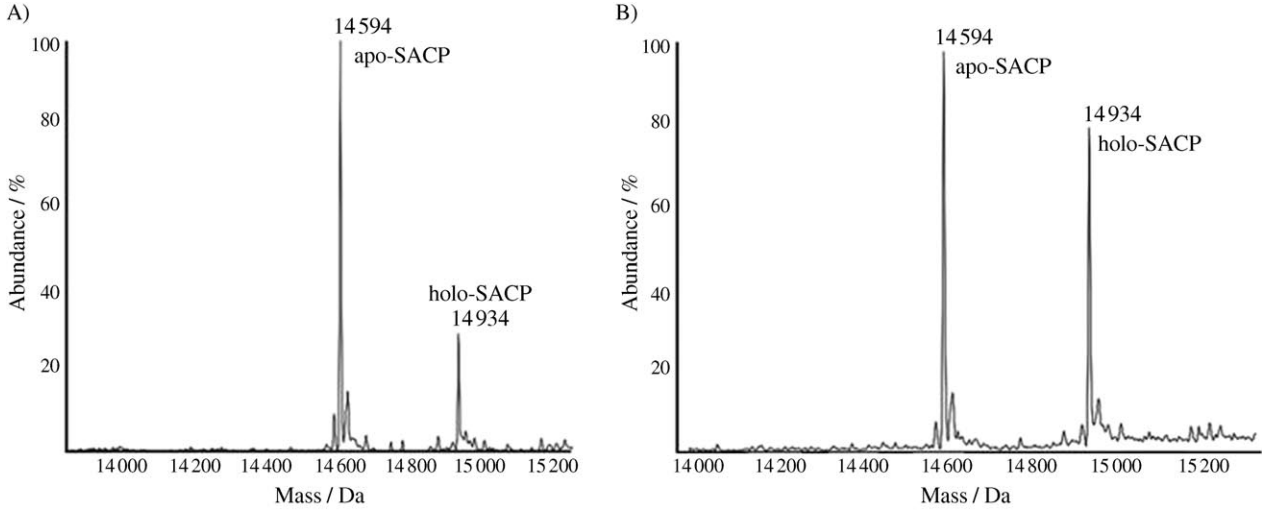

Figure 4. Phosphopantetheinylation of SACP by Sppt. A) Mass spectrum of SACP; B) mass spectrum of SACP after phosphopantetheinylation assay with Sppt.

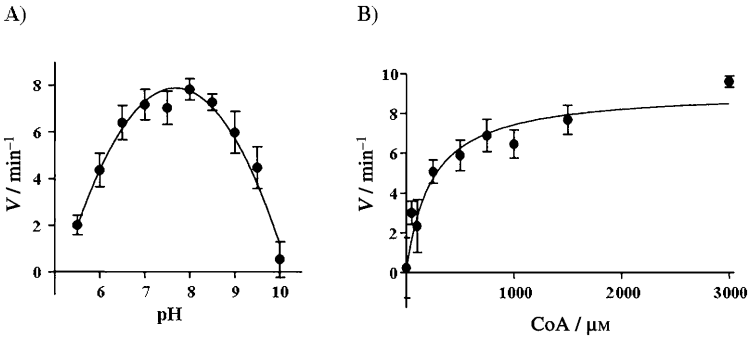

Figure 5. Enzymatic characterisation of Sppt with respect to SACP3. A) Effect of pH on phosphopantetheinyl transferase activity as measured by HPLC assay; B) effect of CoA, the 4′-phosphopantetheinyl group donor, on Sppt activity.

activity (Figure 8, lane 8). This confirms the role of Sppt as the only enzyme capable of phosphopantetheinylation in *Synechocystis* sp. PCC6803. Furthermore, disruption of *Sppt* in the engineered *Synechocystis* sp. PCC6803 *NsPPT*$^{+}$ strain indicated that the broad-range NsPPT enzyme is capable of activating a noncognate carrier protein, SACP, in vivo.

Discussion

Characterisation of the cyanobacterial PPTase, Sppt, was achieved by multiple approaches. Firstly, protein modelling suggested that Sppt was a Sfp-type PPTase, as supported by previous phylogenetic data.[19,34] Secondly, phosphopantetheinylation assays and kinetic analysis revealed that Sppt was able to activate its cognate fatty acid carrier protein from *Synechocystis* sp. PCC6803, however, the enzyme efficiency was relatively low and more similar to that of AcpS-type PPTases. These results prompted the assaying of Sppt's activity towards noncognate carrier proteins from various other cyanobacterial species including a glycolipid synthesis carrier protein from *N. punctiforme* ATCC 29133, an NRPS carrier protein involved in microcystin synthesis in *M. aeruginosa* PCC7806 and a PKS carrier protein from nostopeptolide synthesis in *N. punctiforme* ATCC 29133. Sppt was unable to effectively activate any of these secondary metabolism carrier proteins in contrast to the broad-range PPTase positive control NsPPT from *N. spumigena* NSOR10. This deficient broad-range PPTase activity correlates with the lack of natural products synthesised by *Synechocystis* sp. PCC6803. However, the presence of a Sfp-type PPTase in a species that only encodes a carrier protein for FAS raises interesting questions regarding the evolution of PPTases and secondary metabolite synthesis in cyanobacteria.

Bioinformatics analysis has shown that, with the exception of *Gloeobacter violaceus* PCC7421, cyanobacteria do not encode an AcpS-type PPTase, as exemplified by the single Sfp-type Sppt in *Synechocystis* sp. PCC6803.[19] Finking et al. hypothesised that the Sfp-type PPTases arose from a duplication of the ancestral AcpS PPTase and was subsequently maintained in genomes for the phosphopantetheinylation of secondary metabolism pathways.[35] The broad specificity of Sfp-type PPTases and their ability to complement AcpS activity could then have allowed the loss of the AcpS-type PPTases in species also harbouring a Sfp-type PPTase. Some organisms support this theory, such as *Pseudomonas aeruginosa* PAO1, which produces multiple secondary metabolites requiring phosphopantetheinylation but which harbours a single Sfp-type PPTase. However, it is surprising that *Synechocystis* sp. PCC6803 would harbour a Sfp-type PPTase when the genome does not encode any NRPS/PKS gene clusters.

One hypothesis to explain this phenomenon is that an early cyanobacterial ancestor might have originally produced NRPS/PKS products, therefore, requiring a Sfp-type PPTase. Mutation in this ancestral PPTase gene might have resulted in the inability of Sppt to activate NRPS/PKS carrier proteins; subsequently, *Synechocystis* sp. PCC6803 might have lost the biosynthetic

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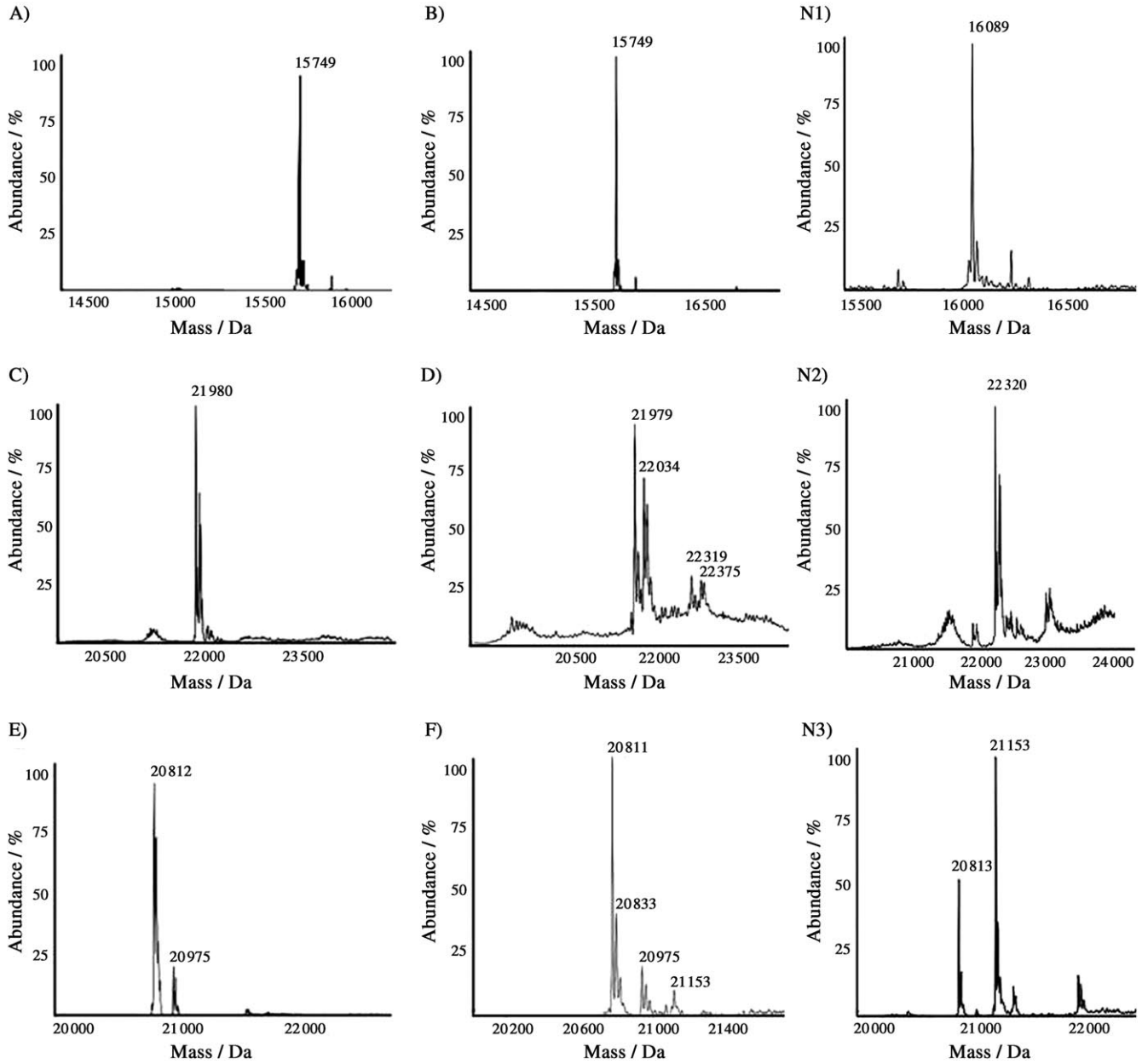

Figure 6. Phosphopantetheinylation of noncognate carrier proteins by Sppt. Mass spectra of: A) NpArCP control; B) NpArCP after incubation with Sppt; C) MPCP control; D) MPCP after incubation with Sppt; E) NpACP control; F) NpACP with Sppt. N1, N2 and N3 indicate the NsPPT positive control assay with the NpArCP, MPCP and NpACP carrier proteins, respectively.

Table 1. Phosphopantetheinylation of noncognate carrier proteins by the *Synechocystis* sp. PCC6803 PPTase, Sppt.

Carrier protein (CP)	Mass [Da]			holo-CP[b] [%]	NsPPT control holo-CP[b] [%]
	Expected	Observed			
		apo-CP	holo-CP[a]		
NpArCP glycolipid synthase	15751	15749	n.d.	0	100
MPCP microcystin NRPS	21978	21978	22319	8	100
NpACP nostopeptolide PKS	20812	20812	21153	5	69

[a] The holo-CP was detected by the mass addition of a phosphopantetheinyl moiety (340 Da) by using mass spectrometry. [b] The percentage holo-CP was estimated by comparison of holo- and apo-CP abundance in mass spectra as previously described;[23] n.d.: not detected.

gene clusters required for nonribosomal peptide and polyketide compound production. This correlates with the widespread absence of these gene clusters in *Synechocystis* and *Synechococcus* species.[36] The low catalytic efficiency of Sppt for SACP, with respect to CoA also supports this theory, as it resembles the relatively low activity of the *B. subtilis* Sfp towards primary metabolism ACPs.[20,35] A comparison of

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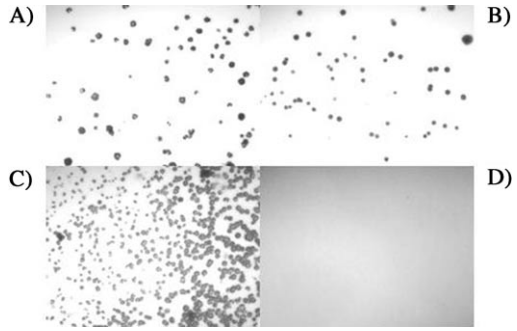

Figure 7. Complementation of *Sppt* knockout with NsPPT. A) *Synechocystis* sp. PCC6803 WT *Sppt::Cam*^{R}; B) *Synechocystis* sp. PCC6803 WT transformed with chloramphenicol resistance plasmid as a positive control; C) *NsPPT*$^+$ *Sppt::Cam*^{R}; D) *NsPPT*$^+$ negative control.

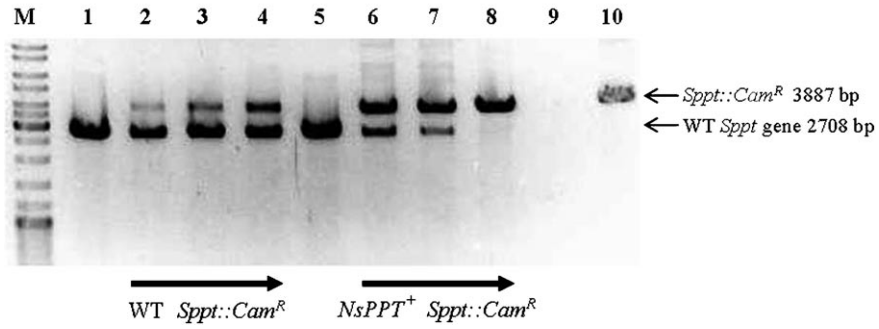

Figure 8. PCR screening of *Sppt*-disrupted and NsPPT-complemented *Synechocystis* sp. PCC6803 strains. M: marker; lane 1: *Synechocystis* sp. PCC6803 WT *Sppt* gene; lanes 2–4: *Synechocystis* sp. PCC6803 WT *Sppt* knockout heterozygous mutant with repeated subculturing on selective media; lane 5: *NsPPT*$^+$ complemented *Sppt*$^-$ gene; lanes 6–7: *NsPPT*$^+$ complemented *Sppt*$^-$ heterozygous mutant with repeated subculturing on selective media; lane 8: *NsPPT*$^+$ fully segregated *Sppt* knockout mutant; lane 9: negative PCR control; lane 10: knockout plasmid, pGCSIr, with chloramphenicol resistance cassette (*Cam*^{R}) inserted into the *Sppt* gene. Arrows indicate segregation of *Sppt* into multiple chromosomal copies of the genome with repeated subculturing.

the Sppt and Sfp CoA/Mg^{2+} binding pockets revealed several changes in key CoA- and Mg^{2+}-binding amino acids, which could attribute to Sppt's poor activation of secondary metabolite carrier proteins. For example, E28 and Q109, which are present in the Sppt binding site but not in Sfp, likely change the electrostatic interaction of the residues with CoA and Mg^{2+} from stabilising hydrogen bonds to destabilising repulsion forces (Figure 2). Furthermore, the variation at positions A89 and F44 might reduce the number of hydrogen bonds interacting with CoA, while the larger Phe residue in Sppt could increase steric hindrances compared to Tyr in Sfp. These differences could be a factor in the poor observed activity and low catalytic efficiencies of Sppt for CoA. Crystallisation of Sppt or mutagenesis of its binding pocket might clarify this hypothesis and determine whether these mutations contribute to the functional deficiencies of the enzyme. An alternative hypothesis could suggest that *Synechocystis* sp. PCC6803 acquired a Sfp-type PPTase by horizontal gene transfer that was more efficient than the original AcpS-type PPTase, which was subsequently lost due to the lack of biosynthetic gene clusters in that species. However, this theory seems unlikely as all characterised cyanobacterial species harbour a Sfp-type PPTase.[19]

The inability of Sppt to phosphopantetheinylate cyanobacterial secondary metabolite carrier proteins might not only be due to electrostatic considerations, but could also be due to the poor accessibility of active sites in larger recombinant proteins.[23] NRPS and PKS carrier proteins rarely exist as discrete domains and are typically integrated into large biosynthetic multienzymes. Therefore, these larger NRPS/PKS multidomains, which encompass carrier proteins, might not be as structurally accessible as some smaller recombinant counterparts used for in vitro studies. In an initial attempt to understand the effect of carrier protein size on the activity of Sppt towards secondary metabolite carrier proteins, a smaller recombinant NRPS protein, TycC PCP,[34] which is involved in tyrocidine biosynthesis, from *Bacillus brevis* ATCC 8185, was tested with Sppt, in vitro. Sppt was able to partially phosphopantetheinylate this 9970 Da carrier protein, which is less than half the size of NpACP and MPCP (data not shown). This suggested that carrier protein size could be an important factor and revealed the potential problems associated with the utilisation of small recombinant carrier proteins for in vitro analysis of PPTase activity. Such experiments can propose the recognition and activation of substrates in vitro, but might not necessarily be indicative of PPTase activity toward carrier proteins in vivo and subsequent utility for heterologous expression of NRPS/PKS compounds. However, Sppt was unable to activate the NpArCP carrier protein, which is only 1 kDa larger than the Sppt-activated full-length SACP. This could indicate that the size of the carrier protein is not critical. To clarify these issues, in vivo studies or experiments with various sized carrier proteins are required.

Despite in silico evidence, Sppt's behaviour is more similar to AcpS-type PPTases with specific carrier protein activation, a high pH optimum and low catalytic efficiency with respect to CoA. Although most characterised PPTases show significantly higher catalytic efficiencies, Sppt has similar values to AcpS from *E. coli*, which has reported K_m values of 50 or 150 μM and a k_{cat} value of approximately 10 min^{-1}.[21,37] Sfp-type PPTases, such as the *B. subtilis* Sfp and *P. aeruginosa* PcpS, have pH optima between 6–7 while the Sppt optimum is more similar to the *B. subtilis* and *E. coli* AcpS PPTases with pH optima between 8–9. This high pH requirement could allow for appropriate electrostatic interactions between Sppt and the acidic SACP, and may explain its poor activation of the neutral MPCP.[23] Optimum Sppt activity at higher pH values could also reflect the environmental conditions (pH 7–9) that favour cyanobacterial bloom formation over phytoplankton growth.[38] Sppt's pH range is broader than other characterised PPTases; this might reflect the dynamic pH of cyanobacterial environ-

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ments during eutrophication and subsequent bloom cycles.[39,40]

This research also has significant implications for the use of *Synechocystis* sp. PCC6803 as a heterologous expression host. A valid, proven expression host for cyanobacterial secondary metabolite production and manipulation would be invaluable for natural compound production. *Synechocystis* sp. PCC6803 is an ideal host due to its natural transformability, well-characterised genetic manipulation techniques, sequenced genome and null background of NRPS/PKS compounds, which would allow the rapid identification of compounds produced by heterologous expression.[5,31,41] However, the low level of secondary metabolite carrier protein phosphopantetheinylation by Sppt indicates that this species would require the coexpression or chromosomal integration of an additional PPTase to be suitable as a heterologous host for NRPS/PKS expression.

The broad-range specificity PPTase, NsPPT, from *N. spumigena* NSOR10 has previously been shown to activate noncognate NRPS and PKS carrier proteins in vitro.[29] In this study, NsPPT was introduced into *Synechocystis* sp. PCC6803 and was shown for the first time to be able to activate a noncognate carrier protein, SACP, in vivo. This NsPPT-complemented strain could provide a valuable biotechnological platform for the activation of entire secondary metabolite pathways in vivo for the biosynthesis of natural and novel cyanobacterial-derived compounds.

Experimental Section

Protein modelling of Sppt: The percentage identity and similarity of Sppt (Genbank accession number: BAA10326) and Sfp (Genbank accession number: CAA46561) were determined by using BlastP[42,43] (basic local alignment search tool) by aligning the two amino acid sequences. The protein sequence of Sppt (Swiss-Prot accession number: Q55185) was modelled on the solved crystal structure of Sfp from *B. subtilis*[34] (PDB ID: 1qr0) by using the SWISS-MODEL[44] automated comparative protein modelling server (http://swissmodel.expasy.org). The model and the corresponding Sfp crystal structure were viewed by using PyMOL (DeLano Scientific LLC) to determine structural similarities and variations between the two proteins.

Expression and purification of recombinant Sppt and SACP proteins: To determine the activity of Sppt with phosphopantetheinylation assays, Sppt and SACP were expressed and purified. *Sppt* (slr0495, Genbank accession number: NC_000911) was amplified with the primer pair SynPpF (5′-TGT TTA AAC TCA CCT G) and SynPpR (5′-CCC AAG GTT ACG AAA C). The resulting PCR product was cloned into pGEM-T (Promega), digested with the restriction enzymes SacI and EcoRV and ligated into pET30 (Novagen). The *Synechocystis* sp. PCC6803 FAS ACP, designated SACP (AcpP BA000022), was amplified with the primer pair ssl2084Efw (5′-GGA ATT CTG AAT CAG GAA ATT T) and ssl2084Hrv (5′-CTC GGC TCC AAA AAG CTT TGG G), digested with the restriction enzymes EcoRI and HindIII, and cloned into pET30. A truncated SACP, SACP3, was cloned and expressed for HPLC kinetic analysis by using the primers 2084NdeF (5′-GCA TAT GAA TCA GGA AAT TT) and 2083XhoR (5′-CCT CGA GTA ATT TAC TTT CGA TAT GCT CAA C). The constructs were transformed into BL21(DE3) Rosetta (Novagen) for expression. Sppt was expressed at 37 °C by induction with isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) for 3 h. SACP and SACP3 were expressed at 37 °C with IPTG (1 mM) for 20–40 min. Cultures were harvested at 5000 *g* and cell pellets were stored at −80 °C. Cell pellets were resuspended in HEPES buffer (HEPES 50 mM, NaCl 150 mM pH 7.4) and passaged three times through a cooled French pressure cell at 1380 kPa (Paton Scientific). After centrifugation at 20000 *g* for 30 min, proteins were purified by using HiTrap affinity columns (Amersham Biosciences) with an imidazole (300 mM) gradient as previously described.[29] Fractions containing purified proteins were desalted with Amicon centrifuge columns (Millipore) and snap frozen with glycerol (8%) for storage at −80 °C. Protein concentrations were determined based on the calculated extinction coefficients: SACP, 6970 $cm^{-1} M^{-1}$; Sppt, 53110 $cm^{-1} M^{-1}$. Due to the absence of aromatic amino acids in SACP3, the concentration of this protein was established by using molecular imaging (FUJIFILM LAS-3000) and compared to a standard curve of known BSA concentration.

Phosphopantetheinylation of SACP by Sppt, in vitro: Sppt activity towards SACP was determined in a 10 min phosphopantetheinylation assay at 37 °C as previously described.[29] Briefly, the reactions (100–200 μL) comprised of Tris-HCl (50 mM, pH 7.4), $MgCl_2$ (12.5 mM), CoA (0.5 mM), DTT (2 μM), carrier protein (100 μM) and Sppt (300 nM). Reactions were incubated for 10 min at 37 °C, terminated by the addition of trichloroacetic acid (1 mL) and precipitated, overnight, at −20 °C before centrifugation for 15 min at 18000 *g*. Samples were dissolved in water/acetonitrile/formic acid (50:49:1). Phosphopantetheinylation was observed by an increase in mass of 340 Da as visualised by ESI-MS. Spectra were acquired by using an API Qstar Pulsar i hybrid tandem mass spectrometer (Applied Biosystems) as previously described.[29]

Kinetic analysis of Sppt activity: The CoA inhibition and pH activity range for Sppt was determined by using in vitro phosphopantetheinylation assays with the truncated SACP3. Assays were carried out as described above, in reactions (200 μL) comprising of MES (75 mM, pH 5.5–6.5), Tris (pH 7–8.5) or CAPSO (pH 9–10) and CoA (1–3 mM). After precipitation, the pellets were resuspended in 30 μL 55% solvent B (solvent A: water, 0.1% trifluoroacetic acid; solvent B: acetonitrile, 0.1% trifluoroacetic acid). An Alltech 5 μm Nucleosil C18 column (250×4.6 mm) was equilibrated with 55% solvent B at 45 °C before sample injection. Samples were eluted with a linear gradient from 55% to 68% solvent B over 15 min with 0.9 mL min^{-1} flow rate. The absorbance at 200 nm was constantly monitored. The amount of holo-SACP3 formed was determined by comparative analysis of the holo-ACP peak area of control assays without PPTase. The holo-SACP3 present after heterologous expression in *E. coli* was subtracted from the total amount phosphopantetheinylated.

Phosphopantetheinylation of noncognate carrier proteins by Sppt, in vitro: The *M. aeruginosa* PCC7806, peptidyl carrier protein, MPCP, and the *N. punctiforme* ATCC 29133 aryl carrier protein, NpArCP, and acyl carrier protein, NpACP, were expressed and purified as previously described.[29] Protein concentrations were determined based on the calculated extinction coefficients: NpArCP, 8250 $cm^{-1} M^{-1}$; NpACP, 13940 $cm^{-1} M^{-1}$; MPCP, 6970 $cm^{-1} M^{-1}$. In the phosphopantetheinylation assays, various conditions were utilised in order to optimise Sppt activation of noncognate carrier proteins. These included a range of Sppt (2–1000 nM) and carrier protein concentrations (5–100 μM) as well as incubation times (15 min to overnight). The other parameters remained constant. NsPPT control assays were performed in reactions (100–400 μL) comprising Tris-HCl (5 mM, pH 7.4), $MgCl_2$ (12.5 mM), CoA (0.5 mM), DTT (2 μM), the respective carrier proteins (10–100 μM) and NsPPT

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(300 nM). The control assays were incubated at 37 °C for 30 min before precipitation and ESI-MS analysis.

Engineering of a broad-range PPTase activity *Synechocystis* sp. PCC6803 heterologous host: The *N. spumigena* NSOR10 PPTase, NsPPT, was introduced into *Synechocystis* sp. PCC6803 in order to develop a cyanobacterial heterologous host with broad-range PPTase activity. *NsPPT* (Genbank accession number: AY646183) was expressed under the control of a nitrate inducible promoter, PnirA, from *Synechococcus* sp. strain PCC7942.[45] PnirA was amplified with the primers nirAF (5′-TTC TAG ATC CCT CTC AGA TCA AAA AG) and nirAR (5′-TGC ATA TGG GAT TCA TCT GCC TAC) and the fragment was cloned into pET30a with XbaI and NdeI to yield pPnirA (Novagen). *NsPPT*, was amplified with the primers NpptF (5′-TGC ATA TGA CGG CGC TTA ATC ATT) and NpptR (5′-TCT CGA GTC AGT AT TGC CAA CAC) and was subsequently cloned into pPnirA with NdeI and XhoI. *NsPPT*, complete with the PnirA promoter and T7 transcriptional terminator, was released from this construct with DraIII and SphI and blunt-ended with Klenow enzyme (Promega). The resulting fragment was ligated into PstI-linearised pKW1188, which harbours the *slr0168* photosystem II gene flanking regions and a kanamycin resistance cassette.[46] The final construct was then naturally transformed into *Synechocystis* sp. PCC6803 for homologous recombination into *slr0168*.[47] Briefly, exponentially growing *Synechocystis* sp. PCC6803 cells (approximately 2 mL) were centrifuged and washed in BG-11 media. After resuspension in BG-11 (300 μL), plasmid (3 $\mu g\,mL^{-1}$) was added and the cells were incubated at 25 °C in 30 $\mu mol\,m^{-2}\,s^{-1}$ constant light for 6 h. The cells were spread onto sterile nitrocellulose filters (0.45 μm, Millipore) on non-selective BGTS plates (BG-11, 10 mM TES, 0.3% sodium thiosulfate, 1% agar). After 36 h, the filters were transferred to selective BGTS plates supplemented with kanamycin (10 $\mu g\,mL^{-1}$). The *NsPPT*-harbouring strain was designated *Synechocystis* sp. PCC6803 $NsPPT^+$ ($NsPPT^+$).

Gene disruption of *Sppt*: *Sppt* was insertionally inactivated in order to confirm that the introduced broad-range PPTase, NsPPT, was active in vivo and was able to modify the noncognate FAS ACP from *Synechocystis* sp. PCC6803. To generate the knockout plasmid, a 2.7 kb fragment including *Sppt* and flanking regions was amplified with the primers slrup (5′-GTA AAC TCC ATT AAC GCT GGC) and slrdn (5′-GGT GCA AAT CCG TTA CAT GGA). This fragment was cloned into pGEM-T-Easy (Promega) and digested with the restriction enzyme AvaI. A chloramphenicol (Cam^R) resistance cassette was ligated into this site and the resulting construct, pGCSlr, was naturally transformed into both the WT and the engineered strain *Synechocystis* sp. PCC6803, $NsPPT^+$, to insertionally inactivate *Sppt*. The *Synechocystis* sp. PCC6803 WT was also transformed with a chloramphenicol resistance knockout plasmid as a positive control for strain viability while ddH_2O was transformed into $NsPPT^+$ as a negative control. Transformants were selected on BGTS plates supplemented with chloramphenicol (10 $\mu g\,mL^{-1}$). Positive colonies were subcultured ten times on antibiotic selection plates or until complete segregation was achieved. Complete segregation of *Sppt* gene disruption throughout the multiple chromosomal copies was detected by colony PCR screening with the primers slrup and slrdn. Knockout experiments were carried out three times in duplicate.

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