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Two Functionally Redundant Sfp-Type 4′-Phosphopantetheinyl Transferases Differentially Activate Biosynthetic Pathways in *Myxococcus xanthus*

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Phosphopantetheinyl transferases (PPTases) represent a superfamily of enzymes that are essential for the post-translational modification of carrier proteins involved in the biosynthesis of primary and secondary metabolites, such as fatty acids, polyketides and nonribosomal peptides.[1] These carrier proteins are activated by the transfer of a 4′-phosphopantetheine (Ppant) cofactor from coenzyme A to a conserved serine residue of the *apo* form of the protein, in a Mg^{2+}-dependent reaction. Chain-extension intermediates are bound to the protein by a thioester linkage to the Ppant prosthetic group; this enables them to be shuttled around the individual active sites of the multienzyme complexes. Several organisms are known to possess multiple types of PPTase enzymes, which exhibit specificity for distinct biosynthetic pathways.[1] Acyl carrier protein synthase (AcpS)-type PPTases usually activate fatty acid synthases (FASs) and type II polyketide synthases (PKSs). These enzymes consist of approximately 120 amino acids, and exhibit a homotrimeric structure.[2,3] In contrast, Sfp-type PPTases (named after the prototype PPTase Sfp from *Bacillus subtilis*[4]) typically modify carrier proteins that are responsible for the biosynthesis of secondary metabolites, such as type I PKS, nonribosomal peptide synthetase (NRPS) systems and their hybrids. Furthermore, Sfp-type PPTases have also been shown to participate in fungal lysine biosynthesis, β-alanine conjugation and cyanobacterial heterocyst differentiation.[5–8]

Sfp-type PPTases are approximately twice the size of AcpS-type PPTases, which suggests that they evolved by gene duplication from an AcpS ancestor.[9] Sfp exhibits broad substrate tolerance towards different types of carrier proteins,[10] a feature that has enabled its exploitation in various biotechnological applications.[11–15] Indeed, some genomes contain only a Sfp-type PPTase; this strongly supports its function in both primary and secondary metabolism.[16] For example, inactivation of the single PPTase-encoding gene *pcpS* in *Pseudomonas aeruginosa* could only be accomplished when a copy of the *E. coli acpS* gene was simultaneously introduced in trans on the chromosome.[17] In contrast, disruption of the Sfp-type PPTase gene *mtaA* in *Stigmatella aurantiaca* DW4/3-1 was not lethal, due to the presence of at least one additional (AcpS-type) PPTase (Figure 1C), but production of all known secondary metabolites was abolished.[18] In *E. coli*, the defect caused by a mutated, dysfunctional AcpS could be restored by over-expression of a second *E. coli* PPTase, YhhU, the exact function of which has yet to be determined.[19]

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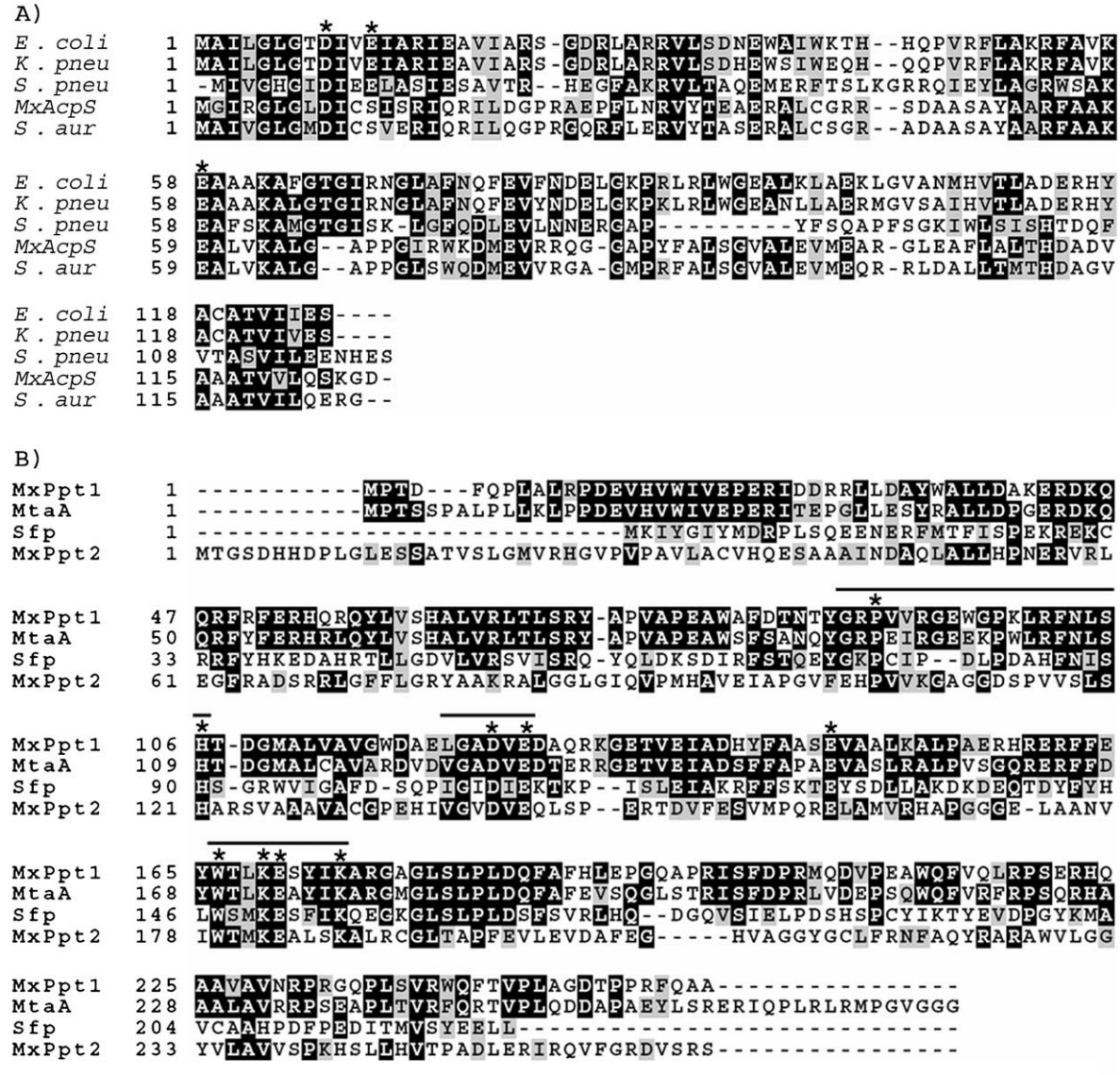
M. xanthus DK1622 employs two Sfp-type PPTases

The genome of the recently sequenced myxobacterial model strain *Myxococcus xanthus* DK1622[20] includes at least three genes that encode PPTases. MXAN_4350 (renamed here to MxAcpS) is 126 amino acids in size and shows the highest sequence similarity to PPTases from the AcpS-type family (32% sequence identity on the amino acid level to AcpS from *E. coli* K12; 73% identity to AcpS from *S. aurantiaca* DW4/3-1), and it contains the highly conserved residues Asp10 and Glu60[3] (Figure 1A). MXAN_3485 (258 amino acids, renamed to MxPpt1) and MXAN_4192 (266 amino acids, renamed to MxPpt2) are homologues of Sfp-type PPTases, although MxPpt1 exhibits significantly higher sequence similarity to known PPTases from this class, including MtaA from *S. aurantiaca* DW4/3-1[21] (MxPpt1 shows 62% sequence identity/71% similarity to MtaA, whereas MxPpt2 exhibits 19% identity/29% similarity to MtaA, and 18% identity/28% similarity to MxPpt1). Both MxPpt1 and MxPpt2 include all of the important residues that have been shown to be essential for PPTase structure and activity, and may be grouped into the W/KEA subfamily of Sfp-type PPTases (Figure 1B).[9,22] Analysis of PPTases from other sequenced myxobacterial species, revealed that both *Sorangium cellulosum* So ce56[23] and *S. aurantiaca* DW4/3-1 also encode one AcpS-type and two Sfp-type PPTases. In contrast, *Anaeromyxobacter dehalogans*[24] possesses two (AcpS and Sfp) and *Plesiocystis pacifica*[25] has only one (Sfp-type) PPTase (Figure 1C).

We failed to generate mutants of MxAcpS by insertional mutagenesis, most likely due to its essential function in fatty acid biosynthesis; evidently, neither MxPpt1 nor MxPpt2 can completely complement the function of MxAcpS. We therefore targeted genes MXAN_3485 (MxPpt1) and MXAN_4192 (MxPpt2) for inactivation by using a homologous recombination strategy. The inactivation fragments were amplified by PCR by using primers PMMXAN3485_1 and PMMXAN3485_2 for MXAN_3485 and primers PMMXAN4192_1 and PMMXAN4192_2 for MXAN_4192, each of which introduced a stop codon (Table 1, underlined), and the PCR products were cloned into plasmid pCR2.1-TOPO (Invitrogen). The plasmids were then introduced into *M. xanthus* DK1622 by electroporation, as described previously.[26] Clones were selected for kanamycin resistance and were verified by PCR by using a plasmid-specific primer pair and two primers that bind outside of the inactivation fragment, as described earlier.[27] The PPTase mutants and *M. xanthus* wild type were grown in quadruplicate in CTT medium[28] in the presence of Amberlite XAD-16 resin (1%), and were harvested during the same stage of logarithmic



C)

Strain	PPTase/Acc.No	Length [aa]	Type	Identity [%] to: MxAcpS	MxPpt1	MxPpt2
Myxococcus xanthus DK1622	MxAcpS[a]	126	AcpS	100	7.0	7.9
	MxPpt1[b]	258	Sfp	7.0	100	18.2
	MxPpt2[c]	266	Sfp	7.9	18.2	100
Sorangium cellulosum So ce56	Sce0320	162	AcpS	32.9	8.1	14.3
	Sce5058	246	Sfp	6.5	47.5	17.6
	Sce4887	244	Sfp	11.5	15.8	18.6
Stigmatella aurantiaca DW4/3-1	ZP_01466494	125	AcpS	73.0	7.0	7.9
	MtaA	277	Sfp	7.2	62.5	18.6
	ZP_01459466[d]	278	Sfp	7.6	19.1	15.1
Anaeromyxobacter dehalogans 2CP-C	ABC81274	128	AcpS	55.0	7.0	9.4
	ABC83662	239	Sfp	11.2	17.4	20.3
Plesiocystis pacifica SIR-1	PPSIR1_19799	290	Sfp	7.6	24.8	15.9

[a]Acc. No YP_632523.1, [b]Acc. No YP_631680.1, [c]Acc. No YP_632367.1, [d]Acc.No ZP_01459466 of truncated PPTase (Het I protein, 225 aa)

Figure 1. Sequence alignment of *M. xanthus* PPTases with bacterial A) AcpS-type and B) Sfp-type PPTases from different species. Black shading indicates identical residues, while grey shading shows similar residues. Consensus motifs are indicated by black bars, and conserved residues that are essential for activity and structural stability are indicated by an asterisk (alignment created with ClustalW2[47] and BOXSHADE 3.21). C) PPTases from sequenced myxobacterial species.

Table 1. List of strains and primers that were used in this study.

Strain or primer	Genotype or sequence[a]
M. xanthus strains	genotype description
DK1622	wild type[46]
PM3485	DK1622 *MXAN_3485::kan*
PM4192	DK1622 *MXAN_4192::kan*
PM3485tn5	DK1622 *MXAN_3485-tn5_3485::kan*
PM4192tn5	DK1622 *MXAN_4192-tn5_4192::kan*
Primers	
PMMXAN3485_1	5′-GCGTCCGGATTAGGTCCAT
PMMXAN3485_2	5′-CGCCTTGATCTAGGACTCC
PMMXAN4192_1	5′-CGACGGTGTAGCTCGGCA
PMMXAN4192_2	5′-CGCCGCCAGCTAGCCGCC
Mch71	5′-TGGACAGCAAGCGAACCGG
Mch72	5′-CATAATCTGTACCTCCTT
PM3485_Pr1	5′-GATAAGGAGGTACAGATTATGCCGACAGACTTCCAGCC
PM3485_Pr2	5′-CTACGCCGCCTGGAACCG
PM4192_Pr1	5′-GATAAGGAGGTACAGATTATGACCGGCTCTGACCATCA
PM4192_Pr2	5′-CTAGCTTCGAGACACGTCGC

[a] The introduced stop codons are underlined.

growth (as assessed by measuring the optical density). The cells and XAD resin were extracted with methanol and subsequently analyzed by HPLC–MS in positive-ionisation mode (Agilent 1100 HPLC series coupled to a Bruker HCT plus ion trap). The major metabolite from each compound family known from *M. xanthus* DK1622 was chosen as a target compound for quantitative analysis: DKxanthene-534,[29] myxalamid A,[30] myxovirescin A[31] and myxochromide A_3[32] (Figure 2). The myxalamides and DKxanthenes comprise the main secondary metabolites in *M. xanthus*, whereas the myxovirescins and myxochromides are produced as minor components. We chose to omit the myxochelins[33] in this analysis as their production level varied significantly even in wild-type cultures; this precluded reliable quantification. Quantification of each secondary metabolite was performed by collecting the respective parent ion (Table S2 in the Supporting Information) and subjecting it to fragmentation in mass spectrometric analyses. The intensities of the particular fragment ions were summarised and integrated by using the Bruker QuantAnalysis v1.5 software. The overall yield of each secondary metabolite was calculated with respect to cell density by dividing the total amount of secondary metabolite by the optical density. The secondary metabolite profile for all of the strains that were analyzed in this study was calculated as a percentage of the wild-type *M. xanthus* DK1622, which was set to 100% for each natural product (Figure 3, wild type not shown). Additionally, the fatty acid composition of all mutants generated in this study was determined as described previously.[34]

Single mutants that carry inactivated copies of either PPTase retained the ability to produce secondary metabolites, but the specific production profiles differed from each other and from wild-type *M. xanthus* DK1622. The secondary metabolite profile of mutant PM4192 in which MxPpt2 had been inactivated, showed a strong overall resemblance to that of the *M. xanthus* wild type. However, a moderate decrease in production level was observed for all of the examined natural products; this effect was most pronounced in the case of myxochromide A_3 (60% of wild type). In contrast, inactivation of MXAN_3485 (encoding MxPpt1) in strain PM3485 led to significant changes in the secondary metabolite pattern (Figure 3). The production of DKxanthene-534 and myxovirescin A was significantly reduced in mutant PM3485 in comparison to the wild type and all other mutant strains investigated in this study that expressed an active MxPpt1. This result indicates that MxPpt1 plays a dominant role in the activation of the assembly lines for myxovirescin and DKxanthene biosynthesis. In the cases of myxochromide A_3 and myxalamide A, the inactivation of MXAN_3485 resulted in a four- to sixfold increase in production level per cell, respectively, when compared to the natural producer *M. xanthus* DK1622. We attribute this increased production to the higher availability of precursors, due to reduced competition from other biosynthetic pathways.

As inactivation of both of the identified PPTases led to a shift in metabolite production profiles, we decided to test whether the introduction of a second copy of the respective PPTases into *M. xanthus* DK1622 would cause further changes in product levels. The over-expression of single genes or multigene transcriptional units in *M. xanthus* was already successfully performed in preceding experiments, and the constitutive *Tn5*-derived *npt* promotor was shown to work very efficiently in myxobacteria (S. C. Wenzel and R.M., unpublished results). To do this, the promotor was amplified from the pCR2.1-TOPO vector by using primers Mch71 and Mch72, and the product was fused to the 5′ end of the target gene(s) by overlap-extension PCR.[35] The resulting fragment was cloned into pCR2.1-TOPO, transferred into *M. xanthus* and allowed to integrate into the chromosome by homologous recombination. Over-expression of

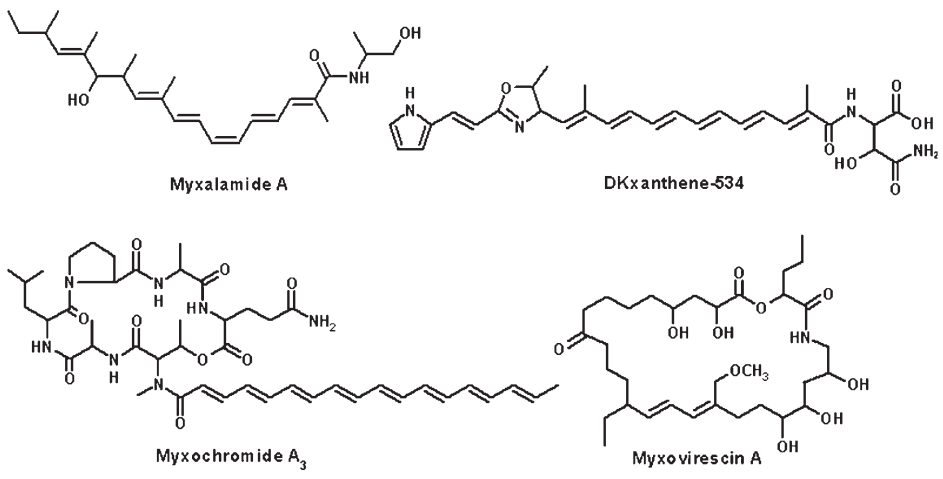


Figure 2. Chemical structures of analyzed natural products from *M. xanthus* DK1622.

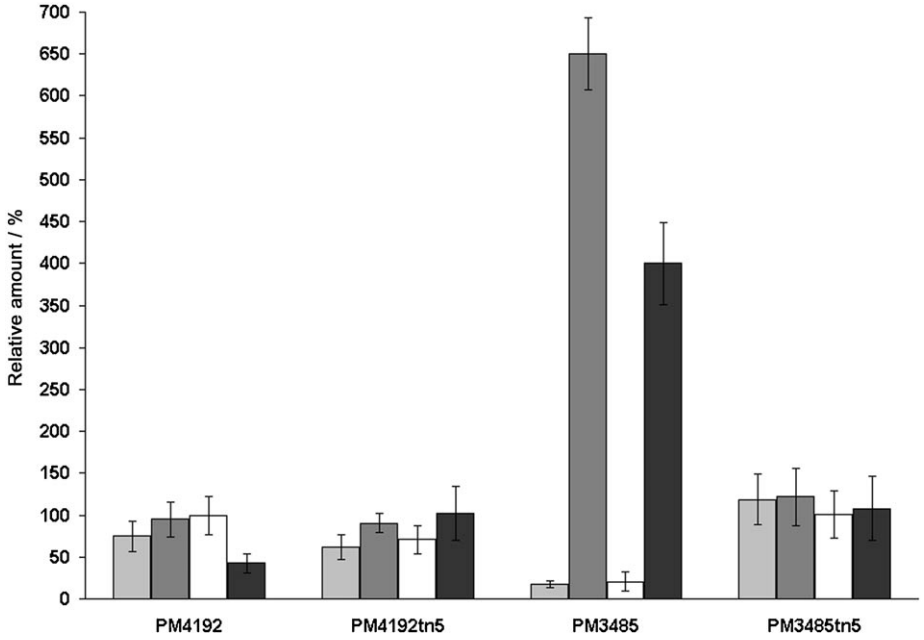


Figure 3. Relative amounts (%) of secondary metabolites produced by *M. xanthus* strains PM4192, PM4192tn5, PM3485 and PM3485tn5 in relation to wild-type DK1622, which was set to 100% for each analysed natural product. Secondary metabolites were quantified as described in the text; DKxanthene-534: light grey; myxalamid A: dark grey; myxovirescin A: white; myxochromide A_3: black.

the PPTases was achieved by using an analogous procedure: genes MXAN_3485 and MXAN_4192 were amplified by PCR (primer pairs PM3485_Pr1 and PM3485_Pr2; PM4192_Pr1 and PM4192_Pr1, respectively; Table 1). The forward primer in each PCR contained a fragment that was homologous to the 3′ end of the *Tn5*-derived *npt* promoter; this allowed the subsequent fusion with the promotor fragment that was generated with primers Mch71 and Mch72 (S. C. Wenzel, R.M., unpublished results).

After cloning of the final PCR product into vector pCR2.1-TOPO and verification of the sequences, the constructs were introduced into *M. xanthus* DK1622 by electroporation.[27] The genotypes of the obtained mutants (PM3485tn5 and PM4192tn5) were confirmed by amplifying the promotor–PPTase fragment from genomic DNA. After homologous recombination, both mutants contained two active copies of the respective PPTase genes, with one copy under the control of the natural promoter and the second copy under control of the *Tn5*-derived *npt* promotor. The secondary metabolite pattern observed for both mutants was similar to that of wild-type DK1622, although PM3485tn5 gave slightly elevated yields of all metabolites relative to PM4192tn5.

Taken together, our results indicate that both Sfp-type PPTases MxPpt1 and MxPpt2 are required for full activation of proteins involved in known secondary metabolite pathways in *M. xanthus*. In contrast to other strains in which PPTases exhibit strong specificity for a particular PKS or NRPS system,[18,36] the *M. xanthus* enzymes show functional redundancy; they can complement, at least in part, a loss of activity of the other PPTase. This redundancy might be vital for the complex life cycle[37] of *M. xanthus* DK1622 as both fatty acids and at least one secondary metabolite (DKxanthene) have been shown to play important roles for the organism during fruiting-body formation.[29,38] Indeed, despite extensive effort, we did not succeed in generating a double mutant in both MxPpt1 and MxPpt2. Experiments aimed at the generation of a double mutant were pursued by the introduction of a plasmid that conferred tetracycline resistance and contained the inactivation fragment for MxPpt1 into mutant PM4192; subsequently clones that were resistant against kanamycin and oxytetracycline were selected. Our failure to generate mutants in which both Sfp-type PPTases are inactive suggests that at least one functional PPTase is required for cell viability. However, the PPTases do exhibit some specificity towards activation of the biosynthetic pathways analyzed, and thus are not functionally identical; this bias is most evident in the decreased yields of DKxanthene-534 in mutant PM3485 and myxochromide A_3 in mutant PM4192. The introduction of a second copy of either PPTase into *M. xanthus* did not produce a significant change in the secondary metabolite profile relative to the wild type. These data suggest that each PPTase is expressed in the strain at sufficient levels to activate all target carrier proteins.

The natural products analyzed in this study represent all secondary metabolites that are known from *M. xanthus* DK1622. To date, no additional metabolites have been observed, despite extensive screening by using various media and conditions (unpublished results). Consistent with this, close inspection of the mutants that were generated in this study did not provide any indication for the presence of unidentified natural products, despite the possibility that decreased competition from alternative biosynthetic pathways might lead to increased production of compounds that had previously been overlooked. However, such an increase would only be expected if these pathways were supported by a single PPTase.

In vitro experiments that investigate the efficiency of both PPTases to activate all secondary metabolite gene clusters in *M. xanthus* would thus be helpful. However, the reported approach exemplifies an easily achieved shift in production pattern and thus might in future pave the way for an easier purification procedure of known or even unknown compounds.

In addition to the changes in secondary metabolite pattern, the fatty acid profile of the PPTase mutants differed from that of the wild-type strain; this indicates that MxPpt1 and MxPpt2 might have additional functions in fatty acid biosynthesis. For example, the fatty acid 16:1ω5c is significantly decreased in PM4192 and increased in PM4192tn5, whereas the major fatty

acid iso15:0 is slightly decreased in PM3485 but increased in PM4192 (Table S1). Conversely, the involvement of the *M. xanthus* AcpS in the activation of secondary biosynthetic pathways cannot be excluded from our data. Moreover, nothing is known about the post-translational activation of the thirteen other secondary metabolite assembly lines that have been identified in the genome,[39,40] many of which appear to be active.[41] Post-translational activation is a critical feature of assembly line biosynthesis on PKS and NRPS multienzymes, and is particularly relevant for attempts to produce the metabolites in heterologous hosts.[21,42] From the data presented here, it appears that MxPpt1 and MxPpt2 are among a selected group of PPTases that exhibit broad substrate tolerance.[4,18] This finding is strongly supported by the fact that the complex PKS/NRPS hybrid pathways for myxothiazol and epothilone from *Stigmatella aurantiaca* and *Sorangium cellulosum*, respectively, could be successfully expressed in *M. xanthus* DK1622 without the need to coexpress a broad spectrum PPTase.[43–45] Both enzymes might therefore find utility in the enzymatic "tool boxes" that are aimed at the in vitro production or heterologous expression of these giant, biosynthetic proteins.

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Keywords: biosynthesis · carrier protein · *Myxococcus xanthus* · natural products · phosphopantetheinyl transferase

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