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The Phosphopantetheinyl Transferases: Catalysis of a Posttranslational Modification Crucial for Life

Joris Beld[1,**], **Eva C. Sonnenschein**[1,**], **Christopher R. Vickery**[1,**], **Joseph P. Noel**[2], and **Michael D. Burkart**[1,*]

[1]Department of Chemistry and Biochemistry, University of California-San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0358, USA

[2]Howard Hughes Medical Institute, The Salk Institute for Biological Studies, Jack H. Skirball Center for Chemical Biology and Proteomics, 10010 N. Torrey Pines Road, La Jolla, CA 92037, USA

Abstract

Although *holo*-acyl carrier protein synthase, AcpS, a phosphopantetheinyl transferase (PPTase), was characterized in the 1960s, it was not until the publication of the landmark paper by Lambalot *et al*. in 1996 that PPTases garnered wide-spread attention being classified as a distinct enzyme superfamily. In the past two decades an increasing number of papers has been published on PPTases ranging from identification, characterization, structure determination, mutagenesis, inhibition, and engineering in synthetic biology. In this review, we comprehensively discuss all current knowledge on this class of enzymes that post-translationally install a 4′-phosphopantetheine arm on various carrier proteins.

1. Introduction

Phosphopantetheinyl transferases (PPTases)[1] are essential for cell viability across all three domains of life: bacteria, archaea and eukaryota. PPTases posttranslationally modify modular and iterative synthases acting in a processive fashion, namely fatty acid synthases (FAS), polyketide synthases (PKS), and non-ribosomal peptide syntethases (NRPS). The central component of these chain elongating synthases is non-catalytic and either a translationally linked domain of a larger polypeptide chain or an independently translated protein. Regardless, this protein component is referred to as a carrier protein (CP), or alternatively a thiolation domain.[2] CP is responsible for timing and efficiency in shuttling the rapidly changing chemical intermediates due to chain elongation between the structurally diverse multienzyme complexes of these pathways. The CP tethers the growing intermediates on a 4′-phosphopantetheine (PPant) arm of 20 Å through a reactive thioester linkage. PPants are thought of as “prosthetic arms” on which all substrates and intermediates of these pathways are covalently yet transiently held during the orderly progression of enzymatic modifications to the extending chain. PPTases mediate the transfer and covalent attachment of PPant arms from coenzyme A (CoA) to conserved serine residues of the CP domain through phosphoester bonds. These essential posttranslation protein modifications convert inactive *apo*-synthases to active *holo*-synthases (Fig. 1). Recently, mechanistically distinct classes of enzymes have been identified that require PPant arms for biosynthetic catalysis. These include enzymes involved in the biosynthesis of lipid A, D-alanyl-lipoteichoic acid, lipo-chitin nodulation factor, β-alanine-dopamine conjugates, carboxylic

*Corresponding author. mburkart@ucsd.edu. Phone: +1 858 434 1360.
**Authors contributed equally

acid reductions, and dehydrogenation of α-aminoadipate semialdehyde (AASDH) (lysine biosynthesis) and 10-formyl-tetrahydrofolate (fTHF-DH). In short PPTases are essential for the biosynthesis of fatty acids and lysine, many bioactive secondary metabolites, and a variety of other central biosynthetic pathways in both primary and specialized metabolism.

The essential enzymatic role of PPTases in general fatty acid biosynthesis was recognized in the groundbreaking work of Vagelos et al.[3] Since then, many other PPTases have been discovered to play the same role in a wide diversity of secondary metabolism.[1, 4] Many of these PPTases have been described on the gene and protein levels providing for intense biochemical characterization. With the mapping of their active sites, their interactions and catalytic mechanisms accompanying CoA and CP recognitions provided quantitative clarity, in some cases delineating predictable strategies for their molecular engineering for an assortment of basic and applied applications.

Due to their key metabolic positions in metabolism, PPTases are considered a prime drug and antibiotic target in medicine as well as agriculture. Being essential for the biosynthesis of natural products with antibiotic, immunosuppressive, and cytotoxic activities, as well as valuable hydrocarbons, efficient and selective PPTases serve pivotal roles for metabolic engineering efforts in the pharmaceutical and biofuel industries.

This is the first comprehensive review of PPTases. We have structured this overview first on published results over the last two decades with goals of understanding the identity, activity, and functions of PPTases across the three domains of life. Where appropriate, we also identify knowledge gaps for future investigations.

2. Types of PPTases

FAS, PKS or NRPS are either assembled from one or more multi-domain polypeptides (type I and possibly originating from ancient gene fusion events) or from separate protein partners (type II). In general, prokaryotes utilize type II FASs and eukaryotes utilize type I FASs. CPs of FASs and PKSs are called acyl carrier proteins (ACPs), whereas CPs of NRPSs are referred to as peptidyl carrier proteins (PCPs). In order for these repeating synthases to function in primary and secondary metabolism of iteratively produced intermediates and products, conserved serine residues of the CPs must be functionalized by PPTase catalysis with a PPant arm. Given the diversity of CP sequences and structures, the PPTase superfamily can be divided into three related yet distinguishable families, based on amino acid sequence conservation and alignments, three dimensional structures, and their target elongating synthases.

Holo-ACP synthase (AcpS) is the archetypical enzyme of the first family of PPTases recognized (Fig. 2). It encompasses 120 aa, forms a homo-trimeric quaternary structure with active sites shared across each homotypic interface, and acts on type II FAS ACP (AcpP). Surfactin phosphopantetheinyl transferase (Sfp) represents the second family of PPTases. Sfp is the PPTase necessary for installing PPant on the PCP of surfactin synthase. In contrast to AcpS, Sfp exists as a pseudo-homodimer of ~240 aa, resembling two AcpS monomers with one active site at the pseudo-dimer interface, and possesses a much broader substrate acceptance. The third family of PPTases are translationally fused C-terminal transferases residing in the megasynthases as one of several catalytic domains acting in type I yeast and fungal FAS megasynthases. This third family of PPTases post-translationally modify *apo*-ACPs prior to assembly of the megasynthases. In this section, we review these three families, first focusing on their discovery, divergent primary sequences and their biochemistry (note: structures will be discussed in Section 5).

2.1 Family I: *holo* acyl carrier protein synthase (AcpS-type PPTases)

The discovery of AcpS in the late 1960s was instigated by studies on ACP and CoA in *Escherichia coli*.[7] Feeding radioactive pantothenate to a pantothenate-requiring auxotroph revealed that 90% of the radioactivity was associated with the protein fractions, and 90% of that total amount was associated with FAS ACP (AcpP). Radioactive CoA was also observed early, correlating with the amount of pantothenate fed, but then dropped. However, the concentration of radioactive ACP was stable under a broad range of conditions, suggesting that CoA formation preceded radioactive incorporation into ACP.[7] Indeed, Elovson and Vagelos partially purified AcpS from *E. coli* and showed that AcpS catalyzed the incorporation of radioactivity into *apo*-ACP using CoA and *apo*-ACP as substrates to form *holo*-ACP and 3′,5′-ADP. The authors also demonstrated that the reaction requires either Mg^{2+} or Mn^{2+}, and described AcpS as being an unstable protein.[3] Years later, Polacco and Cronan mutagenized the pantothenate auxotroph used earlier by Elovson and Vagelos, then selected strains that only grew on very high levels of exogenous pantothenate. One strain in particular had no measurable AcpS activity after cell lysis and the site of the genetic lesion was mapped to the genomic region later shown to include the gene encoding AcpS (*acpS*).[8]

Later work on plants, which synthesize their fatty acids primarily in the chloroplast using a type II FAS similar to the bacterial FAS, afforded AcpS isolations from a second domain of life, the eukaryota. AcpS was isolated from spinach leaves and developing castor bean endosperm. Both AcpS fractions exhibited similar biochemical characteristics as the enzyme isolated earlier from *E. coli*.[9] Moreover, in plants, AcpS activity appeared to reside in the cytosol, and the PPant attached to *apo*-ACP before *holo*-ACP translocated into the chloroplast.[9] In 1995, Lambalot and Walsh purified AcpS from *E. coli* 70,000-fold. Curiously, although they achieved a 70,000-fold purification, the protein was still not homogeneous, suggesting it is expressed at very low levels. Later heterologous overexpression in *E. coli* of the *dpj* gene provided pure AcpS, which appeared to span 125 aa, and purify as a 28 kDa dimer. The original *dpj* gene was renamed *acpS*.[10] Subsequent work in the Walsh lab described AcpS as a trimer not a dimer and that it accepted not only bacterial AcpP but a variety of CPs from type II elongating systems including *Lactobacillus casei* D-alanyl carrier protein, *Rhizobia* protein NodF and *Streptomyces* ACPs involved in frenolicin, granaticin, oxytetracycline and tetracenomycin polyketide biosynthesis.[11] Ironically, CPs from type I elongating systems were not substrates for AcpS, as shown by the inability of *E. coli* AcpS to install a PPant arm on *apo*-EntF, the bacterial enterobactin synthase, or *apo*-TycA, the *Bacillus brevis* tyrocidine synthase. Although AcpSs exhibit a moderate level of CP substrate permissiveness in type II elongating systems, AcpSs are primarily used for post-translational modification and activation of the CPs of FASs (primary metabolism) across a diversity of organisms making them the most commonly found PPTase.

2.2 Family II: Sfp-type PPTases

In the early nineties, Grossman et al. identified genes involved in the biosynthesis of the siderophores enterobactin and surfactin from *E. coli* and *B. subtilis*, respectively.[12] These genes, called *entD* and *sfp*, were soon grouped with the *gsp* gene from *Bacillus brevis*, which plays a role in gramicidin S biosynthesis.[13] In 1996, a landmark paper co-authored by five different labs was published. The work comprehensively described the identification of many PPTases using bioinformatics, the characterization of family II proteins by heterologous expression, purification and biochemical characterization and the clarification of the link between Sfp, EntD and Gsp in natural product biosynthesis.[1] This paper and previously published work marked the widespread discovery of many *acpS* and *sfp* genes across all three domains of life.[14]

Sfp expresses well in *E. coli* and other heterologous hosts and shows highly permissive catalytic activity towards CPs using not only CoA but CoA-like substrates. These properties now afforded many labs with the ability to delve deeply into the biosynthesis of many natural products. Until recently, Sfp was also the only family II PPTase for which the three dimensional structure was reported affording an atomic resolution understanding of substrate recognition and CP maturation.[15] Sfp quickly became the go-to PPTase used in many *in vitro* assays requiring PPTase activity for holo-CP synthesis. Also, its utility in metabolic engineering was widely recognized because it relieved a major bottleneck associated with the concentration of bioactive holo-CPs needed for *in vivo* production of targetted metabolites.

The workhorse bacterium *E. coli* K12 afforded the identification of even a greater variety of PPTases from a single organism. As described earlier, the PPTase AcpS (family I) was first identified in *E. coli* as responsible for phosphopantetheinylating AcpP; however, feeding radioactive pantothenate to *E. coli* revealed two other proteins that incorporated radioactivity. One was later identified as EntF,[16] the CP of the enterobactin synthase complex. This discovery then showed that EntF is phosphopantetheinylated by the family II PPTase EntD. The other protein is EntB, a small isochorismate lyase-carrier protein fusion involved in the initiation of enterobactin synthesis. Bioinformatic tools and the newly uncovered *E. coli* genome revealed another PPTase-like sequence. Originally named o195 (after ORF195), it was later renamed AcpT. AcpT showed sequence similarity to EntD and Sfp. AcpT exhibited poor *in vitro* PPTase activity when using either ACP or EntF as CP acceptors.[1] Surprisingly, AcpT rescued an *E. coli* strain with defects in YejM, a membrane protein with unknown function. Even more baffling AcpT did not need to be a catalytically active enzyme to rescue the *yejM* defective *E. coli* strain (see Section 3.1).[17]

More broadly, in family II PPTases, the genes encoding the PPTase often reside in close proximity to, or part of, a synthase operon. Nevertheless, there are also many cases where the PPTase genes are found far removed from their CP substrate genes and the other synthase operon genes. With improved bioinformatic tools, family II PPTases could be grouped based on phylogenic distributions and sequence alignments.[1, 14, 18] For example, sequence alignments of PPTases identified two highly conserved regions, called ppt-1 and ppt-3, now generalized as the bipartite sequence, (I/V/L)G(I/V/L/T)D(I/V/L/A)$(x)_n$(F/W)(A/S/T/C)xKE(S/A)h(h/S)K(A/G), where x are chemically disparate amino acids, n is 42–48 aa for AcpS (family I) and 38–41 aa for Sfp-type (family II) PPTases, and h is an amino acid with a hydrophobic side chain. Moreover, the sub-motifs WxxKEA or FxxKES are linear fingerprints for at least two different subclasses of Sfp-type PPTases (family II) discussed phylogenetically in Section 3.9 and structurally in Section 5.

2.3 Family III: type I megasynthase PPTases

In the eukaryote Saccharomyces (yeast), three different PPTases have been identified: PPT2 that activates a recently discovered type II mitochondrial FAS ACP, a PPTase dedicated to α-aminoadipate semialdehyde dehydrogenase (AASDH) activity, and a translationally fused PPTase domain sitting at the C-terminal end of a cytosolic type I FAS. Curiously, this translational fusion in yeast and other fungi differs from another eukaryotic system, the cytosolic type I mammalian FAS. In these latter FASs, their translationally fused CPs are phosphopantetheinylated by independently translated family II PPTases.

The mammalian and fungal FASs arrange as strikingly different oligomeric structures. Type I mammalian synthases are multidomain single polypeptide monomers that organize physiologically as 540 kDa dimers. In contrast, type I fungal synthases contain one (or sometimes two) multidomain polypeptides that assemble into large 2.6 MDa hexameric or heterododecameric complexes.[19] In the yeast *S. cerevisiae*, its FAS megasynthase consists

of two related proteins, FAS1 and FAS2 that form a hexameric $\alpha_6\beta_6$ oligomer. It is thought that the PPTase of one α-subunit installs the PPant arm on ACP of the second α-subunit, suggesting that the α2 dimer needs to be assembled before post-translational modification. This is supported by the previous observation that activation of *apo*-FAS was more efficient after *in vitro* re-association.[20] However, the crystal structure of the fungal FAS revealed that the PPTase domain does not associate with other protein modules, sitting 60 Å away from the nearest ACP. Moreover, the PPTase module is located on the outside of the barrel-like $\alpha_6\beta_6$ oligomer, whereas fatty acid biosynthesis takes place inside the barrel.[21] This static structural arrangement suggests that the formation of a transient dimer of two PPTase modules forms first to allow for, post-translational modification of ACP modules which is then followed by full barrel assembly.[22]

When the integrated PPTase domain of *S. cerevisiae* FAS was subcloned and independently expressed from the entire megasynthase, the PPTase formed a trimeric complex similar to AcpS (family I), with three active sites formed by the inter-subunit interfaces. Unexpectedly, both the full length FAS retaining the PPTase domain and the excised PPTase trimer were equally active *in vitro*. Surprisingly, the fully assembled $\alpha_6\beta_6$ FAS was still able to phosphopantetheinylate free *apo*-ACP *in vitro*, suggesting that dynamics of the $\alpha_6\beta_6$ oligomer occur and play critical roles in ACP maturation and FAS function.[22] Recently, cryo-electron microscopy of the FAS particle again showed the PPTase domain resided on the outside of the barrel, but substantial flexibility in the wall of the barrel was also inferred from multiple particle reconstructions.[23] Nevertheless, how the PPTase domain of this family forms an active enzyme, when does this catalytic activity arise during assembly of the megasynthase, and how the mature FAS particle retains phosphopantetheinyl transfer activity remain major unanswered questions.

3. Importance in primary and secondary metabolism

Since the discovery of the large superfamily of PPTases,[1] many homologs have been discovered in various organisms, and the past 25 years has seen a surge of literature on both the characterization of PPTases and identification of PPTase genes in (newly uncovered) biosynthetic clusters. Here we summarize the discovery, and the identification and the target(s) of nearly all PPTases that have been found to date across diverse species. Bacteria and cyanobacteria commonly produce secondary metabolites, thereby representing a rich spectrum of PPTases (Table 1). Since AcpS-type (family I) PPTases are found in many species, and in all cases responsible for FAS activation, this class of PPTases is only briefly mentioned in this section. PPTases activate secondary metabolite biosynthetic clusters, and these clusters along with their products are discussed in detail. Further, in some cases, the family II PPTases are not translated due to genetic inactivation. This mutational event leads to altered secondary metabolism and interesting phenotypes likely under selective pressure. Finally, we assembled large sets of PPTase sequences and constructed phylogenetic trees, showcasing fascinating evolutionary groupings of PPTase natural diversity. Finally, this section aims to emphasize the ubiquity of PPTases, their broad function and diverse catalytic specificities, and their critical role in many secondary metabolism pathways.

3.1 Bacteria

Bacteria are notable producers of CP-mediated secondary metabolites that include pigments, siderophores and antibiotics. Presumably, this arsenal of molecules are produced to scavenge nutrients (e.g. siderophores) and/or provide the host organisms with enhanced fitness in the face of challenging biotic and abiotic environments. In bacteria, pigments are generally not produced for color-mediated recognition, but instead serve as UV protectants, antioxidants, siderophores or antibiotics. Soil-dwelling bacteria, like *Streptomyces* and *Bacilli*, produce many diverse secondary metabolites produced by elongating synthases, and

therefore encode a variety of PPTases that activate synthase-mediated pathways. Even the most common laboratory bacterium, *E. coli* K12, hosts more than one PPTase gene.

Escherichia coli—The genome of *E. coli* K12 contains three genetically encoded PPTases: AcpS, EntD and AcpT. AcpS was identified by Elovson and Vagelos in 1968,[3] shortly after the discovery of the FAS ACP (AcpP).[89] Overexpression of AcpS allowed for detailed characterization of this enzyme.[90]

EntD is the PPTase of the enterobactin biosynthetic cluster (Fig. 3). Enterobactin[91] is a siderophore that is secreted to scavenge iron from the environment for bacterial viability. After biosynthesis, enterobactin is exported from the cytosol to the periplasm, from the periplasm to the outside of the cell, and after binding of of iron, imported back into the bacterium.[92] The biosynthetic Ent cluster and the natural product, then called enterochelin, were isolated and studied in the early 1970s.[93] At that time, it was known that the proteins encoded in the gene cluster associate and require ATP and Mg^{2+} for activity. The biosynthesis starts with the conversion of chorismate to 2,3-dihydroxybenzoate catalyzed by EntA, B and C. Serine and 2,3-dihydroxybenzoate are then assembled into enterobactin by EntD-EntG, which encompass an NRPS cluster. Later, EntD was overexpressed by two laboratories independently and found to be membrane localized, but no function could be assigned to the protein.[94–96] EntD was identified as a PPTase by Lambalot et al.[1] and shown to modify the PCP EntF. The C-terminal carrier protein domain of EntB, a bifunctional isochorismate lyase, also served as a substrate for EntD.[97] The assembly line enzymology of enterobactin and other bacterial siderophores has been reviewed by Crosa and Walsh.[98]

The gene *entD* has been knocked out in *E. coli* (e.g. strain AN90-60).[99] The resulting strain does not produce enterobactin, based upon visualization using low-iron CAS indicator plates, on which wild type strains show a yellow halo and siderophore-deficient strains do not. Overproduction of AcpS cannot compensate the absence of EntD.[100] Conversely, overexpressing *entD* on an inducible plasmid could not complement the absence of *acpS*.[100, 101] However, *in vitro* EntD seems to modify *apo*-AcpP from *E. coli*, albeit at a very slow rate.[1] It is noteworthy that in *acpS* knockout *B. subtilis* strains, fatty acid biosynthesis is maintained, presumably due to the latent activity of Sfp in fatty acid biosynthesis.[102] Surprisingly, the specificity of EntD for carrier proteins or CoA analogs has not been examined in detail. Chalut et al.[36] observed that when expressing PKS modules from mycobacteria in *E. coli*, some synthases were phosphopantetheinylated. To discover the protein responsible for modifying mycobacterial synthases in *E. coli*, an *entD* knockout mutant was constructed, which did not show 4′-phosphopantetheinylation of PKS modules. So, although EntD is part of an NRPS (activating PCPs), the enzyme also seems to be active on other elongating synthases carrying ACPs affording complementation of synthase activity in the absence of their cognate PPTases.

The third PPTase from *E. coli*, AcpT, was identified by Lambalot et al.,[1] but has been difficult to characterize.[35] In *E. coli* O157, AcpT lies in the O-island 138 gene cluster which contains fatty acid biosynthesis-like genes, including two putative carrier proteins Z4853 and Z4854. When these ACPs were expressed in *E. coli* K12 (which lacks the O-island 138 gene cluster), 4′-phosphopantetheinylation was independent of the presence of AcpS but dependent on the presence of AcpT. In AcpS knockout strains, AcpT is only able to restore very slow growth suggesting realization of a latent but low level permissiveness to other non-cognate substrates. The gene cluster present in *E. coli* O157 between *yhht* and *acpT*, which reside next to each other in *E. coli* K12 (thus lacking the O-island 138), consists of a set of interesting FAS-related enzymes (Fig. 4). Sequence alignment of the ACPs Z4853 (or Ecs4328) and Z4854 (or Ecs4329) reveals the conserved serine for PPant modification, but

with a motif that contains “DSI” instead of the typical AcpP DSL motif. This motif is also found in PUFA (poly-unsaturated fatty acid) synthases as well as in type I PKS synthases.[2]

Another pathogenic *E. coli* strain of the phylogenetic group B2 produces a compound that induces DNA double strand breakage in eukaryotic cells.[103] The biosynthetic origin of this toxin was found in a hybrid PKS-NRPS cluster, (also known as “PKS island”) producing colibactin (Fig. 5). Within this cluster, clbA was identified as a PPTase.[103, 104] Colibactin has never been isolated and its structure remains unknown. However, recently, advances have been made in the elucidation of this natural product structure. ClbP (Fig. 5) was identified as a member of a unique group of D-asparagine peptidases involved in the maturation of non-ribosomal peptides.[105, 106] ClbP was shown to be membrane bound and its enzymatic activity located in the periplasm, suggesting biosynthesis of pre-colibactin in the cytosol, export to the periplasm and subsequent activation (thereby preventing toxicity to *E. coli* itself).[107] The NRPS ClbN and the NRPS portion of ClbB were recently characterized *in vitro*.[108] ClbN synthesizes myristoyl-D/L-asparagine and ClbB(NRPS) condenses L-Ala or L-Val onto this scaffold. ClbP cleaves off myristoyl-D-Asn. Recently, *in vivo*, the *clb* gene cluster was knocked-out and the metabolites analyzed. The only natural product found in the wildtype strain, which was not present in the knock-out strain, was myristoyl-D-Asn; the structure of the natural product remains elusive.[109]

Other *E. coli* strains harbor this cluster, including the non-pathogenic probiotic *E. coli* Nissle 1917.[110, 111] Screening 1565 isolates by PCR revealed that the PKS island is also present in *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Citrobacter koseri*.[112] There is considerable interest in colibactin, since it might be that its biosynthesis is associated with colon cancer.[113] *E. coli* Nissle 1917 is used as a probiotic and improves chronic inflammatory bowel disease, but its mode of action is unknown. Interestingly, deletion of the PPTase *clbA* gene causes abolishment of its DNA damage activity, but at the same time also loss of its probiotic activity.[114]

The PPTase ClbA was recently characterized in more detail.[115] Besides enterobactin and colibactin, some *E. coli* strains also produce yersiniabactin. Yersiniabactin is encoded by the high-pathogenicity island and in contrast to *Yersinia pestis* (in *Yersinia pestis* YbtD is the dedicated PPTase)[116] no PPTase is found in the *E. coli* genome that seems to activate this synthase. *In vitro* and *in vivo*, EntD and ClbA can activate yersiniabactin and enterobactin synthases, but the colibactin synthase cannot be activated by EntD. *In vitro*, YbtD, Sfp and PptT can activate colibactin synthase. Interestingly, the PKS island and the high-pathogenicity island are strongly associated and it is hypothesized that this feature is selected in virulent *E. coli* species because the PPTase ClbA can activate both siderophore and genotoxin biosynthesis (Table 2). [115]

Burkholderia—*Burkholderia* are prolific Gram-negative bacteria that are animal, human and plant pathogens. *Burkholderia cenocepacia* produces the siderophores ornibactin and pyochelin by NRPSs. The *pobA* gene was identified as a PPTase and shown to efficiently complement an *E. coli entD* mutant.[18]

Burkholderia pseudomallei is the causative agent of melioidosis, a serious infectious disease in humans, and is resistant to many antibiotics. Su et al. identified bacterial antigens that are immunogenic in the human host, with the hope that these bacterial proteins were upregulated during infection. Interestingly, one of the 109 proteins upregulated during infection was the PPTase BPSS2266.[119]

Burkholderia rhizoxinica is an intracellular symbiont of the plant pathogen *Rhizopus microsporus* and produces the antimitotic polyketide rhizoxin for its fungal host. Rhizoxin is

biosynthesized by a PKS-NRPS hybrid and is also made by *P. fluorescens Pf-5*, which uses a similar gene cluster. In *B. rhizoxinica*, the rhi synthase is encoded on the chromosome and not on one of its several megaplasmids, as previously thought. However, it seems remnants of the synthase cluster are present on one of the megaplasmids, including the PPTase Brp (genbank: RBRH_02776).[120]

Burkholderia thailandensis produces a series of quorum sensing quinolones bearing an unsaturated medium chain fatty acid tail. The *hmq* gene cluster has been shown to mediate the biosynthesis of these 4-hydroxy-3-methyl-2-alkylquinolones, showing close homology to the *pqs* gene cluster in *Pseudomonas aeruginosa*, which is responsible for 4-hydroxy-2-alkylquinoline production. Recently, HmqF was identified as the source of the unsaturated fatty acid, and contains adenylation and dehydrogenase domains along with an ACP.[121] The PPTase that post-translationally modifies this ACP is so far unknown, but at least four PPTases (apart from AcpS) have been annotated in the genome of *B. thailandensis*.

In *Burkholderia* K481-B101,[122] glidobactin is synthesized by a PKS-NRPS hybrid.[123] Glidobactin is an N-acylated depsipeptide with a 12-membered macrolactam ring that inhibits the proteasome. The unsaturated fatty acid chain of glidobactin is installed by a part of the synthase called GlbF consisting of a condensation-, adenylation-, and PCP domain, requiring 4′-phosphopantetheinylation.[122] Here again, the PPTase that is necessary for this post-translational modification is currently unknown.

Streptomyces—*Streptomyces*, the largest genus of actinobacteria, are famous for their large variety of natural products produced ribosomally, by PKS, NRPS or other pathways.[124] For example, *Streptomyces coelicolor* is a Gram-positive bacterium that contains 25 biosynthetic secondary metabolite clusters that produce natural products, including actinorhodin, coelichelin, coelibactin, TW95a, EPA, methylenomycin (encoded on a giant linear plasmid), prodiginines and the antibiotic CDA (Fig. 6).[124] All of these natural products are produced by synthases that require phoshopantetheinylation. Cox et al. identified an AcpS-type PPTase in *S. coelicolor*, which shows a surprising level of permissiveness for CoA substrates as well as carrier proteins.[50] For example, type I mammalian FAS, type I fungal PKS, and several type II bacterial ACPs were efficiently phosphopantetheinylated. The genome of *S. coelicolor* contains two other PPTases: RedU, which is a dedicated PPTase for undecylprodigiosin biosynthesis, and SCO6673 required for CDA biosynthesis.[125] Further *in vivo* studies reveal that both RedU and SCO6673 have specific carrier protein targets, whereas ScAcpS shows broad activity. Recently, the crystal structure of ScAcpS was determined, showcasing the basis for relaxed substrate selection (discussed in Section 5).[126]

The existence of many biosynthetic pathways in *Streptomyces* species led to some controversy over whether certain peptidic natural products were ribosomally produced (lantibiotics) or non-ribosomally synthesized. For example, *Streptomyces actuosus* produces the antibiotic nosiheptide,[128] part of a large family of cyclic thiopeptides,[129, 130] thought to be non-ribosomally synthesized. However, it was recently shown that these macrocylic peptides are ribosomally synthesized and post-translationally modified by a nosiheptide biosynthetic cluster (Nos A to P).[131] In 1990, Li et al. discovered a protein called NshORFC that sits upstream from the Nos cluster,[132] which Lambalot et al. computationally identified as a PPTase, renaming it NshC.[1] Since these thiopeptides are made ribosomally, their biosynthesis does not require a carrier protein or PPTase; so why does this *Streptomyces* species have an EntD/Sfp-like PPTase in its genome closely located to nosiheptide production? Closer evaluation of the Nos biosynthetic cluster reveals the presence of unassigned protein NosJ with a predicted length of 79 aa. It contains the highly conserved DSL motif of carrier proteins, which suggests that the ribosomally produced pre-nosiheptide

is loaded onto a Nos carrier protein before it is post-translationally modified. NosI, directly upstream of NosJ, is indeed annotated by Yu et al.[131] as an AMP-dependent acyl-CoA ligase, which may be capable of loading the pre-peptide onto the carrier protein in an adenylation-type of reaction.

The Nos gene cluster shows similarity to nocathiacin (Noc), thiostrepton (Tsr) and siomycin (Sio). In these three, no NosJ homologs have been annotated as of yet. However, psi-blast analysis of NosJ reveals homologous hits in the large putative hydrolase/transferase proteins NocK, TsrI and SioP (see sequence alignment in Fig. 7), which all contain a similar sequence to NosJ and are directly neighboring the putative adenylation domain NosI (and its homologs). Although speculative, it seems that these biosynthetic clusters have both ribosomally encoded and non-ribosomally encoded synthase characteristics. Future studies will tell how PPTases, in particular Nsh-ORFC, fit into this story.

Streptomyces vertilicus produces bleomycin, and no PPTase is associated with its biosynthetic gene cluster. By similarity to other *Streptomyces* PPTases, the PPTase Svp was identified, cloned and overproduced in *E. coli*. Svp is a catalytically promiscuous PPTase, active on both type I and type II ACPs as well as some PCPs.[56]

Streptomyces noursei produces nystatin using a type I modular polyketide synthase. At the 5′ border of the biosynthetic cluster, a putative PPTase gene, *nysF*, was discovered. However, upon deletion of this gene, an increase in nystatin production was observed, suggesting a regulatory role for this protein.[54] Since *nysF* has not been characterized in more detail, it is unclear whether this protein is a bona fide PPTase, a regulatory gene product, or both.

Streptomyces collinus Tue 365 produces kirromycin, a potent antibiotic that blocks translation in bacteria by interfering with the elongation factor Ef-Tu.[52] Kirromycin was discovered in 1974 by Wolf et al.,[133] and >200 publications have discussed its biosynthesis and mechanisms of action. In 2007, Weber et al. characterized the PKS-NRPS hybrid in detail and identify the PPTase KirP, essential for phosphopantetheinylating the large number of acyl- and peptidyl carrier proteins.[134] Interestingly, inactivation of KirP does not result in the total abolishment of kirromycin biosynthesis.[52]

Streptomyces griseus produces fredericamycin, and *fdmW* has been identified as a PPTase, within this PKS gene cluster.[53] Inactivation of FdmW resulted in a 93% reduction of fredericamycin production. *Streptomyces antibioticus* produces the amino-coumarin antibiotic simocyclinone, using 38 ORFs.[48, 49] Interestingly, two different PPTases (simA11 and simC8) are associated with the biosynthesis of this natural product. SimA11 is similar to JadM and simC8 is similar to NysF (Table 1), but so far the carrier protein targets of these PPTases are unknown.

The genome of *Streptomyces avermitilis* contains a stunning 85 separate carrier proteins, from which some are involved in >70 PKS/NRPS synthase biosynthetic systems.[135, 136] The *S. avermitilis* genome also reveals four distinct PPTases, which have low sequence similarity and seem to represent different subclasses of the Sfp-type family of PPTases.

Recently, dedicated PPTases have also been found associated with pactamycin biosynthesis (PctR) in *S. pactum*,[137] A74528 biosynthesis (SanW) in *Streptomyces* sp. SANK61196,[138] tirandamycin biosynthesis (TrdM) in *Streptomyces* sp. SCSIO1666,[139] jadomycin biosynthesis (JadM) in *S. venezuelae*,[55] oviedomycin biosynthesis (OvmF) in *S. antibioticus* ATCC 11891,[140] griseorhodin biosynthesis (GrhF) in *S. lividans*,[141] and natamycin biosynthesis in *S. chattanoogensis* L10.[142]

Bacilli—Bacilli are Gram-positive bacteria that are used extensively in agriculture for crop protection, due to their ability to produce natural products that protect germinating seeds. This group of protective molecules are generally made by NRPSs, which require 4′-phosphopantetheinylation. The PPTase Sfp was first identified serendipitously by Nakano et al.[143] in attempts to transform the non-surfactin producing strain *B. subtilis* JH642 into a surfactin producer. Interestingly, although *B. subtilis* JH642 does not produce surfactin, the surfactin gene cluster is transcribed and expressed. It was shown that a frameshift mutation led to expression of a truncated, inactive, Sfp protein of 165 aa.[144] In 1994, Borchert et al.[13] identified a gene, *gsp*, located directly upstream from the gramicidin synthase gene, with 34% identity and 54% homology to Sfp. Gsp complements a Sfp deficiency in a *B. subtilis* mutant, but until 1996 its function remained unknown.[1]

Bacilli species produce several non-ribosomal peptides, including surfactin and iturin A.[145] Before the identification and characterization of PPTases, Huang et al.[30] discovered an open reading frame in *B. subtilis* RB14 that regulated the production of iturin A. This ORF named lpa-14, showed homology to Sfp. Another *B. subtilis* strain, YB8, produces the lipopeptides surfactin and plipastatin B1, both biosynthesized by their respective NRPSs, and phosphopantetheinylated by lpa-8, which is better known as Sfp.[29] *B. subtilis* strain B3 produces fengycin using another NRPS, which is activated by lpa-B3 (lpa-14).[31] The *B. subtilis* strain RP24, isolated from the rhizoplane of a field of pigeon pea, possesses antifungal activity. Iturin A, surfactin and fengycin were identified in this agricultural isolate, which also contains a homolog to lpa-14 found to be the PPTase.[146] *B. lichenformis* produces bacitracin by an NRPS, and Bli is the PPTase that phosphopantetheinylates the PCP domain of this elongating synthase.[33, 34] Bli was later expressed in *E. coli* and used to phosphopantetheinylate a domain from tyrocidine synthase *in vitro*.[32] Finally, the *B. pumilus* A-1 gene *psf-1* was found to regulate the production of surfactin and later shown to encode an active PPTase.[27]

Many bacilii species, including *B. anthracis* and some marine bacteria, produce the siderophore petrobactin, which is biosynthesized by a hybrid NRPS/non-NRPS siderophore synthase.[147] The synthase-encoding cluster contains a stand-alone PCP domain, AsbD, which is phosphopantetheinylated by an unknown PPTase. There is no PPTase present in the gene cluster itself and it has been suggested that BA2375, an EntD homolog present in the enterobactin gene cluster, serves as the PPTase that installs the 4′-PP arm on AsbD.[148] *Holo*-AsbD is loaded with 3,4-dihydroxybenzoic acid by AsbC and this AsbD conjugate functions as the substrate for AsbE. AsbE, together with the stand-alone synthases AsbA and AsbB, catalyze the formation of petrobactin.

Mycobacteria—Like many bacteria, mycobacteria secrete siderophores to harvest iron from their hosts. These siderophores are peptide-based and fall into two categories, the water-soluble exochelins and the membrane-associated mycobactins. These molecules have very similar core structures, but differ in the length of their fatty acid side chain (the water soluble siderophores have a short C_5–C_9 chain and the membrane-associated a long C_{18}–C_{21} chain). Both of these siderophores are found in numerous bacteria, and in all cases are produced by the expression of NRPS gene clusters. During the characterization of the biosynthetic cluster of mycobactin from *M. tuberculosis*, PptT was identified as the PPTase for the mycobactin synthase.[38]

In addition to the mycobactin synthase, more than 18 type I polyketide synthases and two FAS genes have been annotated in the genome of *M. tuberculosis*, enabling this bacterial species to genetically encode a large variety of unusual membrane lipids for protection.[149] For example, mycolic acid production relies on two FASs and one PKS. The presence of two FASs in one organism is not common across all three domains of life, but is more

prevalent in eukaryotes and protista. For *M. tuberculosis*, the second FAS (dedicated to mycolic acid biosynthesis) is discussed in more detail in Section 4. The large number of carrier proteins in these synthases are post-translationally activated by PPTases. MtAcpS is responsible for phosphopantetheinylation of the two FASs and PptT for the phosphopantetheinylation of the NRPS and PKSs.[36] Interestingly, a PptT knockout strain in *M. bovis* BCG is not viable, suggesting that this PPTase is essential for organismal viability (although AcpS is present).

X-ray crystal structures have been published of MtAcpS,[150] showing that the protein undergoes conformational changes at pHs >6.5, with resultant decreases in AcpS activity.[151] The intracellular pH of mycobacteria is between 6.1–7.2, even when exposed to acid or base,[152] and although purely speculative, it might be that siderophore or other natural product production is regulated by the activity of the PPTase.

Recently, LeBlanc and co-workers delved deeper into the requirement of mycobacteria to express PptT.[37] Two conditional *pptT* mutants in *M. bovis* BCG and *M. tuberculosis* H37Rv showed retarded growth and persistence. Mutants in which the PPTase gene was controlled by a tetracycline promoter, were constructed, allowing for conditional regulation of the PptT expression. A 95% depletion of PptT was required to inhibit growth of *M. bovis*. Although the constructs in *M. bovis* and *M. tuberculosis* were identical, much higher concentrations of tetracycline were required for growth of *M. tuberculosis*, suggesting either different tetracycline uptake rates, different regulation of expression or higher levels of PptT are required for growth of *M. tuberculosis*. Nevertheless, PptT is necessary for *in vitro* growth of mycobacteria, but the authors point out that although essential *in vitro*, mycobacteria have been shown to require enzymes *in vitro* that are not required *in vivo*. Thus, PptT knock-down strains were also tested for viability in macrophage and mice infections. Both *M. bovis* and *M. tuberculosis* fail to multiply *in vivo*. Although PptT appears to be an excellent anti-mycobacterial target *in vitro* and *in vivo*, the cumulative effects of PPTase depletion are still unknown, since mycolic acid, mycobactin, polyketide-derived lipids, fatty acids, siderophores and some yet to be discovered natural products all depend on PPTase activity for biosynthesis.

Recently, we identified a PPTase in *M. ulcerans* (unpublished data), MuPpt, presumably responsible for phosphopantetheinylating mycolactone synthase. *M. ulcerans* is a human pathogen and the causative agent of Buruli ulcer. The core of mycolactone,[153] a cytotoxic and immunosuppresive natural product, is biosynthesized by two large PKSs (MLSA1, 1.8 MDa and MLSA2, 0.26 MDa) both of which are encoded on a large plasmid (174 kb), pMUM001.[154] The plasmid does not contain a PPTase, thus the synthases must be modified by MuPpt.

Serratia marcescens—The biosyntheses of the red pigment prodigiosin and the surfactant serrawettin W1 in *Serratia marcescens* depend on the presence of the PPTase PswP.[155] Mutants of PswP are unable to produce pigment or surfactant but are still able to produce 2-methyl-3-n-amyl-pyrrole (MAP) and condense it with supplied 4-methoxy-2,2′-bipyrrole-5-carbaldehyde (MBC) to form prodigiosin (Fig. 8). *Serratia* species appear to employ two PPTases, one to activate the PCP of PigG and one to activate the ACPs of PigH. Similarly, in *S. coelicolor*, which also produces prodiginine, the PPTase RedU is responsible for 4′-phosphopantetheinylation of RedO but not for the other PCP and ACP domains.[51] Besides PswP, PigL has also been characterized as a PPTase.[156]

The insect pathogen *S. marcescens* Db10 also produces another antibiotic, althiomycin, that inhibits growth of both *B. subtilis* and *S. aureus*.[45] Althiomycin is synthesized by a PKS-NRPS hybrid and additional tailoring enzymes. Two Sfp/EntD-type PPTases were identified

in *S. marcescens*, and a knockout mutant for each was constructed. Althiomycin production was eliminated upon deletion of one PPTase gene, SMA2452, whereas the other PPTase gene mutation, SMA4147, had no effect. Sequence alignments and psi-blasting of *pswP* and *pigL*, previously identified as PPTases, shows that SMA2452 is PswP. The althiomycin biosynthetic cluster has previously been found in actinomycetes and *Myxococcus*, evolutionary unrelated bacteria, suggesting that *S. marcescens*, which is closely related to *E. coli*, likely obtained this gene by horizontal gene transfer in either direction.

Recently, three broad spectrum antibiotics were isolated from *S. plymuthica* RVH1, called the zeamines. The gene cluster responsible for their biosyntheses contains FAS, PKS and NRPS characteristics and resembles the *pfa* gene cluster, responsible for PUFA biosynthesis in marine bacteria. A potential PPTase, Zmn5, was identified in zeamine gene cluster, which does not show homology with the dedicated PUFA synthase PPTase PfaE, but instead shows a clear homology with EntD and Sfp.[157]

Vibrio—Some *Vibrio anguillarum* strains produce the siderophore vanchrobactin utilizing the NRPS VabF. VabD is the dedicated PPTase used for 4′-phosphopantetheinylation of VabF.[58, 158] A VabD knockout strain shows no retarded growth under iron-rich conditions, but reduced growth under iron-depletion.[57] *V. anguillarum* strains also contain a second, more dominant, siderophore anguibactin, located on the pJM1 plasmid. Anguibactin is also synthesized by an NRPS and requires the PPTase AngD. Both VabD and AngD can complement one another, but since anguibactin is the more potent siderophore, some strains have lost the vanchrobactin biosynthesis pathway.[159] *V. cholerae* is a bacterial pathogen that biosynthesizes a unique siderophore, vibriobactin, using biosynthetic machinery similar to enterobactin synthase. Both VibB and VibF are phosphopantetheinylated by the PPTase VibD.[158, 160]

Pseudomonas—The genome of *Pseudomonas aeruginosa* contains one Sfp-type PPTase, PaPcpS (previously named ‘“PcpS”), with broad substrate specificity. Like *H. influenzae* and cyanobacteria, no AcpS-type PPTase can be identified in the genome.[41] *P. aeruginosa* is a Gram-negative human pathogen that is resistant to antibiotic treatment. Two siderophores, pyoverdin and pyochelin, are produced by NRPSs and associated with the virulence of these bacteria. Pyoverdine biosynthesis requires phoshopantetheinylation of PvdD, PvdI and PvdJ and pyochelin biosynthesis requires activation of PchE and PchF. PaPcpS was identified by similarity to EntD, AcpS and Sfp, although the 242 aa protein shows only 13% similarity to Sfp, and in contrast to Sfp is not located in close proximity of a synthase. PaPcpS acts on FAS, PKS and NRPS acyl carrier proteins, but the PPTase is optimized for primary metabolism, since its activity drops 30-fold for ACPs or PCPs from secondary metabolism *in vitro*.[41] Construction of a PaPcpS knockout was unsuccessful, suggesting that the protein is essential. PaPcpS can genetically complement mutations in AcpS and EntD in *E. coli*, and it can also complement an Sfp mutant in *B. subtilis*.[100] A PaPcpS knockout *P. aeruginosa* strain could only be constructed in an *E. coli* AcpS (EcAcpS) carrying strain, suggesting that the essentiality of PaPcpS originates from its function in primary metabolism. This mutant strain also shows no siderophore production and does not grow in the presence of iron chelators.

Since *Pseudomonas* species are possibly good heterologous hosts for the production of natural products, Gross et al. screened six different carrier proteins from large synthases for efficient 4′-phosphopantetheinylation by the endogenous PPTases. Indeed, the broad specificity of the single PPTase present in *Pseudomonas* sp. could be used to 4′-phosphopantetheinylate various carrier proteins.[42]

P. syringae produces the toxin coronatine, made by a synthase requiring PPTase activity on two individual ACPs, two ACP domains and one PCP. The single PPTase from *P. syringae* was identified (PspT) and shown to have 62% identity with PaPcpS. Interestingly, this PPTase, although having broad activity, shows preference for secondary metabolism carrier proteins, in contrast to PaPcpS.[161]

Genome analysis suggests that other *Pseudomonas* species also utilize one PPTase, although substrate specificity might vary.[43] *Pseudomonas fluorescens* produces the antibiotic mupirocin (pseudomonic acid), which is thought to be biosynthesized by four large type I PKS/FASs and a number of modifying enzymes. Eleven ACPs in the type I synthases and five putative type II ACPs require 4′-phosphopantetheinylation, and a putative PPTase in the gene cluster, MupN, has been identified.[162] Indeed, when *mupN* was deleted, mupirocin production was abolished, and it was shown *in vitro* that both type I and type II ACPs were modified by this PPTase.[43] In contrast to *P. aeruginosa*, *P. fluorescens* has two PPTases, PfPcpS and MupN, at its disposal, and it remains a question whether PaPcpS and PfPcpS have different substrate specificity.

Myxobacteria—Myxobacteria are natural product factories.[163] Myxothiazol was found in *Stigmatella aurantiaca*, epothilone in *Sorangium cellulosum* and *Myxococcus xanthus* produces several polyketides and PKS-NRPS hybrid natural products.[164] The Mta gene cluster is responsible for myxothiazol production and the PPTase MtaA for the 4′-phosphopantetheinylation of this PKS-NRPS hybrid. Mutation of MtaA results in abolishment of the production of many metabolites, including myxothiazol.[46] MtaA has a relative broad carrier protein substrate scope.[47] Interestingly, *M. xanthus* DK1622 expresses two functionally redundant PPTases (MxPpt1, having ~60% sequence identity to MtaA, and MxPpt2), both with relatively broad substrate specificity, supported by the fact that active myxothiazol-synthase and epothilone-synthase could be expressed in *M. xanthus* without the need of coexpression of another PPTase.[164]

Xanthomonas albilineans—The plant pathogenic bacteria *Xanthomonas albilineans* is the causative agent of sugar cane leaf scald, and important factors in this disease are the albicidins produced by the bacteria.[165, 166] Albicidins inhibit DNA replication in bacteria and plastids. Albicidin is partially characterized, but due to the extremely low isolation yield, the biosynthesis is better understood than its chemistry.[167] In 2000, a PPTase gene *xabA* was identified in the genome of *X. albilineans* and shown to be essential for production of the antibiotic, suggesting that the compound is made by a synthase.[59] A *xabA* knockout did not produce albicidin, but when EntD was engineered into the mutant strain, production was restored to the same levels as the wild type strain. Albicidin is indeed produced by a large synthase, XabB, which is a 500 kDa PKS-NRPS hybrid synthase.[168] The gene cluster was characterized in detail, and a theoretical backbone structure of the natural product was proposed.[169] Although the PPTase XabA is essential for albicidin production, it is currently unknown whether this PPTase acts on all five carrier proteins. To increase the production of albicidin, the Xab gene cluster was expressed on two plasmids in *X. axonopodis pv. vesicatoria*, and showed a 6-fold increased yield of the antibiotic.[170] Since non-producing albicidin mutants are still plant pathogenic, Gabriel and Royer's groups have systematically studied the other factors that influence pathogenicity.[165, 166] PPTase knockout mutations indeed showed no albicidin production, but did not eliminate sugar cane leaf scald. More dramatic effects were seen when transport proteins, OmpA family proteins or other (hypothetical proteins) were mutagenized. Recently, another three large NRPS clusters have been identified in *X. albilineans* genome, all with unknown function.[171] Since there are multiple PPTases found in the genome of *X. albilineans*, it is

possible that, although hampered by the XabA knockout, other PPTases still phosphopantetheinylate carrier proteins in these NRPSs, contributing to pathogenicity.

Other bacteria—The Gram-negative enterobacteria *Salmonella* produces enterobactin, which is a cyclic peptide siderophore secreted under iron-deprevation.[172] Lambalot et al.,[1] identified three PPTases from *Salmonella* species as EntD-type enzymes, but no actual *Salmonella* PPTases have been expressed or characterized.

The antibiotic erythromycin is made by the bacterium *Saccharopolyspora erythraea*, using a modular polyketide synthase containing seven ACPs. Three PPTases have been identified in the genome of *S. erythraea*, namely SeAcpS, SePptI and SePptII. *In vitro* characterization of these PPTases showed that SeAcpS is responsible for FAS activation, SePptI is an integrated part of a modular PKS unit and SePptII is a stand-alone enzyme activating an ACP-TE didomain of erythromycin synthase.[44] The function of SePptI remains unknown but the genome of *S. erythraea* contains four NRPSs and three PKSs, from which the products are unknown.

The causative bacterium of the plague is *Yersinia pestis*. Nine genes have been identified in the high-pathogenicity island with NRPS/PKS character and yersiniabactin transport. Within the *pgm* locus no PPTases were found but by similarity to EntD, *ybtD* was identified as a PPTase, and deletion of the *ybtD* gene resulted in a strain deficient in siderophore production.[116]

Poly-D-3-hydroxyalkanoates (PHAs) are biopolymers (polyoxoesters) synthesized from CoA thioesters by the *pha* gene cluster, found for example in the bacterium *Ralstonia eutropha*. The *pha* gene cluster consists of PhaA, a β-ketothiolase, PhaB, an acetoacetyl-CoA reductase, and PhaC, the synthase/polymerase. Heterologous expression of the PHA gene cluster in *E. coli* resulted in the production of PHA.[173] Feeding [^{3}H]-β-alanine to a PHA-expressing and β-alanine auxotroph *E. coli* strain gave four radioactive bands, corresponding to EntF, EntB, ACP and the PHA synthase, suggesting that PhaC is phosphopantetheinylated.[173] However, construction of β-alanine auxotroph *R. eutropha* strains and feeding of [^{14}C]-β-alanine did not show any labeled protein, except ACP.[174] Two site-directed mutants of conserved serine residues in PhaC had no *in vivo* or *in vitro* synthase activity.[174] However, when these serines were mutated to alanine [^{3}H]-β-alanine was still incorporated in PhaC.[175] Incubation of purified PhaC with [^{3}H]-CoA and either of the PPTases ACPS, EntD, AcpT or Sfp did not result in labeled protein.[175] It remains thus the question whether this synthase requires PPTase mediated activation.

Recently, more PPTases have been found (but not described in detail) in other bacteria, including those involved in factumycin biosynthesis (FacP) in *Acinetobacter baumannii*,[176] guadinomine biosynthesis (GdnS) in S. sp. K01-0509,[177] pelgipeptin biosynthesis (PlpC) in *Paenibacillus elgii*,[178] aureusimine biosynthesis (AusB) in *S. aureus*,[179–181] emetic toxin production (CesP) in *B. cereus*,[182] yersiniabactin-related siderophore biosynthesis (NrpG) in *Proteus mirabilis*,[183] quorum sensing related metabolite production in *Dickeya dadantii* (VfmJ),[184] siderophore cupriachelin production in *Cupriavidus necator* H16 (CucB),[185] siderophore taiwanchelin production in *Cupriavidus taiwanensis* LMG19424 (TaiQ),[186] and the production of a metabolite involved in tobacco hypersensitive response and grape necrosis (F-avi5813).[187]

Nocardia—Some bacterial species can reduce aromatic carboxylic acids to their corresponding alcohols (Fig. 9). A (promiscuous) carboxylic reductase (CAR) was isolated and purified from *Nocardia* sp. NRRL 5646 and appeared to be a 128 kDa protein that requires Mg^{2+}, ATP and NADPH.[188] When the carboxylic reductase was expressed in *E.*

coli, a 50-fold decrease in activity was observed. Incubation of the enzyme with CoA and cell-free extract resulted in an increase in activity. Combined with the presence of a "DSL" sequence-containing protein between adenylation domain and reductase domain, it was thought that lack of 4′-phosphopantetheinylation of the carrier protein was the reason for the reduced activity. Indeed, in the *Nocardia* strain a putative Sfp-type PPTase was identified, *npt*, cloned and expressed and shown to activate the reductase. Doubly transformed *E. coli* with Npt and the reductase CAR shows efficient transformation of vanillic acid to vanillin, in contrast to the non-transformed strain.[39] Recently, this enzyme has received considerable interest in the realm of biofuels and it was shown that overexpression of Sfp, reductase CAR (from *Mycobacterium marinum*) and an aldehyde reductase in *E. coli* resulted in the production of fatty alcohols.[189]

Photorhabdus luminescens—*Photorhabdus luminescens* is a bacterium symbiotic with the nematode *Heterorhabditis bacteriophora*. Together, they infect insect larvae. The nematode needs the bacteria for pathogenicity, growth and reproduction, and the bacterium needs the nematode for spreading between insect prey. Only active bacteria can provide the factors necessary for the nematode and dead cells or supernatants do not. Mutagenesis of the bacteria and subsequent screening for non-complementing behavior of the nematode revealed a mutant that consistently failed to support the nematode. This mutant was defective in siderophore and antibiotic biosynthesis and contained a mutation in an EntD-like protein.[190] Since the bacteria were still viable, this *ngrA* gene is not essential, but most likely involved in the biosynthesis of some form of hormonal, regulatory or signaling molecules made by an NRPS or PKS. Ciche et al. later showed that the inability of the bacteria to produce siderophores (including the newly identified siderophore photobactin) did not hamper the nematode's growth but a mutation in *ngrA* did, suggesting that the biosynthesis of some other compound(s) requires the PPTase.[191] *P. luminescens* also produces bacteriocins and an antibiotic stilbene compound that is biosynthesized via a unique ACP-dependent biosynthetic pathway, unlike the canonical stilbene pathway in plants that utilizes type III PKSs.[192] Although type II PKS clusters are rare in Gram-negative bacteria, *Photorhabdus* also produces an anthraquinone formed from a heptaketide precursor using this route.[193] Within this cluster a dedicated PPTase, AntB, is found, which cannot be replaced by NgrA.[194] In the genome of *P. luminescens ngrA* sits in between two biosynthetic clusters: *phf* and *hpa*. The *phf* genes show close similarity to fimbrial proteins, which are involved in adhesion. The *hpa* cluster is involved in hydroxyphenylacetate metabolism.[195]

Recently, *P. asymbiotica* has been found in human infections in North America and Australia. Detailed genomic comparison shows that insect virulence factors disappeared from its genome, but new human virulence factors appeared from other human pathogenic bacteria. A close homolog of *ngrA* is present in *P. asymbiotica*, making this an interesting antibiotic target.[196]

Marine bacteria associated with sponges—Many natural products are isolated from marine sponges. Often, these natural product are not produced by the sponge, but by a bacterial ecosystem living in the sponge. These bacteria are notoriously difficult to culture in a laboratory environment. In some cases, however, these bacteria have been studied in more detail. For example, when the metagenome of the sponges *Theonella swinhoei* and *Aplysina aerophoba* were studied, PKSs were found that belong to the bacterium phylum *Deinococcus thermus* and produces methyl-branched fatty acids.[87] The gene cluster *sup* was identified and *supC* was annotated as a PPTase similar to PPTases from a sponge symbiont and the PPTase from cyanobacterium *Synechocystis* sp. strain PCC6803. Later, SupC was cloned and expressed and shown to activate SupA-ACP.[86] SupA-ACP is also activated by

Sfp and Svp, but not by EcAcpS. Recently, two NRPSs were uncovered by genomic mining of marine sponges, one of which is abundant in many sponges and shows affiliation with actinomycetes. The other NRPS was found in *Aplysina aerophoba* and could be attributed to *Chloroflexi* sponge bacterial symbionts. Adjacent to the NRPS, a PPTase was identified (*lubD*), showing similarity to a PPTase from *Methylobacterium nodulans*.[88]

Marine bacteria and algae—Some marine bacteria and algae produce long-chain polyunsaturated fatty acids (PUFAs) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by a unique pathway, different than the traditional FAS or elongase/desaturase pathways. These PUFAs are synthesized *de novo* by a PKS. Genetically, the bacterial and algal PKSs are organized differently,, but both require a PPTase. In the marine bacteria *Shewanella* sp. strain SCRC2738, five open reading frames were identified that were sufficient to produce EPA in *E. coli*.[197] In a landmark paper, Metz et al. characterize these five open reading frames as PKS genes.[198] *Schizochytrium* is a marine algae that produces large amounts of DHA, and genome analysis also revealed similar PKS genes in this eukaryote.[198] The five ORFs responsible for PUFA production in *Shewanella* were later renamed as *pfaA-E* (Fig. 10), from which *pfaE* is a PPTase.[199] *Moritella marina* produces DHA, and the *pfaE* gene cloned out of this organism could complement a PfaA-D expressing *E. coli* strain.[199] Recently it was shown that an EntD overexpressing *E. coli* strain can complement a PfaE deficient PUFA-synthase cluster.[200]

3.2 Archaea

Archaea forms a third domain of life. The cell membranes of archaea are very different compared to bacteria and eukaryotes. Instead of using glycerol-ester lipids, archaea use glycerol-ether lipids; the stereochemistry of the glycerol moiety is reversed, and the hydrophobic tails are isoprenoids (containing cyclopropane and cyclohexane rings). The total amount of fatty acids isolated from archaea is thus minimal, archaea seem to lack a traditional FAS.[201] However, recently, an ACP independent FAS was found, encompassing all of the bacterial FAS enzymes except the ACP.[202, 203] The only published data on the PPTases of archaea is by Copp and Neilan, who identified three PPTases in archaeal species, namely *Methanosarcina acetivorans*, *M. barkeri* and *M. mazei*, clustering closely with Sfp.[14]

For this review, we psi-blasted the PPTases EcAcpS, Sfp, Lys5, EntD, HetI and AASDHPPT against all archaeal genomes deposited to date and found several convincing hits (Fig. 11). To our surprise, we found both Sfp-type and AcpS-type PPTases. We combined this data with identification of PPant binding sites using ArchSchema[204] and Antismash[127] analyses of archaeal genomes for natural product synthases. The archaea that encode AcpS homologs do not seem to encode a traditional elongating synthase, but both *Methanoregula boonei* and *Methanospirillum hungatei* encode a rather unique -AcpS-ACP-'AMP-dependent-ligase'-'acyl-protein-synthetase/LuxE'-'acyl-CoA-reductase'-DH-cluster, all encoded on separate genes. Two of the Sfp-type PPTase encoding archaea, *Methanobrevibacter ruminantium* and *Methanocella paludicola*, encode, large and rather unusual, NRPS clusters. Interestingly, *Methanosarcina* species contain a Sfp-type PPTase but no identifiable synthases. Expression, characterization and identification of target carrier proteins is needed to understand the extent of PPTase diversity in archaea.

3.3 Cyanobacteria

Cyanobacteria are photosynthetic bacteria that, along with eukaryotic microalgae, are responsible for up to 50% of the worldwide photosynthetic production of organic compounds (transversion of atmospheric carbon dioxide into organic compounds).[205] Some cyanobacterial species are a rich source of functionally and structurally diverse natural

products with various pharmaceutical applications.[206] In the environment, these strains are also of global concern due to their ability to form “harmful algae blooms”. Cyanobacteria produce potent toxins such as saxitoxin and paralytic shellfish poisoning toxins that have anegative effect on the environment. Further, many cyanobacteria are known for their unique ability to fix atmospheric nitrogen. This process is located in a dedicated cell type, called heterocysts. Heterocysts are embedded in a glycolipid layer providing the anoxic environment essential for the activity of nitrogenase.[207] Both processes - toxicity and nitrogen fixation - are potentially controlled by PPTases.

The majority of PPTases discovered in cyanobacteria are of the Sfp-type.[14] These Sfp-like PPTases may be solely responsible for primary and secondary metabolism in cyanobacteria since AcpS-type PPTases are completely absent.[14, 60, 66] A large-scale phylogenetic analysis of cyanobacterial PPTases showed that all currently described cyanobacterial PPTases fell within the W/KEA subfamily of the Sfp-type PPTases.[14] A distinct clade of cyanobacterial PPTases are involved in heterocyst differentiation. These include NsPPT in *Nodularia spumigena* NSOR10,[60] HetI in *Nostoc* sp. PCC 7120[61–63] and NgcS in *Nostoc punctiforme* ATCC 29133.[61] These PPTases from heterocyst-forming cyanobacteria were described as phylotype A, in contrast to phylotype B, which includes PPTases found in *Prochlorococcus*, *Synechococcus* and *Gloeobacter*. Besides understanding the native function of cyanobacterial PPTases, they were further evaluated for their ability to activate non-cognate carrier proteins.

Unaware of its function, Wolk et al. reported the first identification of the cyanobacterial PPTase, HetI of *Anabaena* (also known as *Nostoc*) sp. PCC 7120.[63] Knockout attempts were not successful, leading to the hypothesis that HetI may be required for maintaining vegetative growth. Further, the *hetI* gene is associated with a PKS gene cluster necessary for heterocyst glycolipid production.[61, 64, 208] A genome-wide expression study documented increased HetI levels under nitrogen starvation,[209] although overexpression of HetI had no influence on heterocyst formation.[62]

The genome of the closely related species *Nostoc punctiforme* PCC 73102 (ATCC 29133) contains three putative PPTase genes, each embedded within a unique gene cluster (Fig. 12).[61] This expansion of PPTases may be associated with the large genome (9.1 MB, http://www.jgi.doe.gov/), as well as the biosynthetic potential of *N. punctiforme* for natural products. With 21 putative PKSs, NRPSs and hybrid gene clusters, this strain’s biosynthetic capabilities exceed those of any other described cyanobacterium.[210] Thus, the three PPTases are the potential core activators of an extensive natural product biosynthetic machinery.

The first PPTase, NgcS (Genbank acc. no. YP_001863782), shows high sequence similarity to HetI of other cyanobacteria (Fig. 13) and is associated with the glycolipid-related HetMNI gene locus (Fig. 12A). It appears dedicated to its native glycolipid biosynthetic pathway, since it showed little activity towards other cyanobacterial CPs.[61] Despite high sequence similarity to other HetI-type PPTases (Fig. 13), The other two PPTase genes (Genbank acc. no. YP_001865721, YP_001865651) identified in *N. punctiforme* are located within a putative PKS and a FAS gene cluster (Fig. 12B, C). All three proteins are phylogenetically distinct, demonstrating that PPTase phylogeny is not necessarily concurdant with organismal phylogeny.[61]

In *Nodularia spumigena* NSOR10, NsPPT, a homolog to HetI, is the only PPTase present and displays a broad substrate acceptance.[60] It is involved in heterocyst glycoplipid and nodularin toxin synthesis. NsPPT further is able to activate the glycolipid synthase NpArCP and nostopeptolide PKS NpACP of *Nostoc punctiforme*, the microcystin NRPS MPCP of *Microcystis aeruginosa*[60] and SACP, the AcpP of *Synechocystis* sp. PCC6803 *in vitro*.[66]

In comparison to the previously described cyanobacteria, *Synechocystis* sp. PCC 6803 is not known to synthesize NRPS- or PKS-derived natural products.[211] Its lone PPTase Sppt activated the cognate FAS carrier protein SACP *in vitro*, but activity was low for non-cognate ACPs from secondary metabolism or the glycolipid biosynthetic pathway.[66] Although its primary sequence aligns with Sfp, its activity resembles that of AcpS-type PPTases.

Like *Synechocystis*, the well-studied and environmentally omnipresent species *Prochlorococcus* and *Synechococcus* are not prominent for their bioactive compounds, unlike *Nostoc*, *Anabaena* or *Nodularia*.[211] Further, their PPTases seem to be phylogenetically distinct from those associated with heterocysts.[14] Within this group of non-heterocyst-associated enzymes, another PPTase was identified from *Gloeobacter*.[14] Interestingly however, a PPTase with 40% similarity to that of *Gloeobacter violaceus* was identified using an environmental *in vivo* screening, by activating the CP EntF of the *E. coli* enterobactin synthase, thus demonstrating NRPS labeling abilities.[212]

Studying the anatoxin biosynthesis in *Oscillatoria* PPC 6506, the native PPTase OsPPT was functionally described to act on the native PKS-CP AnaD.[65] However, its ability to activate non-cognate CPs has so far not been tested.

Finally, two integrated PPTases have so far been identified in the cyanobacteria *Gloeobacter violaceus* (Genbank acc. no. BAC92166) and *Azotobacter vinelandii* (Genbank acc. no. ZP_00089517) with Sfp-like domain at the C-terminus of a PKS. However these are lacking further description.[14]

3.4 Protista

Apicomplexan parasites show an interesting diversity in fatty acid biosynthesis. *Plasmodium falciparum* contains a type II FAS in its apicoplast, *Cryptosporidium parvum* contains a type I FAS, and *Toxoplasma gondii* uses both. The presence of PPTases corresponds to their respective synthases.[84] In the genome of the malaria parasite *Plasmodium falciparum*, only one PPTase (PfAcpS) can be identified. PfAcpS shows close homology to EcAcpS, is putatively apicoplast targeted, and is fused to a hydrolase domain of unknown function. In contrast, *C. parvum* has a 900 kDa type I FAS and a large polyketide synthase (CpPKS1). The type I FAS has been reconstituted *in vitro*, after expression of fragments in *E. coli*, but appeared to be in its inactive *apo*-form.[213] The native PPTase of *C. parvum* was later identified as an Sfp-type PPTase, shown to activate CpFASI[84] and CpPKS1[214] *in vitro* and *in vivo*. *T. gondii* uses both an AcpS-hydrolase fusion and an Sfp-type PPTase for its type I and type II FAS.

Trypanosoma have adopted an alternative fatty acid biosynthetic route that utilizes dedicated elongases instead of a type I or type II cytosolic FAS.[215] However, *Trypanosoma* also appear to produce fatty acids in their mitochondria using a type II FAS, requiring PPTase activity.[216] So far, no PPTases have been identified in trypanosoma. We conducted detailed Blast analysis to identify putative PPTases in trypanosomal genomes, revealing PaPcpS homologs in *Trypanosoma* (e.g. CCD12699.1) and Sfp homologs in *Leishmania* (e.g. XP_003860424.1) species.

Another interesting case is the amoeba *Dictyostelium discoideum*. The genome contains more than 45 type I polyketide synthases, ranging in size between 1000 and 3000 aa. Two products of these synthases have been characterized: differentiation inducing factor[217] and 4-methyl-5-pentylbenzene-1,3-diol.[218] Both synthases consist of six catalytic domains homologous to type I FASs, but instead of a thioesterase, an iterative PKSIII is used to offload fatty acids. Interestingly, in the case of the PKS that produces 4-methyl-5-

pentylbenzene-1,3-diol, the iterative PKSIII domain alone produces acyl pyrones, but in the presence of the interacting ACP, an alkyl resorcinol scaffold is produced. These >45 PKS and FAS require 4′-phosphopantetheinylation, and recently two PPTases from *D. discoideum* were identified and characterized.[85] One appears to be an AcpS-type and only works on a type II ACP that seems to be targeted to the mitochondria; whereas the other PPTase is an Sfp-type, which was shown to activate type I synthases. Genetic knockouts of *diAcpS* or *diSfp* resulted in 50% and 20% survival, respectively.

3.5 Fungi

The kingdom of fungi is essential in global nutrient cycling due to their ability to decompose organic matter. The ease of cultivation and genetic modification has led to the use of fungi, especially yeasts, in metabolic engineering. As eukaryotes, fungi produce a highly diverse range of proteins compared to bacteria. Furthermore, fungi have immense capabilities to produce a wide variety of natural products. The fungal antibiotics have found various applications from pharmaceutics to agriculture. However, this metabolic potential has also made fungi one of the most difficult to combat pathogens of economically relevant crops. Like bacteria, fungi produce bioactive compounds via PKS and NRPS pathways and thus require a PPTase. Additionally, fungi have implemented an NRPS-like biosynthetic mechanism into their lysine metabolism leading to a completely novel group of PPTases, the Lys5-like enzymes.

Yeasts—The first indications of similarities between lysine metabolism and NRPS systems were given by Morris and Jinks-Robertson in 1991.[78] They identified a high sequence similarity of the protein Lys2 from the baker's yeast *Saccharomyces cerevisiae* to tyrocidine synthetase of *Bacillus brevis*.[78] The proteins Lys2 and Lys5 were previously implicated in the reduction of α-aminoadipate to aminoadipate semialdehyde,[219] but were described as one protein complex. Later, a PPant attachment site was identified in Lys2, leading to the hypothesis that Lys5 activates Lys2 via 4′-phosphopantetheinylation.[1] This mechanism was confirmed by detailed radiolabeling studies.[79]

Mootz and co-workers performed a functional characterization study in *S. cerevisiae* using a *lys5* knockout strain.[220] The PPTases Sfp and Gsp from *Bacillus* spp., both involved in non-ribosomal peptide synthesis, YdcB (AcpS) from *B. subtilis*, involved in fatty acid biosynthesis, the then uncharacterized PPTases q10474 (Lys7) from *Schizosaccharomyces pombe* and NpgA from *Aspergillus nidulans* were evaluated. While Sfp, Gsp, Lys7 and NpgA were able to complement PPTase activity, YdcB could not. We now know that Lys7 corresponds to Lys5 in *S. cerevisiae*, thus is involved in lysine biosynthesis,[82] while NpgA interacts with NRPSs.[67, 68] This led to the hypothesis that the activation of lysine metabolism and NRPSs are related to each other, but not to the FAS system.

Besides Lys5, *S. cerevisiae* contains an integrated PPTase (family III) and Ppt2, which is associated with the mitochondrial FAS complex.[221] The integrated PPTase was first identified via sequence similarity.[1, 80] It is located within the FAS itself (on the gene and protein level) providing autoactivation of the enzyme[20] (see Section 3.5).

The third PPtase of *S. cerevisiae*, Ppt2, interacts with the mitochondrial FAS.[80] Gene disruption led to abolishment of respiration, and Ppt2 could label the native mitochondrial AcpP *in vitro*. This ACP was further labeled by EcAcpS, but not by the type I FAS-specific PPTase Ppt1 from *Brevibacterium ammoniagenes* that was isolated shortly before this study. AcpS is known for its inability to interact with type I FAS enzymes, while Ppt1 can. These results support the hypothesis that Ppt2 acts only on the mitochondrial type II FAS, but not the cytoplasmic type I FAS of yeast.

Lys7 from the fission yeast *S. pombe* interacts with Lys1, which corresponds to Lys5 and Lys2 in *S. cerevisiae*, respectively.[82] However, evaluating heterologous vanillin biosynthesis in both organisms for biotechnological application revealed an endogenous activity mediating 4′-phosphopantetheinylation in *S. pombe* that was absent in *S. cerevisiae*. Vanillin production in *S. cerevisiae* required co-expression of a heterologous PPTase.[222] Additionally, mutational analysis showed that the amino acid residues necessary for the *S. pombe* Lys7 PPTase function were quite different to that of another yeast, *Candida albicans*[82] (see Section 5).

S. pombe is highly diverged evolutionarily from *S. cerevisiae* and *C. albicans*,[223, 224] and this is further demonstrated in lysine metabolism.[82] Lys7 groups with the Sfp-type enzyme from *Clostridium acetobutylicum* instead of Lys5-like enzymes from other yeast species. This might further explain the different characteristics of Lys PPTases, even though they are thought to be mainly involved in lysine biosynthesis.

Two PPTases were initially described in *S. pombe*.[1] Besides Lys7, therein referred to as 1314154, a second PPTase (1842 aa) was grouped with the FAS-integrated enzymes, similar to *S. cerevisiae*. However, experimental characterization is lacking. One further putative PPTase might be new8 (132 aa).[81] Sequence similarity of new8 is higher to the integrated PPTase and Ppt2, than to Lys7 of *S. cerevisiae*. Further, it is closer to AcpS than Sfp and might therefore be described as AcpS-type PPTase. The knockout of new8 caused slow growth in *S. pombe*.[81] This supports the hypothesis of its activity on mitochondrial type II FAS, since these cells would otherwise not be viable.

Further indications for the conservation of the *lys* genes within fungi were given by Guo et al.[225] The Lys2 enzyme of the pathogenic yeast *C. albicans* was only activated *in vitro* upon addition of cell extract. Comparing their results to the previous studies on *S. cerevisiae* and *S. pombe*, the authors concluded this as strong evidence for the existence and requirement of a Lys5 PPTase in *C. albicans*. This hypothesis was confirmed by a detailed characterization study of Lys5.[74] *In vitro*, it activates its cognate Lys2 protein, but also Lys2 of *S. cerevisiae*, and to a lesser extend Lys1 from *S. pombe*. Site-directed mutagenesis could even reveal the essential PPTase residues for Lys2 activation (see Section 5). Besides Lys2, a second PPTase was identified in *C. albicans*[1] which genetically also represents an integrated enzyme.

Recently, a PPTase gene was discovered in the lipid-producing yeast *Rhodosporium toruloides*.[226] Zhu and co-workers used a transcriptomic and proteomic approach to track down those enzymes absent in non-oleaginous yeasts. An AcpS-type PPTase is encoded within a novel FAS system (Genbank acc. no. EMS21268), and they were transcribed simultaneously under the tested conditions.

Aspergillus—Similar to yeast, *Aspergillus nidulans* contains the gene of an integrated PPTase,[1] which has not been further characterized. A second putative PPTase gene *npgA* (null-pigment mutant, npg, also named cfwA) (Table 1 and Fig. 14) was identified during mapping of a melanin-negative mutant.[227] The same phenotype was observed in a *wA*-knockout strain, a gene referring to a PKS that is involved in pigment production,[228, 229] leading to the hypothesis that the ACP domains of PKS WA are substrates of NpgA.

The *npgA* gene was first isolated and further characterized by Kim et al.[230] While the deletion mutant again was defective in growth and pigmentation, overexpression did not result in a variation of the growth or pigmentation phenotype, though the conidiophores were found to be formed at an earlier stage. Conidiophores are specialized stalks presenting the conidia, the asexual spores. Later, NpgA was evaluated for its ability to complement

Lys5 from *S. cerevisiae*.[220] NpgA complemented Lys5 activity, demonstrating its potential function in lysine metabolism. By *in vivo* studies, NpgA has been shown to be essential for penicillin biosynthesis (which is produced by an NRPS).[67] In this study, the two alleles (version of the same gene) of *npgA*, namely *cfwA+* and *cfwA2*, were characterized. *cfwA2* differs from *cfwA+* at two positions, which results in functional changes of the enzyme, and appears to be the more important allele for penicillin production. *cfwA2* is further essential for the production of the siderophores ferricrocin and triacetylfusarinene C.[68] Underlining this versatility, addition of triacetylfusarinene C did[68] (but supplementation of lysine did not) restore the growth deficiency of the *cfwA2* knockout strain.[67] Besides non-ribosomal peptide and lysine biosynthesis, NpgA is essential for polyketide biosynthesis (shamixanthone, emericellin, dehydroaustinol), but dispensable for the sterol (ergosterol, peroxiergosterol, cerevisterol) and fatty acid production.[69]

PptA, a homologue of NpgA, was shown to be essential for polyketide and non-ribosomal peptide biosynthesis in *A. niger*.[231] The homolog in *A. fumigatus* activates the native NRPS Afpes1 and the non-cognate Lys2 from *C. albicans*.[71, 72] *A. fumigatus* further uses the PPTase PptB which is specific for the mitochondrial AcpP.[73]

Penicillium—Like other fungi, *Penicillium patulum* contains the gene of an integrated PPTase,[1] which has not been studied to date. *Penicillium chrysogenum* further contains the PPTase Pc13g04050, which is a homolog of NpgA from *A. nidulans*.[77] The PPTase-defective mutant was auxotrophic for lysine and lacked both pigmentation and penicillin production. Interestingly, biosynthesis of roquefortine C was not influenced by this mutation, even though both penicillin and roquefortine C are produced via NRPS pathways,[77] suggesting further PPTase genes within the genome. Considering fatty acid biosynthesis, the FAS gene (GenBank acc. no. CAP74216) contains an integrated PPTase. This explains why the Pc13g04050 mutant did not require fatty acids. Garcia-Estrada et al. could further show the high conservation of NpgA homologs within representatives of the fungal classes Eurotiomycetes, Ascomycetes, and Sordariomycetes using sequence alignment.[77]

Other fungi—Homologs of NpgA were discovered in other fungal species, such as the plant pathogens *Cochliobolus sativus*, *Colletotrichum graminicola*, *Fusarium fujikuroi* and the root-colonizing fungus *Trichoderma virens*.[75, 76, 83, 232]

Ppt1-deficient mutants of *C. sativus*, *C. graminicola*, and *T. virens* were auxotrophic for lysine, unable to produce melanin, hypersensitive to oxidative stress, and had significantly reduced virulence resulting from the defective polyketide biosynthesis.[75, 83, 232] In comparison to other studies, no morphological defect or germination delay of conidia in *C. sativus* was observed besides the loss of pigmentation and the production of fewer conidia.[69, 75, 232] Conidia of Ppt1-deficient mutants in *C. graminicola* had a reduced size in comparison to the wild type and exhibited string morphological defects.[232] In *T. virens*, spore formation was severely compromised. However, the mycelia grew faster in comparison to the wild type.[83] Ppt1-deficient mutants were still able to colonize plant roots, but could not prevent growth of phytopathogenic fungi *in vitro*.

FfPpt1 of *F. fujikuroi* is essential for viability and involved in lysine biosynthesis and production of some, but not all, natural products, made by this species.[76] Interestingly, the *ffPpt1*-deletion mutant showed enhancement in terpene-derived metabolites and volatile substances. During infection of rice, lysine biosynthesis and iron acquisition are required, but the biosynthetic pathways of other PKS and NRPS seem less important. Further, FfPpt1 was shown to be involved in conidation and sexual mating recognition.

In conclusion, NpgA and its homologues might cover slightly different functionalities within the disparate fungal species, but the conidiation pathways themselves are still not well understood and demand further investigation.

3.6 Type I Integrated PPTases

In most cases, PPTases are stand-alone proteins, separately encoded or genetically part of the biosynthetic cluster. However, in a few cases the PPTase is part of the megasynthase, as already identified by Lambalot et al. in 1996.[1] This became further apparent when yeast *apo*-FAS was isolated and, upon addition of CoA, spontaneously formed *holo*-FAS.[20] Interestingly, the PPTase domain of the FAS megasynthase is located on the outside of the barrel and cannot activate ACP after organization, necessitating post-translational modification of ACP before the synthase is fully formed (see Section 2.3).

Apart from the primary FAS synthase, fungi also express dedicated FAS or PKS for secondary metabolites.[233] For example, norsolorinic acid and enediyne biosynthesis require a FAS or PKS (or combination), using integrated PPTases in the megasynthases. The PPTase from the FAS domain of the norsolorinic acid synthase was cloned out of the synthase and shown to have very narrow substrate specificity (only its cognate ACP was recognized).[234] Recently, it was shown that this PPTase even has a very narrow CoA substrate specificity, accepting only CoA and no acyl-CoAs.[235]

Enediynes are made by iterative PKSs and decorating enzymes. The iterative PKS from the bacterium *Micromonospora echinospora ssp. calichensis* contains a PPTase domain at its C-terminus.[236] Detailed characterization revealed that this 330 aa domain forms a pseudo-trimer, showing similarity to EcAcpS. Furthermore, this PPTase has very low activity for its cognate carrier protein, suggesting that this enzyme is not optimized for secondary metabolism.[237]

Another integrated PPTase has been found in bacterium *S. erythraea* (SePptI) which is most likely part of a PKS megasynthase.[44] Copp and Neilan identified several novel integrated PPTases in large megasynthases: cyanobacteria *Gloeobacter violaceus* (Genbank acc. no. BAC92166) and *Azotobacter vinelandii* (Genbank acc. no. ZP_00089517) have an Sfp-like domain at the C-terminus of a PKS megasynthase, and the plant *Arabidopsis thaliana* (Genbank acc. no. AAC05345) has an Sfp-like PPTase at the C-terminus of a COP1 interactive partner 4 domain.[14]

3.7 Plants and algae

AcpS activity in plants was evaluated directly after the initial discovery of AcpS in *E. coli*.[3, 9] In plants and algae, the fatty biosynthesis is located in the chloroplasts (type II FAS) and mitochondria (type II FAS).[238, 239] For the spinach chloroplast fatty acid metabolism, Elhussein et al. proposed that phophopantetheinylation mainly takes place in the cytosol and the nuclear-encoded chloroplast ACP is phosphopantetheinylated before entering the chloroplast.[9] This was questioned by studies that suggested a dedicated PPTase located within the chloroplast.[240, 241] However, later on, it was shown that both *apo*- and *holo*-ACP could be efficiently taken up by the chloroplast.[242, 243]

In contrast to the large renewed interest in algae- and plant-derived biofuels, no PPTase from these groups has been functionally described. Metabolic engineering of plants have instead investigated co-expression of a foreign PPTase gene (see Section 6).[244–247] One study in tobacco, however, demonstrated that a heterologously expressed PKS did not need the co-expression of a PPTase for successful biosynthesis of the natural product 6-

methylsalicylic acid.[248] The authors propose the presence of an endogenous enzyme that activates the integrated synthase.

Even though current knowledge of PKSs and NRPSs and their function in plants and algae is limited, a growing number of algal and plant genomes have been analyzed with bioinformatic tools ([127] and others) that predict the presence of "green" natural product gene clusters.[249–251] Further research is needed to describe these enzymes functionally as well as their activation mechanism by PPTases, particularly with regard to renewable fuel and chemical production.

3.8 Animals

Although higher eukaryotes, in general, do not appear to produce NRPS or PKS derived secondary metabolites, homologous biosynthetic pathways seem to be conserved. The fruit fly *Drosophila melanogaster* encodes a 98.5 kDa protein called Ebony that exhibits homology to NRPSs and has β-alanyl-dopamine synthase functionality (Fig. 15).[252] It was shown that Ebony activates β-alanine by an adenylation domain and loads it onto a PCP (thiolation) domain. In a second reaction, amines such as dopamine are selected by a small amine selection domain, which then cleaves the activated amino acid from the synthase. Ebony requires 4′-phosphopantetheinylation for activity and, *in vitro*, Sfp can install the PPant arm. Sequence homology between conserved PPTase regions and the genome of *Drosophila* revealed a PPTase. This PPTase (now called Ebony activating protein, NP_729788.1) was cloned and expressed in *E. coli* and shown to have similar *in vitro* activity as Sfp ("unpublished results").[24]

The peptidoamines produced by Ebony are involved in several processes, including β-alanyl-histamine biosynthesis in the eye as part of neurotransmitter metabolism. Although Ebony closely resembles an NRPS, it is not characterized as a classical NRPS, since it does not catalyze the formation of a peptide bond, via a condensation domain, and lacks a thioesterase domain. Ebony-like proteins are found by homology in many higher eukaryotic species, suggesting that the NRPS-like chemistry is evolutionary preserved. [249, 253] However, it remains unclear why (or if) classical nonribosomal peptides are found only in bacteria and fungi. Horizontal gene transfer between species and "inventive evolution" could be an explanation for this phenomenon, although it remains a mystery why some plants and other eukaryotes seem to contain large NRPS- and PKS-like genes in their genomes.[249, 253]

3.9 Homo sapiens

In mammals, 4′-phosphopantetheinylation is required for several enzymes, including type I FAS, type II mitochondrial FAS, lysine metabolism and 10-formyltetrahydrofolate dehydrogenase (Fig. 16). Surprisingly, mammals only encode one unique PPTase in their genome which is called AASDHPPT. This gene was discovered by homology to Lys5 from *S. cerevisiae*.[26] *S. cerevisiae* synthesizes the amino acid lysine by the enzyme Lys2, an α-aminoadipate reductase, which is activated by Lys5, a PPTase.[1, 79] In contrast to yeast, mammals catabolize lysine via two different pathways, involving α-aminoadipate semialdehyde or pipecolic acid. The enzyme responsible for the latter of these processes is the bifunctional enzyme α-aminoadipate semialdehyde synthase (AASS), which transforms lysine into saccharopine and α-aminoadipate semialdehyde, which is then oxidized by AASDH to α-aminoadipate. In a yeast Lys5 mutant, the mammalian AASDHPPT could efficiently complement, suggesting that this enzyme is also involved in a similar reaction in mammals. Indeed, AASDH contains a PCP domain that undergoes 4′-phosphopantetheinylation by AASDHPPT. Later, it was shown that AASDHPPT has a broad substrate scope.[25]

The mammalian fTHF-DH also requires a PPant arm for catalysis (Fig. 16).[254] This enzyme is phosphopantetheinylated by AASDHPPT as well, and siRNA silencing this enzyme completely prevents the modification of fTHF-DH. A mitochondrial homolog of fTHF-DH was found to be activated by the same PPTase.[255] This suggests that there are no other PPTases present in humans.

3.10 Evolution and phylogeny

PPTases have been identified and classified based on their primary sequence. For example, Lambalot et al.[1] proposed that PPTases can be identified by two conserved motifs, namely (V/I)G(V/I)D$(x)_{40–45}$(F/W)(S/C/T)xKE(A/S)hhK, where h is an amino acid with a hydrophobic chain. Recently, a refined analysis of these signature sequences was made and found to be (I/V/L)G(I/V/L/T)D(I/V/L/A)$(x)_n$(F/W)(A/S/T/C)xKE(S/A)h(h/S)K(A/G), in which n is 42–48 aa for AcpS and 38–41 aa for Sfp-type PPTases.[18] Between these two conserved regions resides a third region which is less conserved, but contains a highly conserved glutamate (E127 in Sfp). Additional sequence alignments and analyses[14, 18, 56] give rise to four more signature sequences for different subclasses of Sfp-type PPTases and AcpS-type PPTases. However, PPTases span a large variety of primary sequences, thus making sequence alignments and blast analyses difficult to interpret. We propose that a more powerful tool to compare, identify and classify PPTases is an evolutionary analysis by phylogeny.

The first phylogenetic tree constructed for PPTases showed a close relationship between PPTases involved in secondary metabolism, but also distinct differences, including the evolutionary separation between Sfp, EntD and JadM.[55] In 2003, Joshi et al. identified the human PPTase AASDHPPT and constructed an updated phylogenetic tree, including the primary metabolism (fatty acid) PPTases, showcasing that the three PPTases found in *S. cerevisiae* (Lys5, type I FAS and mitochondrial FAS PPTases) are very distant from each other.[25, 82] Apicomplexan parasites have AcpS, Sfp-type or both PPTases. *P. falciparum* uses an AcpS-type, *C. parvum* uses an Sfp-type, and *T. gondii* both types of PPTases.[84] Phylogenetic analysis of these proteins puts them in separate clades: the Sfp-types close to human and fungal PPTases, whereas the AcpS-types are closely related to bacterial FAS PPTases. Copp and Neilan construct a detailed phylogenetic tree on Sfp-like PPTases, focusing on cyanobacteria.[14] Interestingly, two major Sfp-like PPTases seem to exist, utilizing conserved sequence motifs F/KES and W/KEA.

Here, we construct an unbiased phylogenetic tree from ~1700 unique (putative) PPTase sequences (see Fig. S1), as well as a neighbor-joining tree of the currently characterized (~ 60) PPTases (Fig. 17). To our surprise, several clades show relatively good affinity. For example, Sfp, Gsp, AASHDPPT, MtaA, JadM, HetI, PptT, PcpS, EntD and AcpT form separate branches of the tree, whereas AcpS-type PPTases, including those from mitochondrial fatty acid synthesis, are far removed from the main trunk. Although we cannot discuss in detail, a few observations can be made. Whereas on one branch of the tree EntD, PcpS, PptT and others group together, on the opposite branch AASDHPPT, Lys5, Sfp and MtaA group together. This division crosses Gram-positive/Gram-negative and bacterial families, and so far the origin of the clear phylogenetic division of these two groups of PPTases has been elusive, since both “groups” are involved in secondary metabolism of various natural products (which do not seem to cluster). A more detailed look into the smaller tree (Fig. 17) shows a similar division. Fungal, animal, protista and human PPTases group together, in close proximity to a Sfp clade and a clade that contains dedicated cyanobacterial, *E. coli*, *Pseudomonas* and *Stigmatella* PPTases. Adding more sequences pulls this clade apart in three major branches, characterized by cyanobacterial HetI, *Stigmatella* MtaA and *E. coli* AcpT. Completely opposite, EntD-like PPTases group

together from various bacteria. In between, two branches are far removed from the two described major clades, showing type I FAS integrated PPTases and AcpS-type PPTases.

4. Carrier protein(s)

Carrier proteins represent a large family of small (70–100 aa) proteins essential in primary and secondary metabolism. These proteins are either part of a single chain multi-domain synthase or exist as monomers and shuttle cargo between enzymes that act on the substrate. All carrier proteins have a conserved serine which requires 4′-phosphopantetheinylation by a PPTase in order to tether cargo via a flexible, labile, thioester linkage. In this section we will discuss the variety of carrier proteins and their cognate PPTases, the non-carrier proteins that are phosphopantetheinylated, the amino acids essential in carrier proteins and peptides for PPTase activity and ACP hydrolases (the reversal of PPTase activity).

Specificity and activity for CPs

The kinetic data on PPTases and their carrier protein or CoA substrates is limited to only six different PPTases. Which PPTase acts on what carrier protein (and to what extent) is crucial for *in vitro* applications and understanding reaction mechanism. In Table 3 we summarize kinetic data obtained for several PPTases and partner carrier proteins, showcasing the range of kinetic parameters found *in vitro*.

AcpS is the ubiquitous trimeric PPTase responsible for installing the PPant arm on the ACP involved in prokaryotic fatty acid biosynthesis. AcpS is relatively specific to ACPs involved in FAS, as the polyketide granaticin- (gra), frenolicin- (fren), oxytetracycline- and tetracenomycin- (tcm) ACPs were poorly phosphopantetheinylated when overexpressed in *E. coli*.[11] Overexpression of tcm-ACP and induction in the exponential phase lead to no *holo*-ACP, but induction in the stationary phase lead to a small percentage of the modified protein. The *apo*/*holo* ratio of gra, fren and act-ACP upon overexpression in *E. coli* gave 30% *holo*-gra-ACP, 2% *holo*-act-ACP and no modified *holo*-fren-ACP. *Apo*-act-ACP was converted to 80–90% *holo*-act-ACP when the post-induction period was increased to 12h. However, *in vitro* AcpS was active on the aforementioned ACPs and the transformations go to completion.[11] AcpS also catalyzes the *in vitro* 4′-phosphopantetheinylation of NodF and D-alanyl carrier protein from *Lactobacillus casei*, but does not act on the PCP from tyrocidine A synthase or *apo*-PCPs of *E. coli* enterobactin synthase.

Sfp is a highly promiscuous PPTase from *B. subtilis* surfactin synthase, capable of activating PCPs and FAS *apo*-ACPs alike (Table 3). Interestingly, both Sfp and EntD also show promiscuity towards their CoA substrates,[259] discussed in Section 5. Although Sfp is promiscuous to both its ACP and its CoA substrate, it prefers carrier proteins from secondary metabolism. The genome of *B. subtilis* also contains AcpS. Deletion of the *acpS* gene has no apparent effect on the bacteria, despite the low *in vitro* activity of Sfp on FAS *apo*-ACP, suggesting that AcpS is not essential in *B. subtilis*.

P. aeruginosa has only one PPTase, named PaPcpS.[41] PaPcpS has 13% sequence similarity to Sfp and shows higher similarity to *E. coli* EntD, but is responsible for both primary and secondary metabolism synthase modification.[100] PaPcpS also shows catalytic behavior more typical of AcpSs (Table 3). Both AcpS from *S. pneumoniae* and *B. subtilis* show different catalytic parameters at high and low *apo*-ACP substrate concentrations, presumably due to allosteric regulation (or cooperativity) of its three active sites or some conformational change(s). Sfp does not show this behavior, but the monomeric PaPcpS surprisingly does,[41] possibly making PaPcpS a unique subclass of PPTases.

The human PPTase AASDHPPT shows promiscuous behavior to a variety of *apo*-carrier proteins (Table 3), in line with the various synthases it needs to activate. Recently, the human PPTase has been crystallized and structure solved, shining detailed light on substrate binding and reaction mechanism, discussed in Section 5.[260]

Cyanobacteria are producers of many PKS/NRPS secondary metabolites that require PPTases. Interestingly, cyanobacteria only express one PPTase and seem to be devoid of an AcpS-type. For example, *Nodularia spumigena* NSOR10 expresses a PPTase which can act on the carrier protein responsible for glycolipid biosynthesis, ArCPNp, the PCP involved in mycrocystin biosynthesis, MPCP and the PKS carrier protein for nostopeptolide, ACPNp.[60] In contrast, the PPTase from Synechocystis sp. PCC6803 has very narrow carrier protein substrate specificity.[66]

The stunning range of carrier proteins modified by PPTases is matched by a lack of understanding for what determines specificity or selectivity on either PPTase or carrier protein side. We further discuss this in the context of the published X-ray crystal structures of PPTases in Section 5.

The other phosphopantetheinylated proteins and their PPTases

Not only traditional synthases (FAS, PKS and NRPS) require PPTases, but there are many more carrier proteins and synthases require a PPant cofactor. The biosynthesis of D-alanyl-lipoteichoic acid by Gram-positive bacteria requires the carrier protein Dcp,[261, 262] which is phosphopantetheinylated *in vitro* by EcAcpS. *Lactobacillus casei* contains only an AcpS-type PPTase in its genome, which presumably can install the PPant on AcpP and Dcp.

NodF is another carrier-protein-like protein that is involved in the biosynthesis of lipo-chitin nodulation factor in *Rhizobia*. Although NodF has 25% sequence identity to *E. coli* AcpP, this AcpP cannot replace NodF *in vivo*.[258] Interestingly, EcAcpS and malonyl-CoA-acyltransferase can interact with NodF, but not ketoacyl synthase III. A chimera of *E. coli* AcpP and NodF can complement a NodF deficiency *in vivo*. In the genome of *Rhizobium leguminosarum*, only an AcpS-type PPTase is identified which presumably can phosphopantetheinylate both its AcpP and NodF.

In *Rhizobia*, four other carrier proteins are found, called ACPXL (involved in lipid A biosynthesis), Rkpf (involved in capsular polysaccharide biosynthesis), SMb20651 (unknown function)[263] and SMc01553 (unknown function).[264] These presumably require a PPant arm for activity, show very little sequence identity (Fig. 18), and are modified by its endogenous AcpS. Only upon co-overexpression of *E. coli* or *S. meliloti* AcpS, *holo*-SMb20651 was formed in *E. coli*, whereas the basal expression of EcAcpS was not sufficient to modify the carrier protein. This suggests poor catalysis maybe due to the presence of a DST motif instead of a DSL motif.

Mycobacterium tuberculosis is an example of an organism that contains a wide range of carrier proteins that require 4′-phosphopantetheinylation.[36] These bacteria encode >18 type I PKS, NRPSs and two fatty acid synthases. *De novo* fatty acid synthesis in *M. tuberculosis* is encoded by a bacterial type I FAS,[265] but the second FAS (type II) is responsible for elongating C_{18} to C_{52}-C_{60} fatty acids. These very long chain fatty acids, and additional PKS and acyl transferases, are required for mycolic acid biosynthesis. The second FAS requires a dedicated ACP, called AcpM,[266, 267] which has a 35 aa C-terminal extension which is presumably involved in substrate binding and dimerization.[268] *E. coli* AcpS quantitatively transforms *apo*-AcpM into *holo*-AcpM. The genome of *M. tuberculosis* contains two PPTases, namely AcpS and PptT. Chalut and co-workers constructed AcpS and PptT knockouts in the model bacteria *C. glutamicum*, which is easier to genetically manipulate

than *M. tuberculosis* but lacks the type II fatty acid synthase. The AcpS knockout shows fatty acid auxotrophy but can still make mycolic acid, whereas the PptT knockout shows no mycolic acid, but wild type levels of C_{16}/C_{18} fatty acids.[36] This suggests that AcpS is responsible for phosphopantetheinylating both of the AcpPs and PptT for all the other (>20) carrier proteins in mycobacteria.

The mammalian enzyme fTHF-DH has two independent catalytic domains located at its C- and N-termini, with a carrier protein domain in between. The function of this protein is the conversion of fTHF to the important co-factor THF. One domain is responsible for hydrolysis of the formyl group off fTHF and the other domain for $NADP^+$ dependent oxidation of formyl to CO_2.[269] Only when the domains are fused is activity observed, but how the formyl group is transferred from one buried active site to the next was unknown until 2007, when the presence of a phosphopantetheinylated carrier protein was shown to link the two domains. AASDHPPT installs the PPant arm on fTHF-DH and when the PPTase is silenced, fTHF-DH is inactivated, and cells show reduced proliferation and cell cycle arrest.[254] Recently, a mitochondrial targeted fTHF-DH was identified in mammals. When purified from pig liver and shown to be active, another protein was added to the growing number of substrates of the mammalian PPTase.[270]

Besides the above discussed PPTases and their unusual carrier protein substrates, other substrates include the previously discussed (see Section 3) human AASDH, fungal lysine biosynthesis, cyanobacterial acyl-ACP reductases, *Nocardia* carboxylic acid reductase and *Drosophila* Ebony.

Carrier protein recognition by PPTases

Both protein-protein interactions and protein-substrate interactions are important in type II synthases. In this section we will discuss in detail how a PPTase recognizes a carrier protein and how it is possible that one enzyme recognizes >20 different carrier proteins, as is the case in *M. tuberculosis*. The first co-crystal structure of *B. subtilis* AcpS with its cognate ACP sheds light on these protein-protein interactions (Fig. 19).[271] AcpS first binds CoA and Mg^{2+}, followed by the ACP, which initiates the transfer of PPant to ACP.

AcpS is a homotrimeric protein with active sites on the protein-protein interfaces. All contacts between ACP and AcpS seem to occur between helix I of AcpS and helix III of ACP (Fig. 19 and Section 5). Two hydrophobic residues of ACP (Leu37 and Met44) protrude into AcpS, where Leu37 extends into a pocket formed by Met18, Phe25, Phe54 and Ile15, and Met44 binds into a pocket consisting of Phe25, Arg28 and Gln22. Arg14 forms a salt bridge with Asp35 of ACP (in close proximity to the “active site” Ser36), and Arg21 forms a salt bridge with Glu41. The other end of helix 3 is locked in place by interaction of Arg24 and Gln22 of AcpS with Asp48 of ACP.[271] When 14 aa in helix II of a PCP were mutated into those present in AcpP, AcpS was able to act on this hybrid carrier protein.[41] More elaborate mutagenesis studies show that the closer the sequence of PCP approached that of the ACP, the higher the activity of AcpS is on these hybrid ACP/PCPs.[272]

There are some clear differences between PCPs and ACPs which determine whether AcpS or Sfp-type PPTases can act on these carrier proteins (Fig. 20). For example, position X in the motif (D/H)SLX is in PCPs a positive residue like Lys or Arg, but in ACPs almost always an Asp. Based on the co-crystal structure of AcpS-ACP and overlays with a Sfp-PCP model, mutations were introduced into AcpS. Residues R14 and K44 of AcpS are important for carrier protein recognition, but introduction of point-mutations at those sides did not result in PPTase activity on PCPs.[272] Interestingly, except for R14K, all mutants do not show allosteric activation anymore, which is observed in wild type AcpS (see Section 2.1).

V. harveyi AcpP (VhACP) shares 86% sequence identity with *E. coli* AcpP. Mutagenesis of Asp35 or Asp56 of VhACP has a large effect on the ability of EcAcpS to activate the ACP.[273] Construction of mutants D30N/D35N/D38N, E47Q/D51N/E53Q/D56N and the combination of both, showcases the importance of these residues. The VhACP mutants containing Asp35 mutations are not phosphopantetheinylated by EcAcpS. A properly folded carrier protein also seems to be important for activity, as shown with the I54A mutant of VhACP, which forms a highly dynamic ACP and cannot be activated by EcAcpS.[274]

S. coelicolor produces 22 known natural products that utilizes synthases requiring 4′-phosphopantetheinylation. Despite this large number of CP targets, the genome of *S. coelicolor* contains only three PPTases, SCO5883, SCO667 and ScAcpS. Actinorhodin is a polyketide natural product and it was shown that SCO5883 and SCO667 are not required for its production, suggesting that ScAcpS is responsible for 4′-phosphopantetheinylation of its synthase.[50, 126] Indeed, *in vitro* ScAcpS acts on a range of FAS and PKS ACPs.[50] Further mutagenesis and structural studies shed more light on the source of promiscuity of ScAcpS for different carrier proteins albeit based on modeling.[126]

To this day, Sfp has not been co-crystallized with any carrier protein. However, by mapping the AcpS-ACP structure on Sfp, it was shown that the binding helix of AcpS (K13-Q22) is a loop in Sfp (T111-S124). Mutagenesis of these residues in Sfp led to mutants with 15–24-fold lower K_m values for PCP, whereas CoA binding was only reduced by 3–6-fold.[275] It is speculative but the increased promiscuity of Sfp might arise from the flexibility of that binding loop versus the rigidity of the α-helix in AcpS.

The human PPTase AASDHPPT, which falls in the same subclass as Sfp, has been co-crystallized with its cognate excised ACP mutant Ser2156Ala. The excised ACP binds in the cleft between the two domains of the PPTase, and three hydrophobic patches on the surface of the PPTase are responsible for protein-protein interactions. Only a few polar interactions are observed between ACP and PPTase, and binding seems to be governed by shape complementarity instead of specific interactions.

There appears to be a fundamental difference between type I and type II synthase ACPs regarding their interactions with their PPTases. In type II synthase ACPs, conserved negatively charged surface residues mediate interactions with PPTases, whereas type I synthase ACPs seem to have a lower overall negative charge, and both hydrophobic interactions and shape seem to be the dominant factors in productive protein-protein interactions.[260] With access to two ACP-PPTase co-crystal structures light has been shed on how these proteins interact and how Nature regulates their specificity. However, with such limited structural information, it remains unknown how general are the observations made for these two structures.

Peptide mimics of carrier proteins as substrate of PPTases

Another approach to probe the recognition of carrier proteins by PPTases is the study of small peptides as ACP-mimics. Synthesis of a 19 aa consensus peptide of the SrfB-PCP made it possible to investigate whether the activity of Sfp depends on its partner's primary sequence or requires structure. Even at very high concentrations, the peptide was not a substrate for Sfp, suggesting that a folded *apo*-carrier protein is necessary.

Walsh and co-workers showed that PCPs displayed on the surface of M13 phages (phage display) were substrates for the promiscuous PPTase Sfp.[276] Various other proteins were phage displayed and besides PCPs and ACPs, truncated forms of the 484 aa protein YbbR were found. The shortest phosphopantetheinylated YbbR fragment was 49 aa and has no sequence homology to any ACP or PCP. Synthesis of a shorter fragment identified an 11 aa

YbbR derived fragment (DSLEFIASKLA) that was a substrate of Sfp (Fig. 20). Amino acids can be added to the N-terminus without influencing labeling, but the C-terminus was very sensitive to truncation. Interestingly, the residues ASKLA are not encoded by the YbbR ORF but are a linker introduced between the hexahistidine tag and the peptide. In the phage display-selected clones, ASKLG was present at the C-terminus, but again also part of a linker introduced between phage particle and peptide. The short peptide with the original YbbR C-terminal sequence is not a substrate for Sfp, which could indicate that full length YbbR may not be a substrate for Sfp. Indeed, the original YbbR protein has not been shown to be a substrate for either AcpS or Sfp, and it is unclear whether YbbR is phosphopantetheinylated in Nature.[277]

All identified YbbR-derived peptides that are substrates of Sfp show low catalytic efficiency and strong helical propensity. None of the helical wheel representations of these peptides matches PCPs or ACPs. This suggests that these YbbR derived peptides have no structural relevance to carrier protein – PPTase interactions. Based on the prior YbbR-derived peptide results, phage display was used to select for small (12 aa) peptides as specific substrates for Sfp or AcpS, respectively. After multiple rounds of selection, two short peptide tags (S- and A-peptide) were identified that have a 442-fold greater k_{cat}/K_m for Sfp over AcpS, or a 30-fold greater k_{cat}/K_m for AcpS over Sfp, respectively (Table 3).[257] Interestingly, only the S-peptide shows structured helical propensity. Nevertheless, the authors compare the short S- and A-peptides with helix II of ACPs and PCPs and tentatively assign the differences in catalytic activity to certain residues in these selected peptides. For example, glutamic acid at position 8 of peptide A seems prominent in distinguishing whether AcpS or Sfp can act on the peptide. Further minimization of the peptide gave an eight residue peptide as a substrate for AcpS.[278]

Regulation by 4′-phosphopantetheinylation

PPTases convert the inactive *apo*-form into the active *holo*-form of a synthase. The levels of *apo*- and *holo*-carrier proteins in a living organism can therefore regulate synthase activity.[243]

In vitro, the disulfide-bonded dimeric form of *holo*-carrier proteins is often observed. The crystal structure of *P. falciparum* apicoplastic ACP, involved in FAS, showed that the disulfide-bond is deeply buried and difficult to access, even for small molecule reductants.[279] This raised the question whether this dimeric form is present in the parasite with some regulatory function. However, in blood-stage parasites, no dimeric form is observed, suggesting that the apicoplast is a sufficiently reducing environment to prevent disulfide-bond formation between the two thiols of phosphopantetheinylated PfACP, and thus *holo*-ACP dimerization was ruled out as being involved in regulation.

The presence of large amounts of *apo*- or *holo*-carrier proteins can influence many metabolic processes. The ratio (or presence) of *apo*- over *holo*-ACP is directly linked to the activity (and presence) of PPTase. Early studies on ACPs often found both *holo*- and *apo*-forms in cell lysate. For example, when spinach ACP was overexpressed in tobacco leaves, a 50/50 mixture of *apo*- and *holo*-spinach ACP was found, whereas the native tobacco ACPs were all in the *holo*-form.[243] In *E. coli*, no detectable amounts of *apo*-ACP are found, suggesting that the active/inactive ACP ratio is not the point of regulation in Nature.[280] Upon overexpression of EcACP in *E. coli*, growth rates were severely retarded, suggesting some toxic effect.[281] Indeed, *apo*-ACP is a potent inhibitor of cell growth, somehow regulating sn-glycerol-3-phosphate acyltransferase.[281] Recently, it was shown that C18:1-loaded ACP regulates a plastidic acetyl CoA carboxylase.[282] Taken together, it is still unclear whether PPTases, which directly control the *apo*/*holo* ratio of carrier proteins, regulate cellular processes.

In *E. coli*, acyl carrier protein hydrolase (AcpH, also called ACP phosphodiesterase) removes the PPant arm from the carrier protein (Fig. 21), and it seems likely that this enzyme is activated by decreasing CoA levels.[283] Vagelos and Larrabee describe in 1967 the isolation of AcpH from *E. coli* and study its enzymatic activity in detail.[284] Mn^{2+} appeared to be a requisite for the reaction, although also Mg^{2+}, Co^{2+}, Fe^{2+} and Zn^{2+} showed restoration of activity. Increasing concentration of reductant (DTT, βME) also improved the hydrolytic activity. Interestingly, AcpH seemed to be highly specific for full length ACP, since it was unable to cleave the phosphopanteteine arm from large peptides of proteolytically digested ACP (fragments of 43 or 62 of the total 86 aa). However, it deactivated ACP from *Clostridium butyricum*, but not the ACP of mammalian type I FAS.[284]

The *in vivo* turn-over rate of the PPant arm is higher than the turnover of the ACP itself, both in *E. coli*[7] and in rat liver,[285] suggesting that perhaps PPTase or AcpH activity serves a regulatory function. A crude enzyme preparation from rat liver was able to hydrolyze 4′-phospho[^{14}C]pantetheine from the rat fatty acid synthase.[286] Later, a purified enzyme preparation was unambiguously shown to hydrolyze radioactive-pantetheine from labeled rat type I FAS (*holo*-FAS).[287] This was also the first proof that the large type I FAS only carries one ACP, since the molar ratio of pantetheine released from the protein was 1:1. The purified enzyme preparation was not able to cleave the PPant arm from CoA or from pigeon liver *holo*-FAS, and the expression of the enzyme seems to vary with nutrition: the enzymatic activity was high in 3-day fasted rats, whereas no hydrolase activity was detected in 2-day fasted or normally fed rats.[287]

E. coli AcpH was recently expressed and purified.[288, 289] EcAcpH appears to be a poorly behaving protein. It aggregates and expresses mostly in the insoluble fraction. *Holo*-ACPs from *Aquifex aeolicus*, *Bacillus subtilis*, *Lactococcus lactis*, and the mitochondrial ACP of *Bos taurus* were tested for hydrolysis by AcpH. *A. aeolicus* and *B. subtilis* ACPs were hydrolyzed, but both *L. lactis* and *B. taurus* ACPs were not. This behavior matched that of EcAcpS, suggesting that PPTase and AcpH recognize similar ACP features.

Further, AcpH activity must somehow be regulated *in vivo*, since based on the cellular levels of AcpH and its activity, it would transform all cellular *holo*-ACP into *apo*-ACP within one minute.[289] AcpH is non-essential in *E. coli*, so it remains unclear what physiological role it plays. The *acpH* gene is also found in other Gram-negative bacteria, cyanobacteria, and, surprisingly, in *Ricinus communis* (castor bean). Whether the latter is an artifact or contamination has not been discussed. Protein-blast does not reveal other plant AcpHs, however nucleotide-blast does show a few hits in other plants (e.g. *Oryza sativa* and *Zea mays*).

AcpH is a non-canonical member of the HD phosphatase/phosphodiesterase family.[290] Currently, there is no structure of AcpH available, but sequence alignments identified a protein with homology: the N-terminal portion of SpoT. SpoT also catalyzes the cleavage of a phosphoester and requires Mn^{2+} for activity (Fig. 21). Murugan et al. [291] isolated and expressed the AcpH (Uniprot protein PA4353) from *P. aeruginosa*, which is a soluble protein and hydrolyzes multiple *holo*-ACPs, as well as an acylated ACP in a multidomain polyketide.

Recently, we have utilized this well-behaved AcpH to cleave many different PPant probes from a variety of carrier proteins.[292] Various chain length probes were cleaved from carrier proteins to facilitate facile attachment and detachment, in order to study the behavior of the labeled protein by protein NMR spectroscopy. The plastidic *holo*-AcpP from green

microalgae *C. reinhardtii* is also a substrate for this AcpH and is efficiently transformed into *apo*-Cr-cACP.[293]

Regulation by 4′-phosphopantetheinylation, as predicted by Vagelos et al.[284], remains elusive and might not be the point at which Nature regulates fatty acid biosynthesis and synthase activity. However, the presence of AcpH in some species and its broad activity suggests that there is some control on the activation of carrier proteins.

5. Structures

Structural studies of PPTases began as early as 1999.[271] Since then, 13 PPTases have been structurally described (Table 4). Several of these PPTases were crystallized along with CoA and carrier protein targets, providing a substantial amount of architectural information concerning the residues required for CoA binding and carrier protein recognition. From a structural point of view, the AcpS-type PPTases are better understood that either Sfp-type or Fungal Type I FAS PPTases. Of the 13 known structures, two are Sfp-type PPTases, and only one represents the Fungal Type I FAS PPTases. Further structural characterization of additional PPTases will aid in our understanding of the evolution of function in PPTases generally and the discovery of potent inhibitors as well as modification of substrate profiles for biotechnological use.

Structural features of AcpS type PPTases

The first structurally characterized AcpS-type PPTase is AcpS from *B. subtilis.*[271] It exhibits a trimeric quaternary structure, with each monomer measuring about 15 kDa. The trimer contains three active sites, formed at the interface between monomers. At each interface, a β-sheet “cleft” is responsible for binding the cofactor CoA (Fig. 21).

The active site contains a Mg^{2+} cation that coordinates to the pyrophosphate moiety of CoA (Fig. 23). The Mg^{2+} ion is held in place by two acidic residues, Asp8 located on β-Sheet 1 and Glu58 located on α-helix 4. Glu58 serves a dual purpose, responsible for both coordinating the Mg^{2+} ion as well as deprotonating the conserved serine residue of the ACP. Oligomerization of the trimer is controlled by the interaction of β-sheet 1, the sheet that contains Mg^{2+} coordinating residues, and β-sheet 5 of an adjacent monomer. These interactions are dominated by hydrophobic interactions, with Ile5 on β-sheet 1 contributing many hydrophobic interactions with β-sheet 5. Additionally, the Gln113 residues of each monomer form a hydrogen bonding network with each other in the core of the assembled trimer. The adenine base of CoA is cradled by the opposite side of an adjacent AcpS monomer, with Pro86 on β-sheet 3 supporting the aromatic base. Lys64 on α-helix 4 points at the diphosphate of CoA, most likely donating a proton during PPant-transfer. While all subsequently solved structures of AcpS-type PPTases exhibit similar overall structural features, varying crystallization conditions and ligand complexes result in structural variations that enable a greater understanding of structure-function relationships.

AcpS from *M. tuberculosis* adopts two distinct conformers when crystallized under varying conditions. A structure containing 3′,5′-ADP was initially solved by Dym et al. [150] When compared to other AcpS structures, several regions were absent in the electron density including residues 22–30, 41–44 and 75–78. Additionally, α-helix 3 extends further than other AcpSs. A linker region between α-helix 3 and loop 1, which is longer than the same region of other AcpS structures, adopts an “open” conformation. When later crystallized by Gokulan et al.[151] at a lower pH, this linker region adopted a “closed” conformation, with a 12 Å shift in α-helix 2 and a 9 Å shift in the previously described α-helix 3 region. No absence of electron density was observed, possibly indicating that cofactor-free AcpS is more compact and ordered than when bound to 3′,5′-ADP. This conformational change was

predicted to be pH dependent when Gokulan et. al. obtained an *apo*-AcpS structure that more closely resembled the "open" conformation, crystallized under similar pH conditions to Dym et al.[150] The same helix movements observed by Dym et al.[150] were observed in this new structure.

Comparison of the structures of *apo*-, CoA-bound, and acetyl CoA-bound *S. coelicolor* AcpS revealed large conformational changes upon cofactor binding.[126] Conserved residue Arg44 shifted to bind to the 3′-phosphate of CoA. Additionally, the backbone shifted to accommodate both the PPant moiety and the adenine base of CoA. Mutational studies led to the discovery of an H110A mutant that showed a negligible decrease in CoA binding, but a severe loss of activity. Based on structural data, H110A greatly alters the orientation of D111, which is essential for Mg^{2+} coordination. This observation suggests that loss of activity does not always corroborate with the ability of a PPTase to bind CoA.

Structural features of Sfp-type PPTases

Only two structures of Sfp-type PPTases have been characterized to date: Sfp from *B. subtilis*,[15] the founding member of the enzyme class, and AASDHPPT from *H. sapiens*.[260] These PPTases range from ~200 aa to over 340 aa in size. Unlike AcpS-type PPTases, Sfp-type PPTases exhibit a pseudodimeric fold, consisting of two structurally similar subdomains connected by a short polypeptide loop (Fig. 22). This pseudodimer resembles the interface of two AcpS-type monomers (Fig. 24). It is possible that this pseudodimer is a result of a gene duplication of AcpS to form two tandem copies. Alignment of AcpS with each half of Sfp reveals an interesting conservation pattern. Residues that correspond to the pseudodimer interface, as well as several active site residues, are more highly conserved than the outer residues. Additionally, the C-terminal half of Sfp more closely resembles a monomer of AcpS, which contains the acidic residues that coordinate the Mg^{2+} ion.

An extra loop that contains a short helix is found at the C-terminus of Sfp-type PPTases. Based on the currently solved structures, this confers stability to the pseudodimer, with the α-helix bound between the two pseudodimer halves (Fig. 22). Sfp-type PPTases contain only one active site in which CoA is bound. The structure of Sfp was solved with CoA and a Mg^{2+} ion bound in the active site. Similar to AcpS-type PPTases, an absolutely conserved glutamate residue, which corresponds to Glu151 in Sfp, serves to deprotonate the serine of the incoming ACP to facilitate PPant transfer (Fig. 23). Sfp contains two acidic residues, Asp107 and Glu109 on β-sheet 6, that coordinate the Mg^{2+} ion, as opposed to the single Asp residue found in AcpS type PPTases (Fig. 23). It was observed by Mofid et al. that upon MALDI analysis of Sfp, both the mass for Sfp and the mass for Sfp plus CoA were observed.[297] It was estimated that about 20–30% of the recombinantly expressed Sfp co-purifies with cellular CoA.

AASDHPPT is the only PPTase gene identified in the *H. sapiens* genome, and thus is likely responsible for activating all enzymes that require a PPant modification, which include the ACP from type I FAS,[260] aminoadipate semialdehyde dehydrogenase,[26] and tetrahydrofolate reductase.[269] It is almost 100 residues larger than Sfp, with a long "tail" that wraps around the back of the PPTase (Fig. 25). This tail may be important for recognition of the various target proteins. In contrast to Sfp, AASDHPPT only contains two acidic residues at the Mg^{2+} binding site, more closely resembling AcpS-type PPTases. Sequence analysis of Sfp-type PPTases reveals conservation of three acidic residues in the active site in prokaryotes (resembling Sfp), while only two acidic residues are observed in eukaryotes (resembling AASDHPPT). In the structure of Sfp, two β-sheets of the C-terminal half extend out to form an "arm" that wraps around an adjacent Sfp monomer. While gel filtration of Sfp indicates a monomeric quaternary structure, it is unclear whether this is an artifact of

crystallization or indicative of a native interaction between monomers. AASDHPPT exhibits a more globular overall structure.

AASDHPPT was co-crystallized with human AcpP, sub-cloned from the large type I FAS biosynthetic complex. In contrast to yeast type I FAS, which contains a PPTase at the C-terminus of FASI complex,[21] human type I FAS must be activated before final assembly by AASDHPPT. To obtain a structure with both ACP and CoA bound simultaneously, a Ser to Ala mutation was introduced into the ACP, eliminating the serine residue involved in PPant transfer. This structure differs slightly from the structure of AASDHPPT. Mg^{2+} was not observed in the active site, which may be due to the pH of the crystallization conditions, as the acidic residues responsible for coordinating the Mg^{2+} ion are likely protonated at the acidic pH of crystallization. The active site Glu, responsible for both Mg^{2+} coordination and serine deprotonation, is rotated away from the Mg^{2+} binding site and points into a space that would normally be occupied by the conserved serine of the ACP. The “tail” observed in the CoA bound AASDHPPT structure is largely absent from the AASDHPPT-ACP co-structure.

Structural features of integrated Type I PPTases

The structure of the FAS from *S. cerevisiae* did not exhibit strong electron density for the PPTase located at the C-terminus of the megasynthase, which is located outside of the core of the large FAS assembly.[21] It was unclear how this PPTase interacted with the ACP to convert it from the *apo* to the *holo* form, since this PPTase resembles AcpS-type PPTases that undergo trimerization before they are active. This PPTase domain was excised from the fungal FAS and structurally characterized both with and without CoA.[22] Interestingly, it formed a trimer in solution, similar to AcpS-type PPTases. However, the active site more closely resembles the Sfp-type PPTases, since it contains three acidic residues (E1817, D1772, E1774) that coordinate the Mg^{2+} ion. This excised PPTase showed phosphopantetheinylation activity with the *S. cerevisiae* ACP from the same FAS complex. These data suggest that an assembly event of several megasynthase subunits form either a dimer or trimer that can then activate other synthases. Since both the ACP and the PPTase are located on the α subunit of the FAS, it is possible that these subunits associate and activate the ACP domains prior to $\alpha_6\beta_6$ megasynthase assembly.

Identification of residues important for PPTase activity

Mutational analyses of PPTases based on structural data have elucidated several integral residues that are required for function, many of which are conserved throughout the protein family. In general, acidic residues that coordinate the integral Mg^{2+} ion are essential for activity. Additionally, the lysine positioned behind the diphosphate of CoA is required for catalysis (Fig. 23).

Parris et al. investigated the residues required for oligomerization of AcpS.[271] I5A and I5R mutations led to a decrease in apparent molecular weight, as measured by gel filtration. Activity of these mutants was unobservable. An N113E mutation displayed gel filtration behavior similar to wild type, but with reduced activity *in vitro*, indicating that the hydrogen bonding network important for oligomerization can be formed from the glutamate residues. An N113R mutation led to no activity and reduction of oligomerization. Residues E57, H110, and D111 were mutated in *S. coelicolor* AcpS and assayed for CoA binding (*via* isothermal titration calorimetry, ITC) and activity. E57A led to a 4 to 6-fold reduction in CoA binding, while H110A showed little change when compared to wild type. D111A, however, exhibited uncharacteristic binding behavior during ITC experiments, with CoA binding occurring at lower concentrations of CoA with a stoichiometry of 0.5, and a second binding event at higher concentrations. A K_d could not be accurately calculated for this mutant. E57A completely abolished PPTase activity, further implicating its importance in

the 4′-phosphopantetheinylation reaction. H110A and D111A both showed significantly reduced activity, measured at 5% and 28% of wild type activity, respectively.

A comprehensive panel of active site mutants of Sfp was assayed for carrier protein and CoA binding and activity.[275] H90A and H90N, mutations of a His that facilitates CoA binding by coordinating the diphosphate moiety, significantly reduce the activity of Sfp. K75N, a mutation of the lysine that aids in binding the adenine base of CoA, decreased the K_m for CoA, but did not severely affect activity. The S89L mutation caused a similar effect. Mutation of the highly conserved E151 and D107 to a Glu and Asp, respectively, resulted in a large decrease in activity. Mutations that completely inactivated Sfp include T44S, D109E, D107A, E109D, and E151A. The resulting effect of these mutations corroborate with their proposed functions. Interestingly, substitutions of Glu with Asp (and *vice versa*), which conserve the acidic nature of the natural amino acid, lead to catalytically inactive Sfp mutants. This indicates that both charge and the position of the charge are important for Sfp activity.

A similar panel of mutants were constructed for AASDHPPT based on the structure and proposed catalytic mechanism. The effect of these mutations was observed based on varying the concentration of the substrate acetyl-CoA as well as the magnesium required for catalysis. Mutation of acidic residues D129 and E181 greatly decreased PPTase activity, but has a relatively small effect on the K_m for CoA. Although Gln112 was thought to aid in Mg^{2+} binding, mutation of this residue had little to no effect on the enzyme. Mutation of Arg47, Arg86, and His111 reduced the K_m for both CoA and Mg. This suggests a cooperative binding event in which CoA facilitates the binding of Mg^{2+} to the PPTase. Alteration of these two Arg residues increased the k_{cat}, suggesting the release of the byproduct 3′,5′-PAP is controlled by coordination of the 3′-phosphate with these two arginine residues. Lys185, located behind the diphosphate of CoA, did not affect the affinity of AASDHPPT for Mg^{2+} or CoA, but significantly reduced the activity.

Mutants of Sfp-type PPTases from several species of fungi have been investigated for functional alterations in PPTase activity. The Lys5 gene of *C. albicans* was biochemically characterized, and residues proposed to be important for activity and function were mutated.[74] The two mutations that had the greatest negative effect on Lys5 activity were E198D, K197R, K202R, and D153E. The Glu and Asp are most likely the acidic residues responsible for both coordination of Mg^{2+} and deprotonation of the carrier protein substrate. As previously observed in Sfp, mutations of acidic residues that alter the distance between the backbone and the acid moiety severely altered the natural function of Lys5. This indicates that both charge identity and position are integral for proper PPTase function. The K202R mutation most likely disrupts the key interaction with the diphosphate of CoA. K197R, which may or may not play a role in catalysis, might affect the positioning of the key acidic residue E198. Cfw/NpgA, the Sfp-type PPTase from the fungus *A. nidulans*, is required for the production of several natural products, as well as conidiophore development, and was named for the phenotype of non-pigmented colonies.[67, 68] It was discovered that a single point mutation in this gene, L217R, resulted in a temperature sensitive strain of *A. nidulans*.[69] This residue is possibly required for protein stability, and mutation from a hydrophobic residue to a hydrophilic residue severely disrupts the solubility and stability of the PPTase NpgA.

pH and metal effects on PPTase activity

Activity of PPTases is pH dependent. In general, PPTases are most active at pH values ranging from 6.0 to 7.0, but still maintain some activity at pH values approaching 8.5.[25, 28, 41, 56] This is advantageous to *in vitro* studies that contain other proteins that require higher pH conditions for stability and/or activity.[298]

All PPTases require a Mg^{2+} ion for PPant transfer, and Mg^{2+} is a key component for PPTase reaction buffer conditions. As discussed previously, acidic residues involved in coordinating a Mg^{2+} ion are universally observed in all PPTases. Since the structure of *B. subtilis* AcpS contains a Ca^{2+} ion instead of Mg^{2+}, Mofid et al. investigated the ability of Sfp to utilize other divalent cations for catalysis.[275] Interestingly, only Mn^{2+} showed activity when supplemented to Sfp. In fact, while having a much lower binding affinity for Sfp, it conferred a greater catalytic efficiency to Sfp. PPTase activity with Mg^{2+} was only 40% of the activity seen with manganese, with a k_{cat}/K_m almost 3 orders of magnitude lower. Ca^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} did not confer any PPTase activity to Sfp.

Activity and specificity for CoA donor

As previously described, PPTases catalyze the transfer of the PPant portion of CoA onto the conserved serine residue of carrier proteins. Both AcpS and Sfp-type PPTases utilize CoA *in vivo*. However, the inherent promiscuity of some PPTases enables the utilization of CoA analogs as alternative substrates both *in vitro* and *in vivo* (Fig. 26).[299–301]

The phosphoadenylate portion of CoA is indispensable for PPTase binding. Structural studies suggest strong conservation among closely related organisms of the hydrophobic pocket surrounding this portion of CoA. However, the residues that form this hydrophobic pocket may vary between distantly related PPTases. CoA that lacks a 3′-phosphate on the ribose sugar will not bind to PPTases.[28] 3′,5′-PAP, the byproduct of PPTase labeling, binds strongly enough to inhibit PPTase activity at high concentrations, and is used as a standard for Sfp inhibition.[302]

While the adenylate moiety is integral for binding, current structural data indicates that the PPant portion does not significantly contribute to CoA binding. In several crystal structures, density for the PPant arm of CoA is not completely observed, indicating that it is not locked in a specific “binding conformation.” Additionally, the PPant arm may shift into alternative conformations upon carrier protein binding. The AcpS-type PPTase from *S. coelicolor* is capable of utilizing a variety of acyl CoAs.[50] The co-structure of this PPTase with CoA shows a distinct binding pocket for the PPant arm. However, closer examination of the structure reveals a possible route for the pantetheine arm to extend over the surface of the PPTase that would not interfere with the phosphopantetheinylation reaction.[126] A possible explaination involves the residues Leu70 and Thr72, which are usually larger charged or polar residues in other AcpS-type PPTases. Their small size and lack of charge does not obstruct the exit route for the PPant arm of CoA. In the structure of AASDHPPT (Fig. 25) with the excised human type I ACP, density for the PPant arm was not observed. This suggests that it is projected out between the ACP-PPTase interaction surface through an exit route similar to that observed in *S. coelicolor* AcpS. (Fig. 25). Residues 199–204 border the PPant arm, and consist of small, non-polar amino acids that could allow alternative substrates to protrude from the binding pocket. These observations corroborate with the ability of certain PPTases to modify carrier protein domains with a wide variety of modified CoAs, in which the PPant arm bears unnatural molecules, including substrate mimics,[292, 303] fluorescent molecules,[304, 305] crosslinking agents,[299] and affinity tags[257] (Fig. 26). The ability to utilize unnatural CoA analogs has been important for assessing PPTase activity, especially in the context of drug discovery.[306] Additionally, modification of carrier proteins with substrate intermediates is important for elucidating carrier protein function.[307]

Measuring the activity of PPTases

Methods for measuring PPTase activity are integral for studying PPTase specificity towards carrier protein targets and the ability to use non-natural CoA substrates. Additionally, measuring the activity of mutant PPTases reveals both important catalytic residues and

residues required for PPTase-ACP interaction. Unlike many enzyme reactions, there are no co-factor transformations that can be easily followed for PPTases, nor is the reaction mechanism easily coupled to a reporter system. Thus, the modification of target carrier proteins is measured using various separation methods and CoA substrates.

Initially, PPTase activity was assessed using ^{3}H labeled CoA,[1] which was prepared by exposing CoA to tritium gas. A reaction containing this labeled CoA, *E. coli* AcpS, and *E. coli* ACP was allowed to progress for 30 minutes at 37 °C. The proteins in the reaction were precipitated with 10% trichloroacetic acid and subsequently washed to remove free, labeled CoA. The resulting pellet was redissolved and assessed for radioactivity using liquid scintillation counting. In this way, apparent kinetic values for AcpS were determined. In the same study, the use of urea-PAGE allowed the authors to assess labeling by gel-shift. Urea-PAGE is conformationally sensitive and can be used to distinguish between the *apo* and *holo* forms of carrier proteins.[308] Thus, modification of the carrier protein with AcpS causes a “shift” in the ACP band down the urea-PAGE gel. Carrier protein modifications can also be detected using high pressure liquid chromatography.[41, 102, 309] For example, *apo* and *holo* forms of the PCP from a module of surfactin biosynthesis, SrfB, were separated on a reverse phase HPLC column.[310] This allowed not only for detection of activity, but also for the quantification of *apo*- and *holo*-ACP by integration of each HPLC peak. While these methods are useful for determining kinetic parameters, they are both resource and time intensive. Radiolabeling can pose safety hazards, while gel and HPLC analysis require long runs to complete. New PPTase assays have addressed both the safety and time issues with these older experimental methods.

We have developed several assays based upon PPTase catalyzed transfer of fluorescent labeled CoA, described in the next section.

Duckworth et al. have recently used fluorescence polarization to measure PPTase activity by utilizing the strong association of CoA with Sfp.[311] For this assay, a BODIPY-CoA conjugate was synthesized by reacting CoA with a BODIPY-maleimide construct. Since the free BODIPY-CoA will tumble in solution at a greater rate than an Sfp-bound BODIPY-CoA molecule, a difference in anisotropy between the two states can be measured. Addition of increasing amounts of BODIPY-CoA to a fixed concentration of Sfp causes an increase in anisotropy signal, corresponding to the binding of BODIPY-CoA to Sfp. Validation as an assay for discovery of PPTase inhibitors was performed using 3′,5′-PAP, and an IC_{50} value comparable to previously published results was obtained.

Recently, a single module NRPS protein that is responsible for producing the blue pigment indigoidine was discovered,[312] and subsequently utilized in the detection of PPTase activity.[40, 212] This protein utilized two molecules of L-glutamine to produce a blue, bicyclic molecule that is amenable to visible light detection methods. There is a single PCP domain that requires modification by a PPTase to produce the blue pigment. Since heterologous expression of BpsA in *E. coli* does not yield a protein product bearing a PPant modification on the PCP, it can be used for *in vitro* assessment of PPTase activity.

6. Biotechnological use of PPTases

Some PPTases such as Sfp are promiscuous. Besides their cognate enzyme, they are able to activate a wide range of non-cognate carrier proteins. Concomitantly, the interaction of a PPTase with the carrier protein is very specific. The same is true for the second substrate CoA. While the PPTase explicitly recognizes CoA, it is able to utilize CoA conjugated to a broad range of small molecules.[313] With these characteristics, the unique reaction of a PPTase-catalyzed carrier protein modification has been applied for the development of site-

specific protein labeling techniques *in vitro* and *in vivo* (Fig. 27).[90, 314, 315] For detailed experimental labeling procedures, we refer to Sunbul et al.[316]

In vitro labeling

In 2004, we were the first to utilize the PPTase-carrier protein interaction to visualize, identify and purify modular synthases.[313] This work used Sfp and a modified CoA to attach a non-native PPant onto AcpP, PKS ACP and PCP. Several CoA derivatives were synthesized by attaching fluorescent- and affinity reporter-labeled maleimides on the free thiol group of CoA. The probe palette was extended by generating CoA analogs with different fluorescent dyes.[317] To accelerate the process of high throughput screening of small molecule libraries for drug discovery, Yin et al. utilized the interaction of GrsA-PCP and Sfp in phage display (see Section 4).[318] Using Sfp, small molecules like biotin, fluorescein or glutathione were covalently linked to the serine residue of GrsA-PCP presented on the phage surface. A similar setup of this interaction was applied to identify a specific PCP-tag for affinity purification that can be co-expressed with the target protein.[318]

In vivo tagging and labeling

The concept of PPTase catalyzed carrier protein-tagging was improved and patented for the *in vivo* study of membrane proteins.[315] The specific labeling allows not only localization of proteins such as receptors, but to further study the protein dynamics and intracellular trafficking.[319–322] This technique could be successfully applied to various membrane proteins in *E. coli*, *S. cerevisiae*, HEK293 and TRVb cells and neurons.[320–324] Offering a high signal intensity as well as excellent photostability, quantum dots were evaluated as replacement of fluorescent dyes for imaging of cell surface and transmembrane proteins.[325, 326]

All these methods have been applied *in vitro* or upon the cell surface. Clarke et al. were the first presenting *in vivo* labeling.[304] Using nonhydrolyzable, fluorescent pantetheine analogues, overexpressed VibB-PCP was labeled *in vivo* using co-expressed Sfp. Loading cell lysate directly onto a SDS–PAGE gel, labeled VibB-PCP was visualized by UV. These fluorescent probes were modified for additional surface-based affinity detection and purification.[327] Using a stilbene reporter tag enabled a switchable, antibody-elicited, fluorescent response in solution or on affinity resin. The range of probes was expanded by testing various combinations of linker, dye, and bioorthogonal reporter.[300] This allowed for purification of the carrier protein independent of antibody techniques. Further *in vivo* labelling of native carrier proteins using pantetheine analogues could be demonstrated for Gram-positive and Gram-negative bacteria and in a human carcinoma cell line.[328]

Site-specific protein labeling using the Sfp system was refined using novel protein tags such as YbbR,[276, 329] S6, and A1,[257, 278] which were identified using a phage-displayed peptide library. These tags are very short (11 and 12 aa), and thus cause minimal disturbance to the target protein structure and function. S6 and A1 can even be used as a pair for the sequential labeling of two proteins with different small-molecule probes with very little cross-labeling, using Sfp and AcpS, respectively. To evaluate how far the peptide tag size can be reduced, ^{15}N-HSQC based NMR titration experiments were conducted.[278] The resulting octapeptide could be used for *in vitro* and cell surface labeling. Thus, during this process, it was shown that AcpS is able to convert a PCP that is naturally not modified into a substrate. If this is generalizable, it would be of great importance for natural product engineering.

Powerful tools were generated by combination of the carrier protein-PPTase interaction with other techniques, such as yeast surface display for vaccine development[330] or structural fixation for X-ray crystallographic analysis as demonstrated for a di-domain construct from

EntF.[331] High-throughput assays were developed for the identification of novel PPTases, PKS and NRPS, or improved enzyme activities using *in vitro* (solid phase/phage display)[316, 332, 333] as well as *in vivo* methods (metagenomic libraries).[212, 334]

Other applications

For solid phase applications, Wong et al. used Sfp to immobilize YbbR-tagged protein onto surfaces functionalized with CoA.[332] Immobilization was demonstrated for ACP, luciferase, glutathione-S-transferase and thioredoxin onto PEGA resin as well as hydrogel microarray slides and could even be performed directly from cell lysate. Developing this technology for cell biology and tissue engineering, Mosiewicz et al. could even encapsulate primary mouse fibroblasts into a hydrogel using the PPTase-mediated linkage.[335] In reverse, Stack et al.[333] used an immobilized synthase to measure PPTase activity with fluorescent CoA substrate analogues in a microtiter plate assay.

These advancements in utilization of the PPTase-carrier protein interaction paved the way for the elucidation of modular enzyme biosynthetic pathways, including mechanism, structure and proteomic identification of the synthases.[336] To study the interaction between the carrier protein and the other synthase domains, pantetheine analogues were developed containing terminal moieties serving as irreversible cross-linking reagents.[299] The carrier protein was labeled with the pantetheine probe by one-pot chemo-enzymatic synthesis.[298] This includes two steps: the conversion of the pantothenate into a CoA analog using CoAA, CoAD and CoAE, and attachment of this probe onto the carrier protein by a PPTase. Following, the second domain interacts with the probe, forming an irreversible covalent adduct with the carrier protein. Cross-linking could be successfully demonstrated for the ketosynthase, dehydratase, and thioesterase domains.[293, 301, 337–339]

Fluorescent and affinity reporters were further utilized for activity based proteomic profiling.[340] In combination with the chemoenzymatic methods of carrier protein labeling, the method was applicable for probing inhibitor specificity, assigning domain structure, and identifying natural product producing modular synthases *in vitro* and *in vivo*.

A further example for how to study these enzymatic pathways *in vivo* is demonstrated with GlyPan (disulfide of N-pantoylglycyl-2-aminoethanethiol).[303] GlyPan is a pantetheine analogue containing glycine, and thus one carbon shorter than endogenous β-alanine. GlyPan was efficiently loaded, *in vivo*, onto *E. coli* AcpP presumably by an endogenous PPTase, showcasing another kind of promiscuous behavior of PPTases.

Production of natural products in heterologous hosts

Many non-ribosomal peptides and polyketides are important pharmaceuticals and agro-chemicals. Production of these natural products and possibly “unnatural” natural products (by modification or mutagenesis of the synthase) has been the goal of many research projects over the past two decades. This effort led to significant innovations in the field of metabolic engineering for drug discovery and development.[341]

The first attempts to heterologously express modular natural product synthases in *E. coli* yielded primarily the inactive *apo*-form of the synthase, prohibiting actual biosynthesis of the natural product.[342–346] After the discovery of PPTases and their function,[1] co-expression of a PPTase (Gsp) with a truncated NRPS (gramicidin S synthase) led to *in vivo* activation of the synthase by detection of an intermediate that was absent when only the NRPS was expressed.[347] Co-expression of the PKS 6MSAS (6-methylsalicylic acid synthase) and the PPTase Sfp in *E. coli* and in *S. cerevisiae* by Kealey and co-authors marked the first instance of heterologous biosynthesis of a natural product.[348] Production of 6MSA (6-methylsalicylic

acid) in yeast was even 2-fold greater in comparison to the native producer *Penicillium patulum*.

Since then, multiple polyketide and non-ribosomal peptides have been successfully expressed in further optimized, biologically friendly heterologous hosts, such as *E. coli*, *S. coelicolor*, and yeast.[349–353] Due to their modular nature, these synthases can theoretically be manipulated to yield a wide range of possible biomolecules.[354–356] Within these model organisms, the flux can further be improved by increasing the amount of starting material such as CoA-derivatives.[357, 358] Besides their pharmaceutical application, modular synthases have gained increasing importance in the fields of biofuel and nutrition. The declining availability of fossil fuels has intensified the effort to investigate novel routes to heterologously produce hydrocarbons.[359] Besides *in vivo* modification of the native fatty acid biosynthesis by introduction of additional domains,[293, 360, 361] the actual synthases were evaluated for the production of valuable molecules in more suitable host systems. Towards heterologous production of specific aliphatic hydrocarbons, Akhtar and co-workers engineered the carboxylic acid reductase (CAR) gene from *Mycobacterium marinum* into *E. coli* (also see Section 2.1).[189] In combination with a chain-length-specific thioesterase, this strain was able to convert fatty acids to fatty alcohol and alkanes. Including a fatty acid-generating lipase, *E. coli* even utilized natural oils for this progress. CAR however requires 4′-phosphopantetheinylation, as previously shown (see Section 3).[39] The co-expression of Sfp was the key to get this biosynthetic machinery progressing at maximum activity. Recently, Amiri-Jami and Griffiths produced both EPA and DHA in *E. coli* by heterologous expression of the omega-3 fatty acid synthase gene cluster from *Shewanella baltica* MAC1 (Fig. 10).[362] One fosmid clone from *S. baltica* contained *pfaA-D*, but not the PPTase gene *pfaE*, resulting in a clone that did not produce DHA or EPA. However, when the full *pfaA-E* gene cluster was expressed in *E. coli*, the bacteria were able to produce both omega-3 fatty acids (see Section 3.6). By heterologous expression of PUFA genes in plants, these nutritional fatty acids are produced in crop plants that do natively not provide these compounds.[363] In general, PUFA PKS genes have been shown to require co-expression of a PPTase gene.[199, 244–247, 364, 365] Similarly, the PKS 6MSAS requires activation by heterogously expressed Sfp in *E. coli* and yeast,[348] but was potentially transformed into its *holo*-form by an endogenous PPTase in tobacco.[248] This demonstrates the power of plant expression systems for this kind of production. Besides plants, algae that naturally produce very valuable fatty acids and lipids[366] have recently received attention for their great potential as heterologous host for the biosynthesis of complex molecules,[367–370] and should be further investigated for application in biofuel production.

Drug discovery

Since PPTases activate important metabolic pathays, they represent a new drug target in bacteria that has not been fully explored. Only recently has the PPTase enzyme family been investigated as a potential drug target, with a small handful of potent inhibitors identified to date. (Fig. 28).

A variety of 4*H*-oxazol-5-one derivatives with potent AcpS inhibition were synthesized by modification of 4-chlorophenyl 4*H*-oxazol-5-one, an AcpS inhibitor discovered *via* high-throughput screening.[306] Modification of the oxazol-5-one core with an anthranilic acid moiety led to decreases in IC_{50} values against AcpS, with one compound reaching sub-micromolar activity. The anthranilic acid moiety was again utilized by Joseph-McCarthy et al. to produce anthranilic acid based AcpS inhibitors.[295] Using structure-based drug design, initial HTS hits were optimized based on molecular modeling into the active site of AcpS from *B. subtilis*. The optimized compounds showed a 30-fold decrease in IC_{50} values. Furthermore, four of the lead compounds were co-crystallized with *B. subtilis* AcpS. These

structures confirmed the importance of the anthranilic acid portion of each inhibitor, which binds in the location normally occupied by the adenine base and ribose sugar of CoA. This success underscores the value of structural information for rational design of inhibitors.

The natural product SCH 538415, isolated from an unidentified bacterium, was found to inhibit the AcpS gene.[371] Using a radiolabeled CoA substrate, a HTS utilizing unidentified bacteria extracts identified this product as an AcpS inhibitor. Discovery of inhibitors that target Sfp-type PPTases began only very recently with the development of a fluorescence resonance energy transfer (FRET) assay that relied on the ability of Sfp from *B. subtilis* to label the short peptide YbbR.[302] YbbR was conjugated to fluorescein isothiocianate (FITC) at the N-terminus. Modification of the serine residue of YbbR with a different fluorescent dye enabled the FRET interaction. FITC was chosen for its ability to act as either a fluorescence energy acceptor or donor. This assay was optimized and validated using 3′,5′-PAP as a model inhibitor. This method was later modified to replace the N-terminal FRET molecule on YbbR with a fluorescence quenching dye, BHQ2.[372] When YbbR was labeled with rhodamine-CoA, the rhodamine fluorescence was quenched by the now adjacent BHQ2. This significantly improved the reliability of the signal, increasing the sensitivity of the assay. The $LOPAC_{1280}$ compound library was screened for potential Sfp inhibitors using this assay. Several hits with low IC_{50} values were discovered (Fig. 28). These hits were validated using both the original FITC assay as well as a gel based labeling assay. 6-Nitrosobenzypyrone (NOBP), a hit from the $LOPAC_{1280}$ screen, was further utilized to inhibit phosphopantetheinylation of the single module NRPS BpsA.[40] NOBP was measured to have a K_i ranging from 0.4–5.2 μM.

PptT, the Sfp-type PPTase from *M. tuberculosis*, has recently been identified as a valid drug target for combating tuberculosis infection.[36, 37] Le Blanc and coworkers have developed a high-throughput screening method for PptT, which utilizes scintillation proximity to measure the extent of protein carrier labeling. A biotin-tagged ACP excised from the Pks13 gene of *M. tuberculosis* served as the target for an MBP-PptT fusion, and the labeling substrate for PptT was [^{3}H]-CoA. Once the reaction was allowed to progress, a streptavidin tagged scintillation bead was allowed to bind to the biotin tag of the ACP. Spectral counts were then taken of the reaction samples. This assay, which is tolerable to DMSO and amenable to high-throughput, could also be adapted to PPTases from other organisms by altering the target ACP.

Recent studies of bacteria and fungi reveal that several species require the action of an Sfp-type PPTase for important secondary metabolic pathways, that affect growth, reproduction, and pathogenicity. *Aspergillus nidulans* requires the Sfp-type PPTase NpgA for proper pigmentation and spore formation.[69] Functioning Sfp-type PPTases are required in the bacteria *Agrobacterium vitis*[187] and the fungus *Trichoderma virens*[83] to effectively elicit plant immune responses. Several strains of plant pathogenic fungi contain an Sfp-type PPTase that is indispensible for infection, including maize anthracnose fungus *Colletotrichum graminicola*[232] and the cereal fungus *Cochliobolus sativus*[75]. Inhibiting the Sfp-type PPTase in these fungi is a new strategy for combating plant pathogens, but there are currently no antifungal compounds that target this enzyme.

Detection of the Sfp-type PPTase Lys5 and the closely related lysine metabolic gene Lys1 in *C. albicans via* PCR can be used to specifically identify pathogenic strains of this fungus.[373] Amplification of the Lys5 gene in *C. albicans* enables rapid detection of this opportunistic fungus, which is often found to infect immunocompromised patients. Since the primary sequence of Sfp-type PPTases between organisms can vary greatly, specific detection and targeting of a pathogen and not the infected host is possible.

7. Outlook

Natural products have been an inspiration for chemists and biologists for hundreds of years, and many of our currently used medicines are natural products or based on a natural product.[374] Until 1990, natural product biosynthesis was studied primarily by feeding radioactive precursors to whole organisms, protein preparations or lysates. In 1990 and 1991, Katz and Leadlay separately published the discovery of the polyketide 6-deoxyerythronolide B synthase gene, its modular nature and its assembly line production of the natural product. Marahiel and co-workers discovered a few years later that non-ribosomally encoded peptides are also synthesized in a similar fashion. These discoveries (among others) opened up the field of natural product biosynthesis and allowed for connecting the gene to the natural product.

Although it was already known since the 1960s that the FAS requires a PPant arm on a conserved serine of a carrier protein, and that a dedicated PPTase is responsible for this post-translational modification, it took until 1995 to establish that PPTases are a separate superfamily of enzymes that are essential for the three major metabolic pathways. In other words, without this post-translational modification, these synthases are unable to let the carrier protein ferry cargo from one active site to the next. The family of PPTases now contains many annotated or putative proteins (>1700), and the characterization of ~60 (and kinetic characterization of ~7) enzymes illustrates the diversity of this class of proteins, in oligomerization state, specificity for CoA analogs, specificity for carrier proteins, kinetic parameters, structure and function.

Besides AcpS, which was identified from *E. coli* by Vagelos in the 1960s,[3] Sfp from *B. subtilis* is the archetypical PPTase, used by the bacteria to post-translationally modify surfactin synthase. Since Sfp has a broad substrate scope, both for its CoA as well as for its carrier protein substrate, this PPTase has been the most used for co-expression with engineered biosynthetic pathways. However, Sfp also has its limitations. For example the 19 aa consensus peptide of the SrfB PCP cannot be modified by Sfp, and all FAS carrier proteins are labeled relatively slow by Sfp. To the best of our knowledge however there is no *apo*-carrier protein that is not post-translationally modified by Sfp. Recently, it was shown that a carrier protein from sphingolipid biosynthesis cannot be modified with either C_{16} or C_{18} CoA analogs using Sfp, suggesting that there might be some limitation to the catalytic activity and substrate promiscuity of the PPTase.[375]

In recent years, we are observing a trend of expression of newly identified (by genome mining) biosynthetic clusters in heterologous hosts, and it might well be that Sfp will not always be the ideal PPTase for the job. We hypothesize that examining the detailed phylogeny of the PPTase family could reveal which PPTase to overexpress with the synthase of interest, or even selecting a heterologous host with an endogenous PPTase that modifies the desired synthase. Thus far, biosynthetic clusters have been expressed mainly in model bacteria and fungi. However, many new synthase genes have been recently found in cyanobacteria, plants, and even higher eukaryotes. Expression of these synthases might well require specialized protein expression systems, or even designer heterologous hosts.

Many natural products that are biosynthesized by synthases are essential to bacteria and fungi for virulence and therefore survival. Since these synthases require 4′-phosphopantetheinylation, targeting the PPTase (instead of any other domain) could be ideal to weaken or kill these pathogens. It should be noted that in the late 1990s and early 2000s, targeting FAS by inhibiting AcpS was a hot topic. Two crystal structures of AcpS-type PPTases were published, and inhibitors were discovered and optimized by Wyeth and

Schering-Plough.[295, 306, 371] Since then, industrial interest in PPTase inhibition has dwindled and most likely these projects were terminated.

In the past years, major strides towards the discovery and development of inhibitors that target Sfp-type PPTases have been made. In order to discover these, high-throughput assays first had to be developed. Now with these in place, we expect that antibiotics targeting secondary metabolism will be on the rise in the coming years. Finally, since the discovery of PPTases as a broad family of post-translational carrier protein modifying enzymes, they have been used *in vitro* and *in vivo* for activating carrier proteins and derivatizing carrier proteins with natural and unnatural probes. In the near future, we may see a further expansion of the repertoire of probes used *in vitro* and *in vivo*, including - but not limited by – fluorescence, solvatochromic, FRET, electronic, positron electron tomography (PET), nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR) and purification tags. Utilizing Nature's promiscuity to install these and other probes onto carrier proteins opens up avenues towards drug delivery, studying post-translational modifications and protein-protein interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PPTase	phosphopantetheinyl transferase
FAS	fatty acid synthase
PKS	polyketide synthase
NRPS	non-ribsomal peptide synthase
CoA	coenzyme A
3′,5′-PAP	phosphoadenyl phosphate
PPant	4′-phosphopantetheine
ACP	acyl carrier protein
PCP	peptidyl carrier protein
CP	carrier protein
AASDH	a-aminoadipate semialdehyde dehydrogenase
fTHF	10-formyl tetrahydrofolate
fTHF-DH	10-formyltetrahydrofolate dehydrogenase
PUFA	polyunsaturated fatty acid

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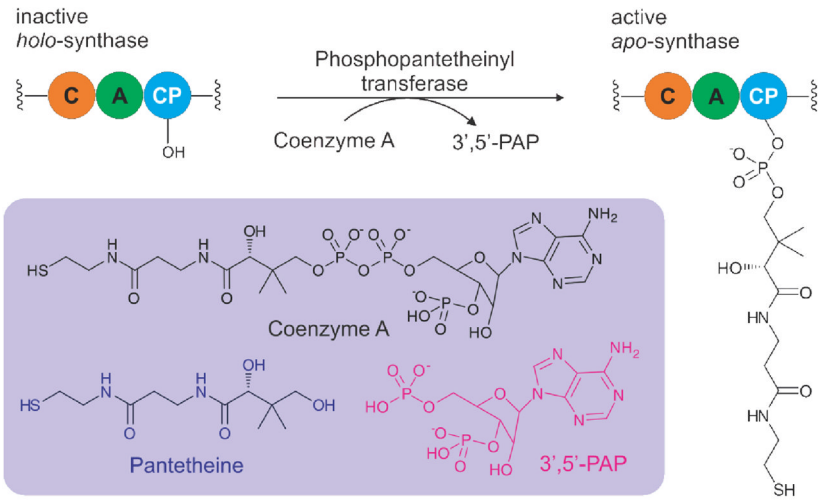

Fig. 1.

General reaction scheme of post-translational phophopantetheinylation by a PPTase. The PPTase transfers the PPant moiety from CoA to a conserved serine residue on the *apo*-CP to produce *holo*-CP, here showcased by a typical NRPS module containing C, condensation; A, adenylation; and CP, carrier protein, domains. 3′, 5′-PAP is 3′,5′-phosphoadenosine phosphate.

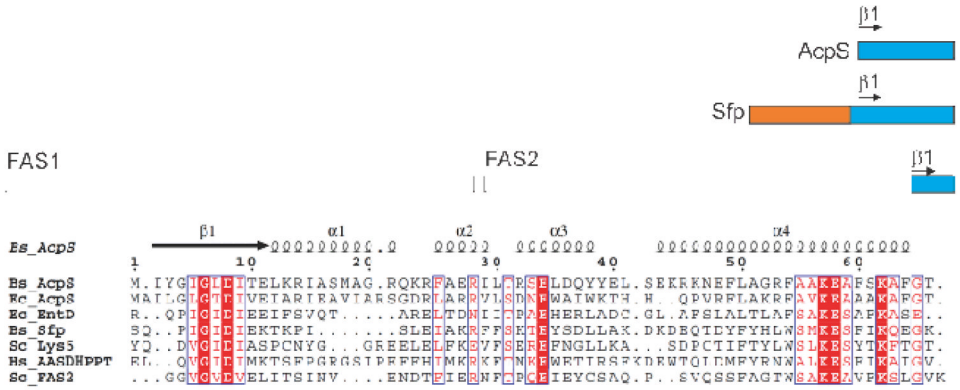

Fig. 2.

PPTases. Overview of the three families of PPTases,[1] typified by AcpS, Sfp and the integrated PPTase domain of *Saccharomyces cerevisiae* FAS2, and a sequence alignment of archetypical PPTases using TCoffee and Espript[5, 6] (Bs, *Bacillus subtilis*, Ec, *Escherichia coli*. Hs, *Homo sapiens* and Sc, *Saccharomyces cerevisiae*.)

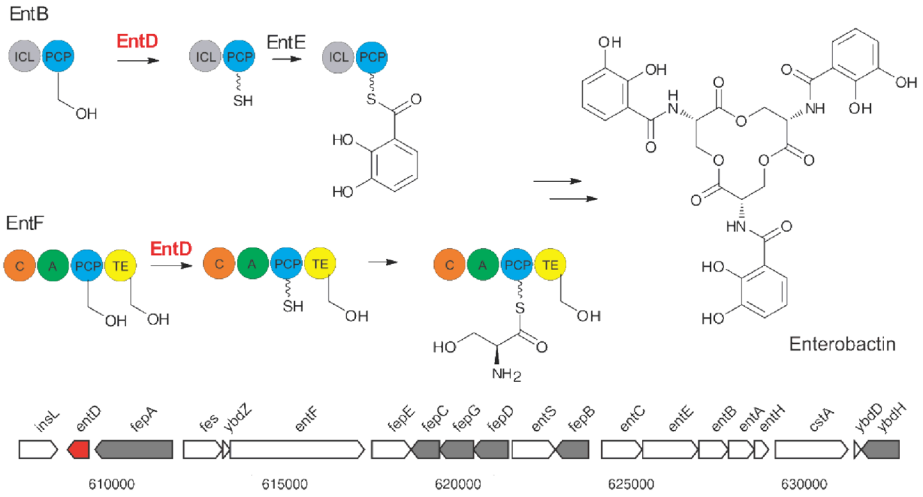

Fig. 3.

Biosynthesis of enterobactin and the corresponding gene cluster including the PPTase *entD*, labelled in red (GenBank acc. no. NP_415115.2) (data extracted from the *E. coli* genome with the GenBank acc. no. NC_000913.2). ICL represents isochorismate lyase,; PCP, peptidyl carrier protein; C, condensation domain; A, adenylation domain; and TE, thioesterase domain.

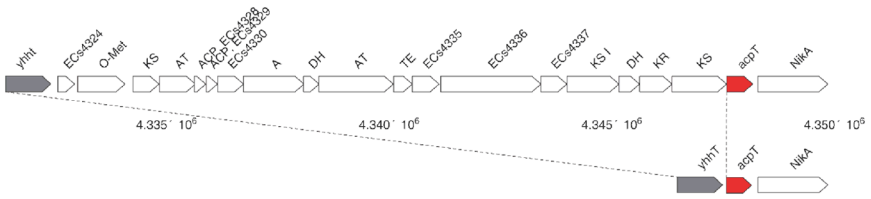

Fig. 4.

Comparison of *E. coli* O157 (top) and *E. coli* K12 (bottom) in the region of the PPTase *acpT* (in red).

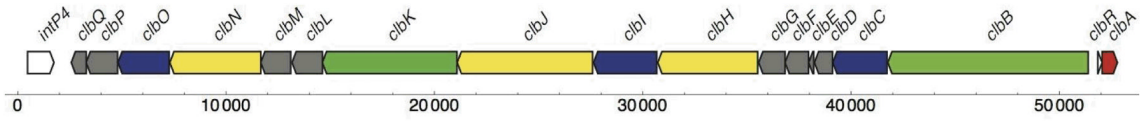

Fig. 5.

Colibactin biosynthetic gene cluster with the PPTase gene *clbA* (GenBank acc. no. AM229678.1). NRPS genes: yellow, PKS genes: blue, PKS-NRPS hybrid genes: green, PPTase gene: red.

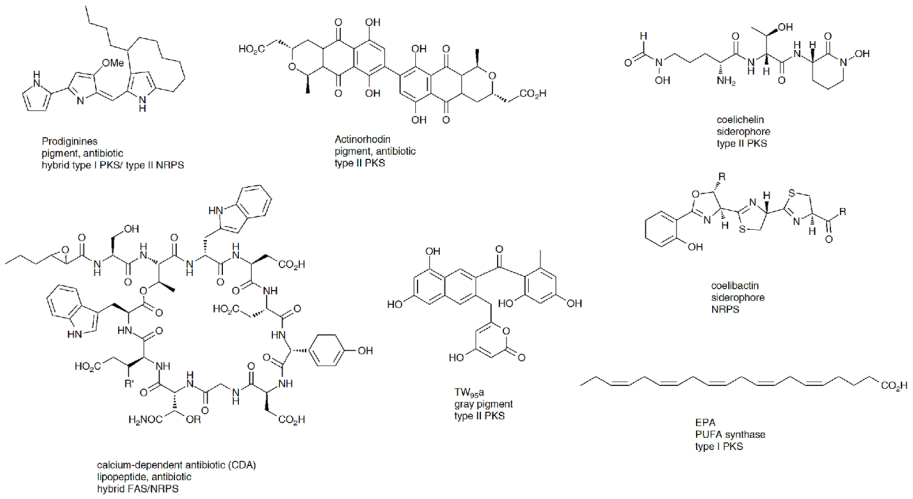

Fig. 6.

Secondary metabolites of *Streptomyces coelicolor* that depend on PPTase activity. Antismash identifies additionally butyrolactone (type I PKS), an NRPS natural product (nrp-cys) and another type I PKS product.[124, 127]

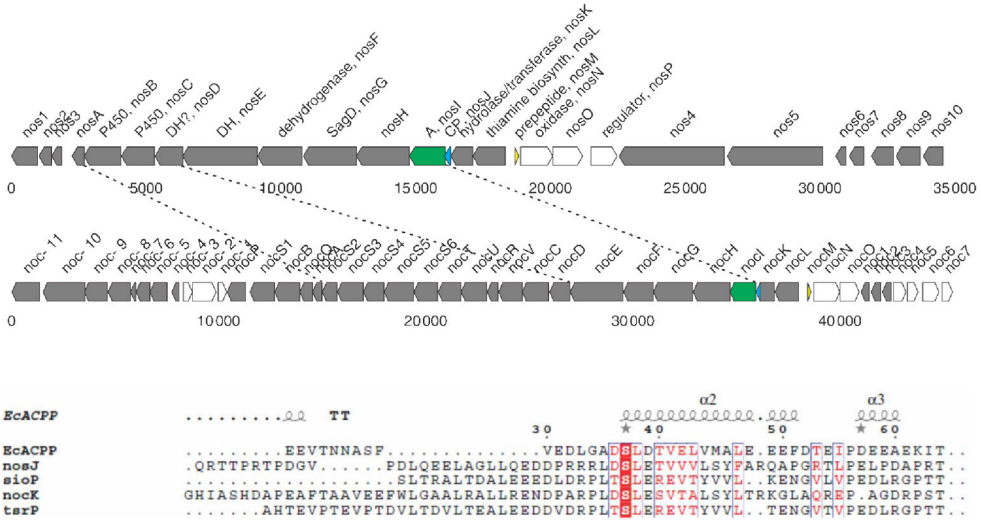

Fig. 7.

Comparison of the nosiheptide and nocathiacin gene clusters. The adenylation domain is shown in green, the putative carrier protein in blue and the ribosomal pre-peptide in yellow. Sequence alignment of *E. coli* AcpP and nosJ (ACR48339.1), sioP (ADR01086.1), nocK (ACN52299.1) and tsrP (ACN80653.1) show the conserved (D/T)SL motif, characteristic of a carrier protein. Sequence alignment was made using TCoffee and Espript.[5, 6]

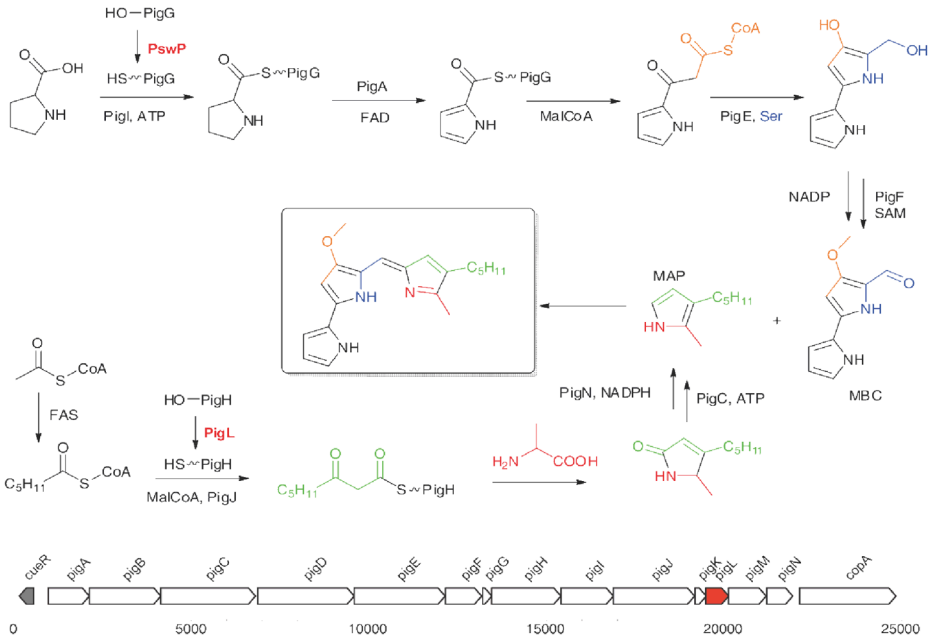

Fig. 8.

Prodigiosin biosynthesis requires both PPTases PswP and PigL.[156] Prodigiosin biosynthetic gene cluster (GenBank acc. no. AJ833002.1) including the PPTase gene *pigL*, labelled in red.

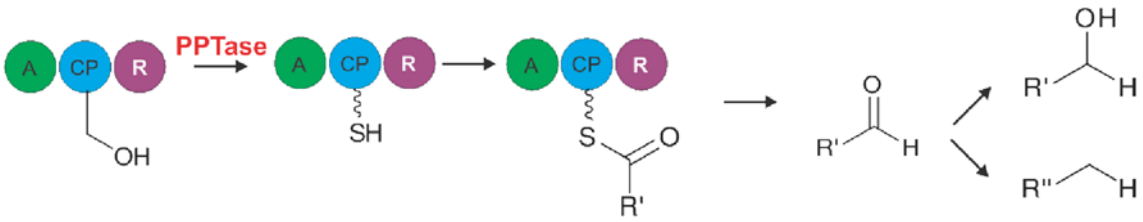

Fig. 9.

Carboxylic acid reductase (CAR). CAR is a promiscuous enzyme that reduces several (R′=) acyl and aromatic carboxylic acids to an aldehyde. Carboxylic acids are first activated by an adenylation domain (A), loaded onto the carrier protein (CP) and in a NADPH-dependent fashion reduced from the carrier protein by the reductase domain (R). The aldehyde product can further be processed to yield alcohols or alkanes.[189]

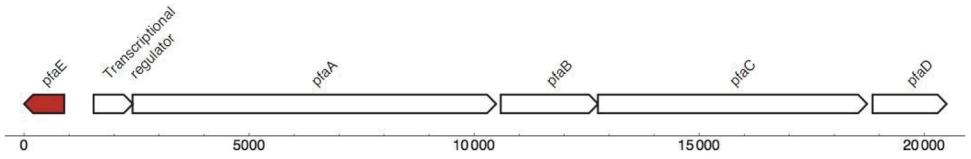

Fig. 10.
PUFA-PKS biosynthetic gene cluster (GenBank acc. no. EU719604.1) including the PPTase gene *pfaE*, labelled in red.

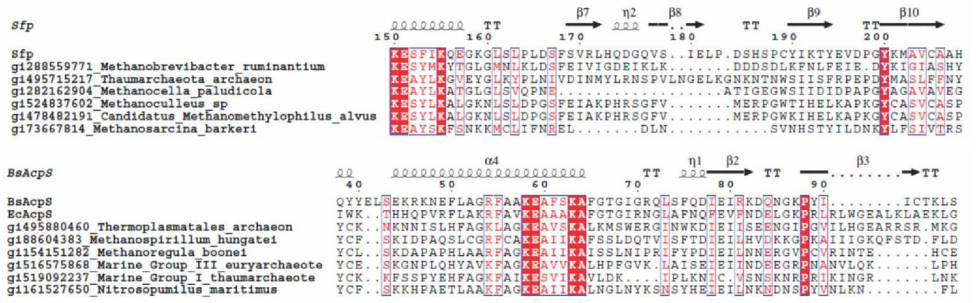

Fig. 11.

Sequence alignments of archaeabacterial PPTases. Only the active-sites are shown.

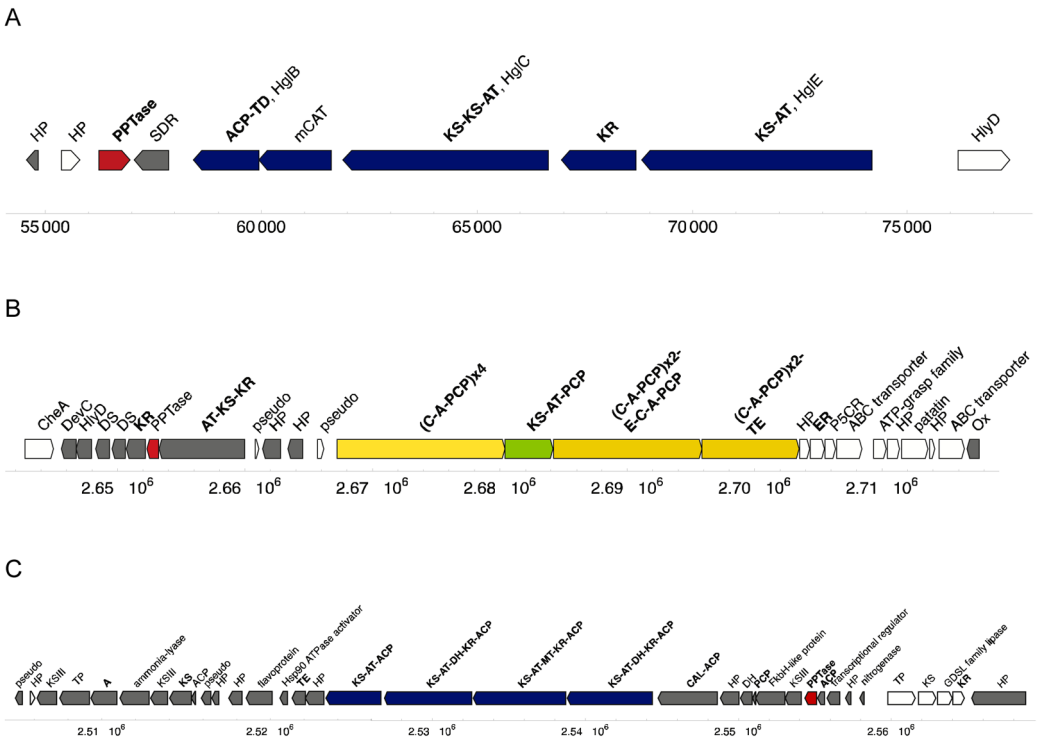

Fig. 12.

Biosynthetic gene clusters surrounding the three PPTase genes of *Nostoc punctiforme* PCC 73102 (ATCC 29133) (GenBank acc. no. NC_010628). A: NgcS (GenBank acc. no. YP_001863782) with glycolipid PKS locus, B: PPTase (GenBank acc. no. YP_001865721) with PKS-NRPS locus, C: PPTase (GenBank acc. no. YP_001865651) with PKS locus. Domains identified with Antismash[127] in bold. NRPS genes: yellow, PKS genes: blue, NRPS-PKS hybrid genes: green, PPTase gene: red. A: adenylation domain, ACP: acyl carrier protein, AT: acyl transferase, C: condensation domain, DH: dehydratase, DS: desaturase, E: epimerization domain, ER: enoylreductase, HP: hypothetical protein, KR: ketoreductase, KS: β-ketoacyl synthase, mCAT: malonyl CoA-ACP transacylase, MT: methyl transferase, Ox: NADH:flavin oxidoreductase, P5CR: pyrroline-5-carboxylate reductase, PCP: peptide carrier protein, PKS: polyketide synthase, SDR: short-chain dehydrogenase/reductase, TD: thioester reductase, TE: thioesterase, TP: thiamine pyrophosphate binding domain containing protein.

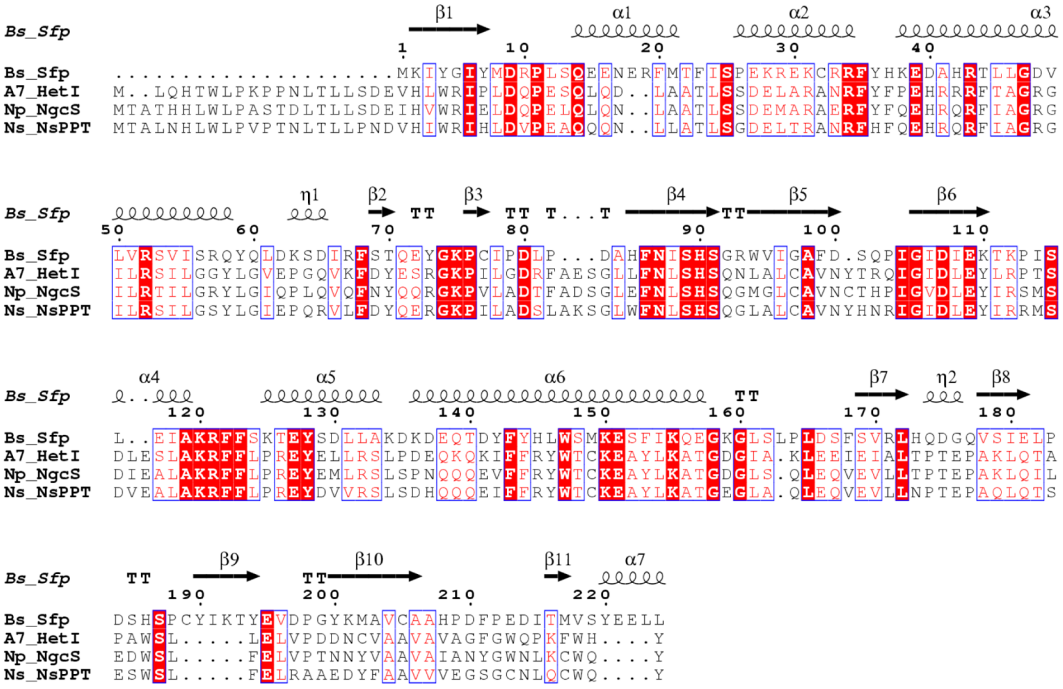

Fig. 13.

Alignment of HetI-like PPTases from *Anabaena* sp. PCC7120 (A7_HetI), *N. punctiforme* PCC 73102 (Np_NgcS) and *N. spumigena* NSOR10 (Ns_NsPPT) against Sfp (Bs_Sfp) (GenBank acc. no. AAA22003, ACC78839, AAW67221, CAA44858). Sequence alignment was made using TCoffee and Espript.[5, 6]

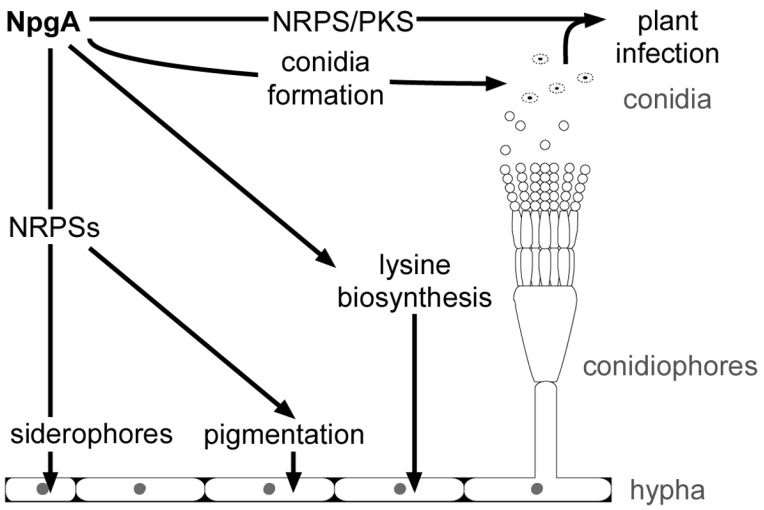

Fig. 14.
Targets of PPTase activity in fungi, showcased by activities of the PPTase NpgA from *A. nidulans*.

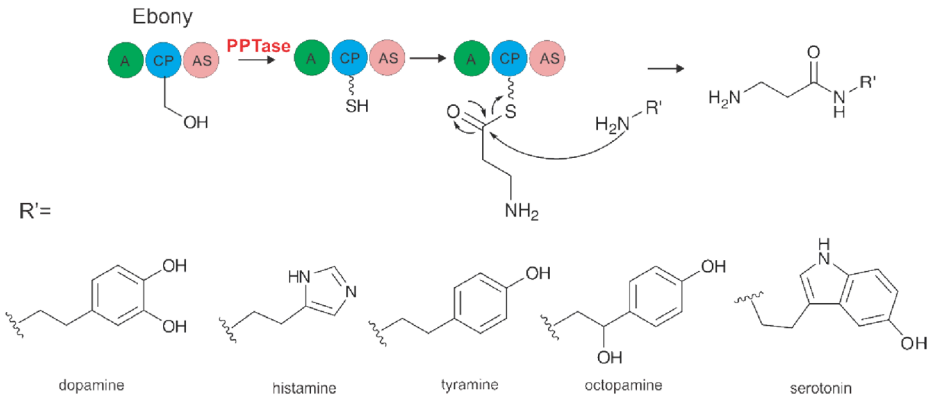

Fig. 15.

Ebony from *Drosophila* resembles an NRPS and catalyzes the formation of β-alanine-amines, like β-alanine-dopamine. Also other biological amines are used to produce β-alanine-amines. AS represents an amine-selecting domain; A, adenylation domain; and CP a carrier protein.

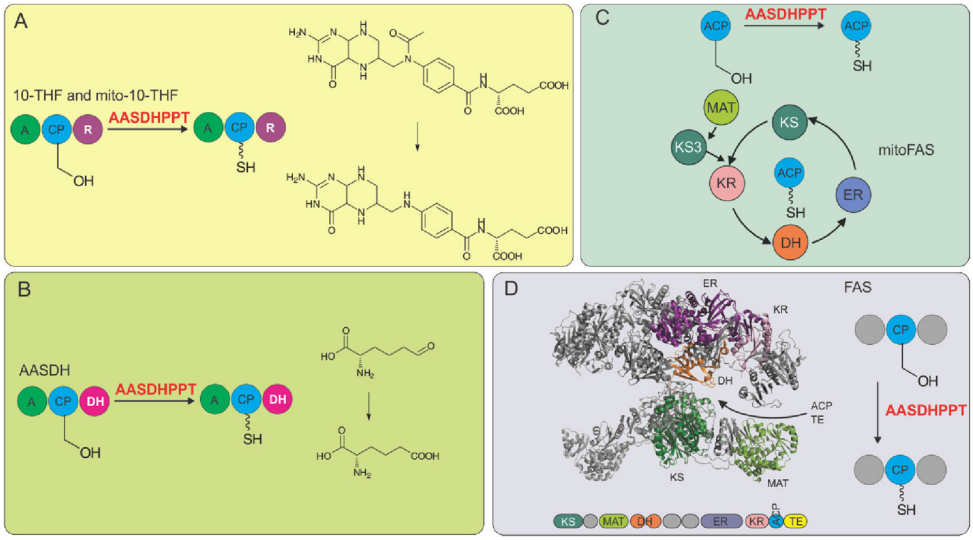

Fig. 16.

Activities of human PPTase AASDHPPT. A) 10-THF involved in 10-fTHF metabolism, B) AASDH involved in catabolism of lysine, C) mitochondrial FAS and D) cytosolic FAS. DH represents a dehydrogenase; A, an adenyltransferase; R, a reductase; ACP, acyl carrier protein; MAT, malonyl-CoA acyltransferase; KS3, ketoacyl synthase III; KR, ketoreductase; DH, dehydratase; ER, enoyl reductase; KS, ketoacyl synthase; and TE, thioesterase.

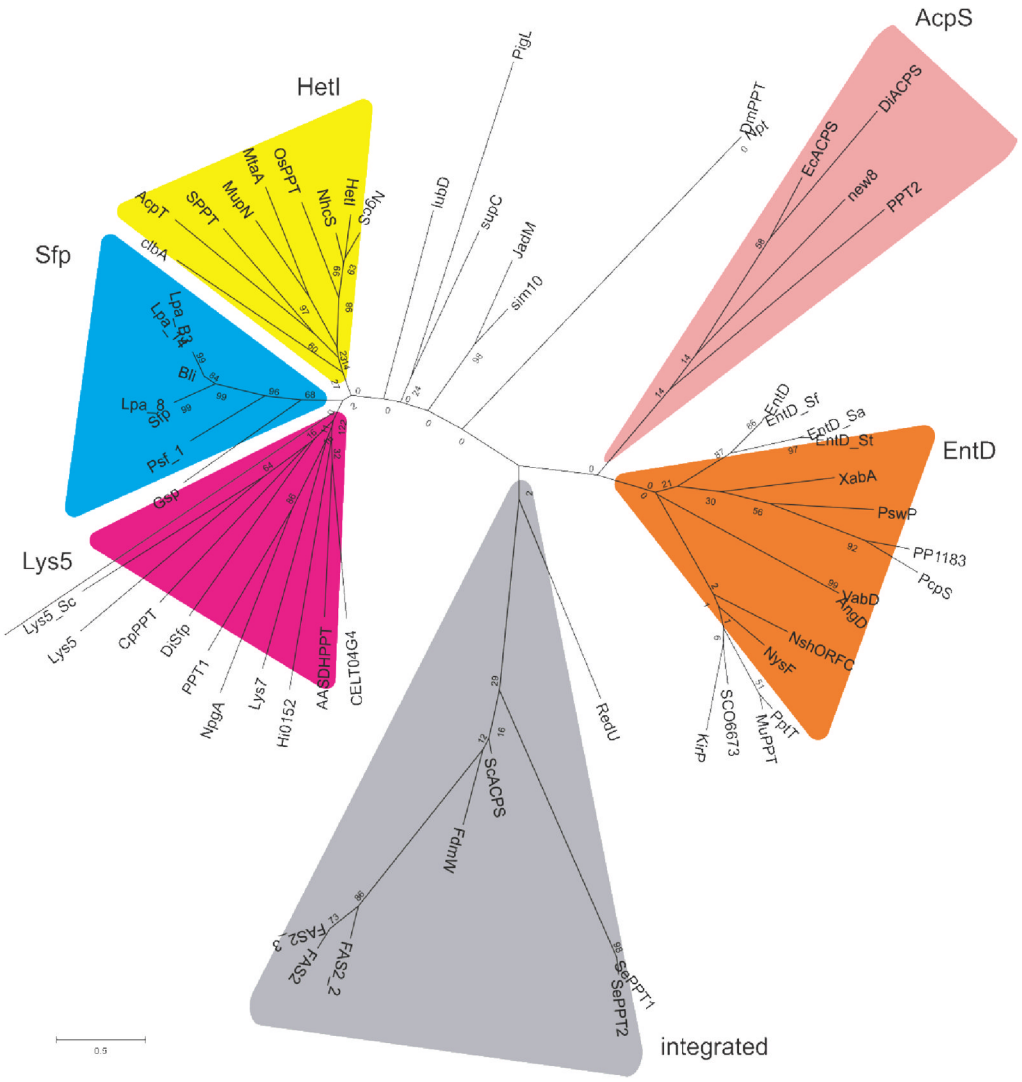

Fig. 17.

Neighbour Joining Method phylogenetic tree of annotated ~60 PPTases (see Table 1), constructed using MEGA.[256]

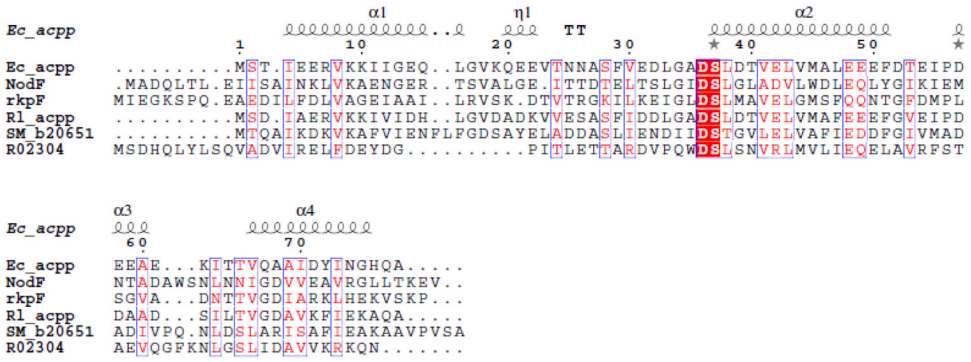

Fig. 18.

Sequence alignment of several carrier proteins from *Rhizobia*. Ec_acpp is *E. coli* ACP, NodF a CP involved in lipochitin nodulation factor biosynthesis, rkpF a CP involved in capsular polysaccharide biosynthesis, R1_acpp the AcpP of *R. leguminosarum*, SM_b20651 and R02304 two other CPs present in *R. leguminosarum*.

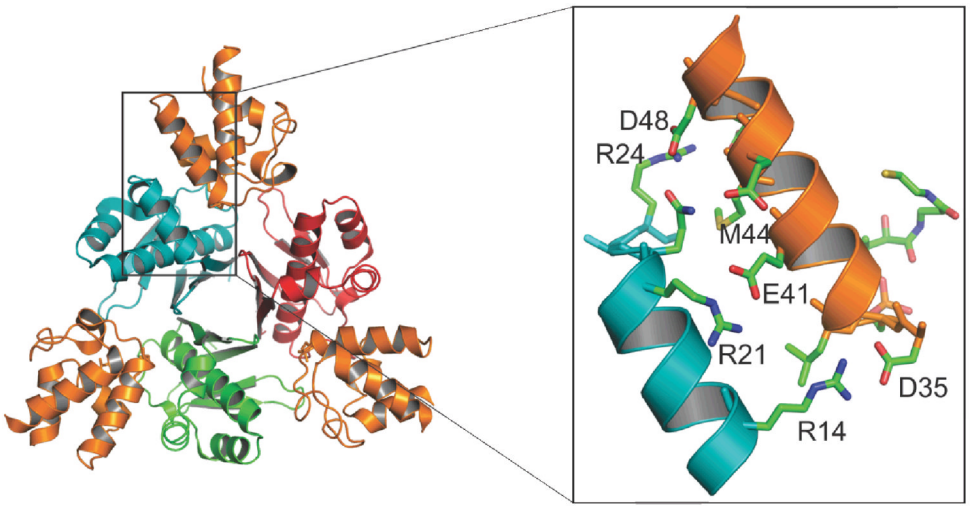

Fig. 19.

Left: AcpS-ACP co-crystal structure, showing the *B. subtilis* AcpS trimer and three *B. subtilis* ACPs (in orange) binding to the PPTase. (PDB:1F80). *Right*: zoom in on the interaction between helix I of AcpS and helix III of ACP. Crucial amino acids are labeled.

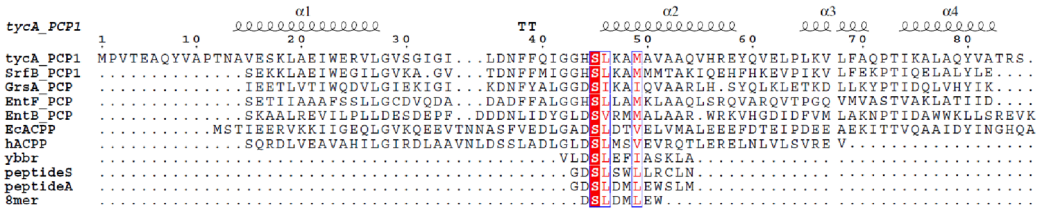

Fig. 20.

Sequence alignment of carrier proteins and peptide mimics. TycA_PCP is the PCP1 from tyrocidine synthase TycA, SrfB_PCP1 is the PCP1 from surfactin synthase SrfB, GrsA_PCP is the PCP from Gramicidin synthase GrsA, EntF and EntB are the PCPs from enterobactin synthase, EcACPP is *E. coli* AcpP, hACPP is the excised human AcpP; YbbR, peptideS, peptideA and 8mer are the short peptides found to be substrates of PPTases.[257, 276, 278]

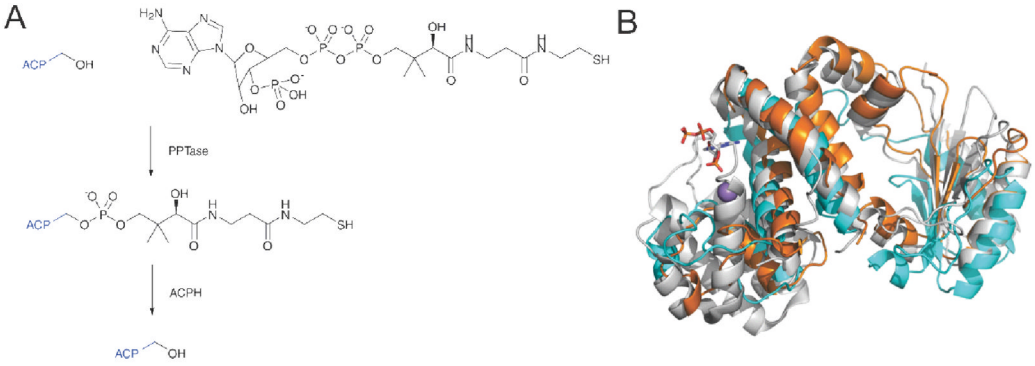

Fig. 21.

AcpH, ACP hydrolase or ACP phosphodiesterase. A) Reaction catalyzed by AcpH. B) Model of *E. coli* and *P. aeruginosa* AcpH (modeled after SpoT).[290] SpoT (PDB: 1VJ7) in grey, EcAcpH in orange and PaAcpH in orange. The natural substrate of SpoT, ppGpp, is shown in sticks and the Mn^{2+} ion as a purple sphere.

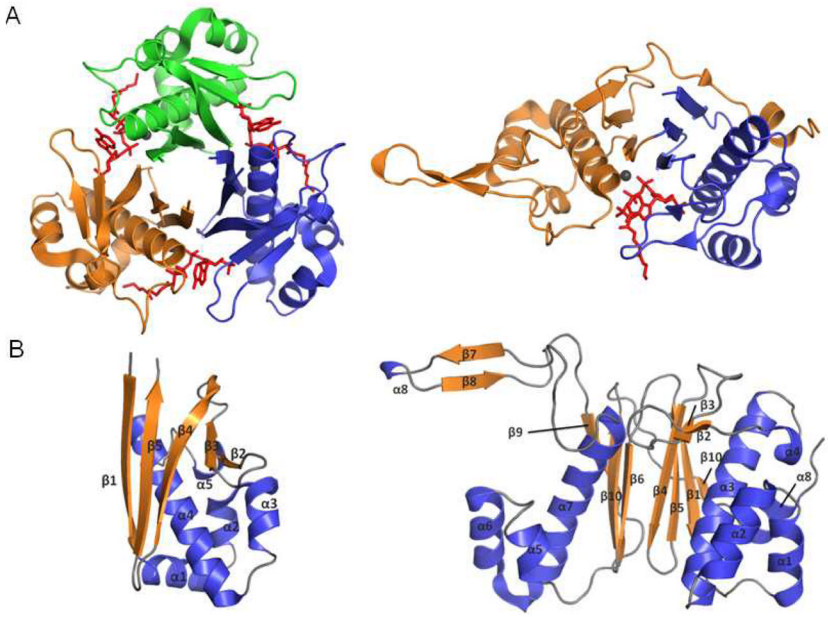

Fig. 22.

Comparison of *B. subtilis* AcpS and Sfp. (A) Top view of both enzymes. CoA is colored red, and divalent cations are shown as black spheres. Each trimer of AcpS is colored differently to show arrangement of monomers, and each "pseudodimer" of Sfp half is colored differently for comparison to AcpS. (B) Assignment of secondary structure to both AcpS and Sfp. The α-helices are shown in in blue, and β-sheets shown in orange.)

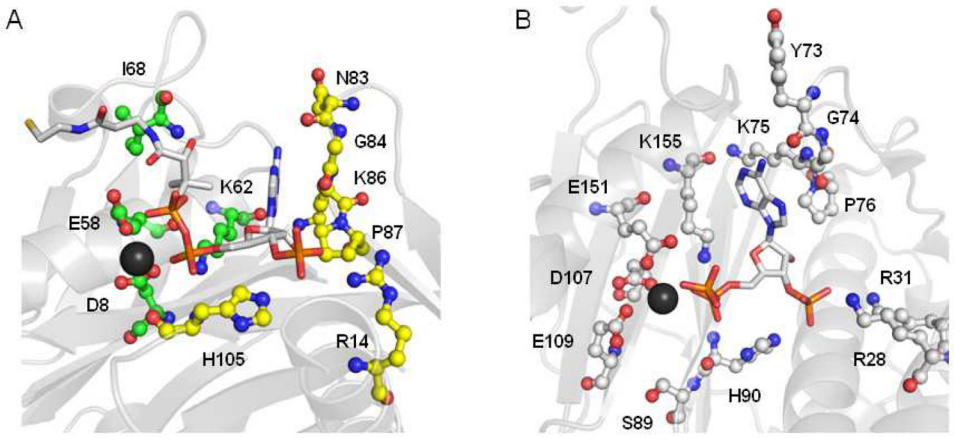

Fig. 23.

Comparison of the active site of *B. subtilis* AcpS and Sfp. CoA is shown in stick form, divalent cations are shown as black spheres, and important residues are shown in ball-and-stick form. (A). Residues on different monomers are colored green and yellow.(B) The PPant moiety of CoA was removed for clarity.

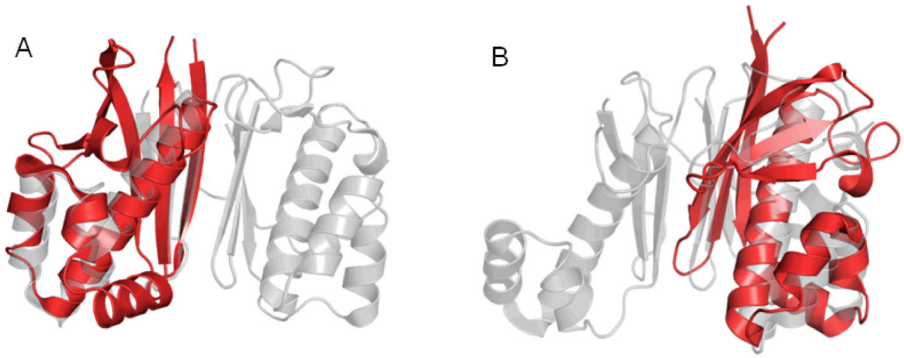

Fig. 24.

Overlay of a monomer of AcpS onto the structure of Sfp, exhibiting the similarity of the pseudodimer to the interface between two AcpS monomers. The “arm” in the C-terminal portion of Sfp has been omitted for clarity. (A) Overlay onto the C-terminal portion of Sfp. Strong secondary structure conservation is observed. (B) While overall shape is conserved, less similarity is observed between AcpS and the N-terminal portion of Sfp.

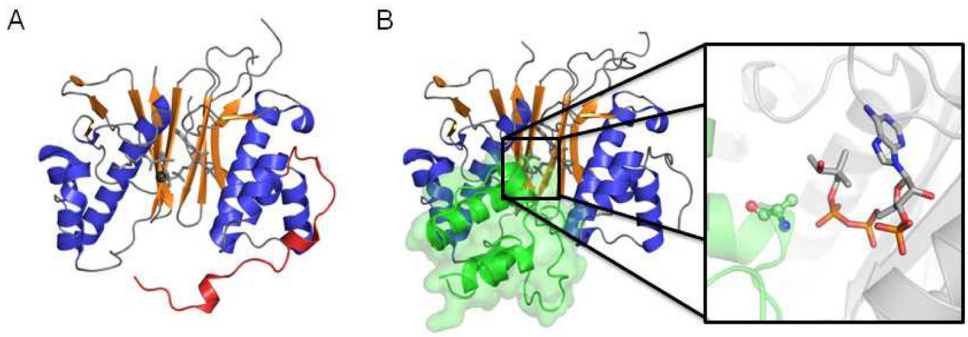

Fig. 25.

Cartoon representation of AASDHPPT. (A) The C-terminal “tail” present in ASDPPT but not in Sfp is depicted in red, with CoA in grey and Mg^{2+} in Black. (B) Human FASI ACP-AASDHPPT co-crystal structure. A Ser to Ala mutation was performed on the ACP to prevent 4′-phosphopantetheinylation. The Ala residue is positioned closed to the phosphate that is attacked by the native Ser. Density for CoA was only observed up to two carbons past the diphosphate.

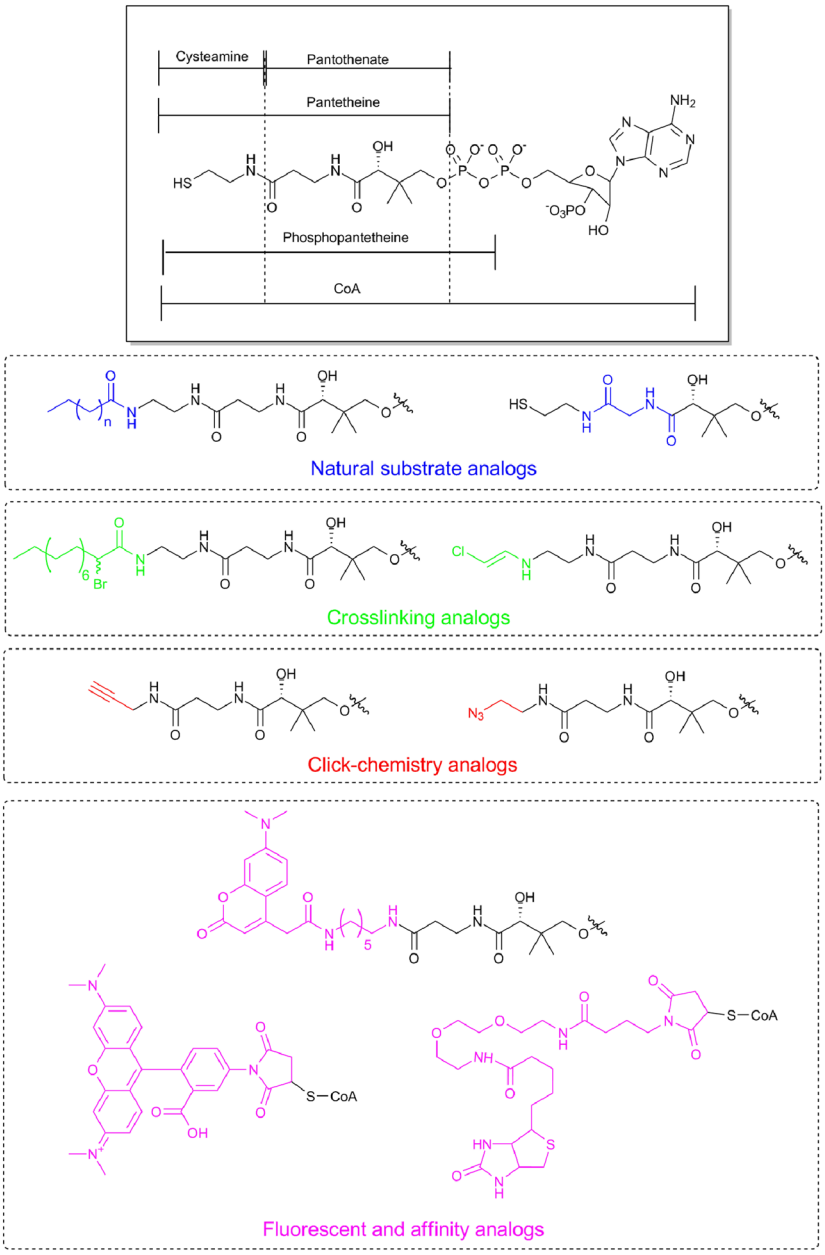

Fig. 26.

CoA Analogs: CoA can be broken down into several parts, including phosphopantetheine, pantetheine, pantothenate, and cysteamine. Sfp and other Sfp-type PPTases can utilize CoA analogs that bear modified pantetheine arms

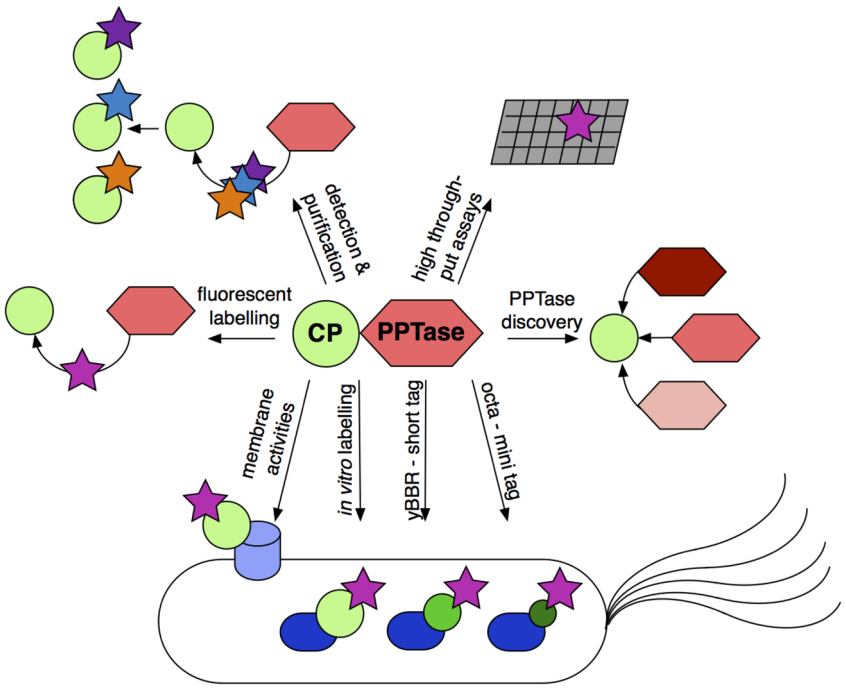

Fig. 27.
Scheme demonstrating the diversity of biotechnological applications of the carrier protein-PPTase interaction.

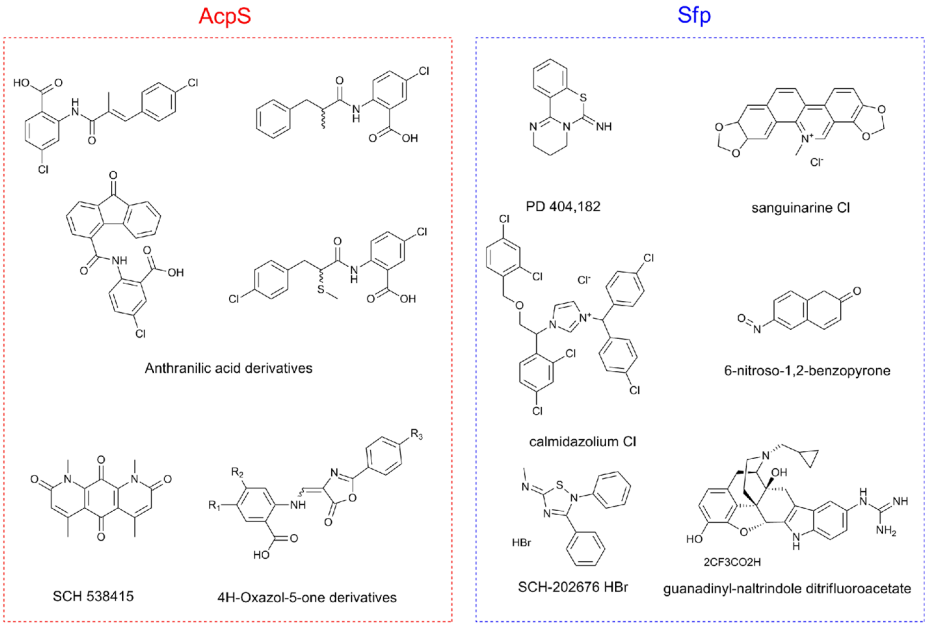

Fig. 28.
Inhibitors of AcpS-type and Sfp-type PPTases. [295, 306, 371, 372]

Table 1

Functionally described PPTases

PPTase	Protein accession no.	Sectio	Phylum	Species	Function/specificity	Citation
CELTO4G9	NP_508153	Animal		*Caenorhabditis elegans*		1
DmPPT	NP_729788	Animal		*Drosophila melanogaster*	FAS/Ebony	24
AASHDPPT	NP_056238.2	*Homo sapiens*		*Homo sapiens*	FAS/mitoFAS/AASDH/THF	25, 26
Gsp	CAA53988	Bacteria	Firmicutes	*Bacillus brevis*	Gramicidin	13
Psf-1	P55810	Bacteria	Firmicutes	*Bacillus pumilus*	Surfactin	1, 27
Sfp, Lpa-8	P39135, BAA09125	Bacteria	Firmicutes	*Bacillus subtilis*	Surfactin, plipastatin B1	28, 29
Lpa-14, Lpa-B3	2113333A, P39144	Bacteria	Firmicutes	*Bacillus subtilis*	Iturin A, surfactin, fengycin	1, 3031
Bli	AAO74604	Bacteria	Firmicutes	*Bacillus. licheniformis*	Bacitracin	1, 32–34
AcpS	P24224	Bacteria	Enterobacteriaceae	*Escherichia coli*	FAS	1
EntD	P19925	Bacteria	Enterobacteriaceae	*Escherichia coli*	Enterobactin	
AcpT, o195	NP_290041	Bacteria	Enterobacteriaceae	*Escherichia coli*		35
ClbA		Bacteria	Enterobacteriaceae	*Escherichia coli*	Colobactin	
HIO152	P43954	Bacteria	Pasteurellales	*Haemophilus influenzae*	FAS	1
PptT	NP_217310	Bacteria	Actinomycetales	*Mycobacterium tubercolosis*	Secondary metabolism	36–38
MuPpt,	YP_906028	Bacteria	Actinomycetales	*Mycobacterium ulcerans*	Mycolactone	
Npt	ABI83656	Bacteria	Actinomycetales	*Nocardia sp. NRRL 5654*		39
PP1183	AAN66807	Bacteria	Pseudomonadales	*Pseudomona putida KT2440*		40
PcpS	BAK88897	Bacteria	Pseudomonadales	*Pseudomonas aeruginosa PAO1*	FAS, siderophore metabolism	40–42
MupN	AAM12928	Bacteria	Pseudomonadales	*Pseudomonas fluorescens*	Mupirocin	43
SePPT1	Q6T710	Bacteria	Actinomycetales	*Saccharopolyspora erythraea*	Erythromycin	44
SePPT2	A4FC68	Bacteria	Actinomycetales	*Saccharopolyspora erythraea*	Erythromycin	44
PigL	Q5W260	Bacteria	Enterobacteriaceae	*Serratia marcescens*	Althiomycin (NRPS-PKS)	45
PswP	Q75PZ2	Bacteria	Enterobacteriaceae	*Serratia marcescens*	Althiomycin (NRPS-PKS)	45
MtaA	AAF19809	Bacteria	Myxococcales	*Stigmatella aurantiaca*	Myxothiazol	46, 47
NshC	Nsh-ORFC	Bacteria	Streptomycetaceae	*Streptomyces actuosus*	Nosiheptide	1
EntD-type	Q53636	Bacteria	Enterobacteriaceae	*Salmonella austin*	Enterobactin	1
Sim10	Q93FA6	Bacteria	Streptomycetaceae	Streptomyces antibioticus	Simocyclinone	48, 49

PPTase	Protein accession no.	Sectio	Phylum	Species	Function/specificity	Citation
ScAcpS	O86785	Bacteria	Streptomycetaceae	*Streptomyces coelicolor*	PKS and FAS	50
RedU	NP_630004	Bacteria	Streptomycetaceae	*Streptomyces coelicolor*	PKS and FAS	51
SCO6673	NP_630748	Bacteria	Streptomycetaceae	*Streptomyces coelicolor*	PKS and FAS	50
KirP	CAN89630	Bacteria	Streptomycetaceae	*Streptomyces collinus*	Kirromycin	52
EntD-type	P0A3C0	Bacteria	Enterobacteriaceae	*Shigella flexneri*	Enterobactin	1
FdmW	AAQ08936	Bacteria	Streptomycetaceae	*Streptomyces griseus*	Fredericamycin	53
NysF	Q9L4X7	Bacteria	Streptomycetaceae	*Streptomyces noursei*	Nystatin	54
EntD-type	Q56064	Bacteria	Streptomycetaceae	*Salmonella typhimurium*	Enterobactin	1
JadM	AAF34678	Bacteria	Streptomycetaceae	*Streptomyces venezuelae*	Jadomycin	55
Svp	AAG43513	Bacteria	Streptomycetaceae	*Streptomyces verticillus*	Bleomycin	56
VabD	ABG82032	Bacteria	Vibrionales	*Vibrio anguillarum*	Vanchrobactin (NRPS)	57, 58
AngD	YP_004566698	Bacteria	Vibrionales	*Vibrio anguillarum*	Anguibactin	58
XabA	AAG28384	Bacteria	Xanthomonadales	*Xanthomonas albilineans*	Albicidin	59
NsPPT, PptNs, NhcS, HetI	AAY42632, AAW67221	Cyanobacteria		*Nodularia spumigena NSOR10*	Glycolipid, nodularin	14, 60
NgcS	YP_001863782	Cyanobacteria		*Nostoc punctiforme PCC73102/ATCC29133*	Glycolipid	61
HetI	AAA22003, BAB77058	Cyanobacteria		*Anabaena (Nostoc) sp. PCC7120*	Glycolipid	61–64
OsPPT	ZP_07109281	Cyanobacteria		*Oscillatoria PCC6506*	Anatoxin-a, Homoanatoxin-a	65
SppT, HetI	BAA10326, NP_442256	Cyanobacteria		*Synechocystis sp. PCC 6803*	FAS	66
NpgA, CfwA	XP_663744	Fungi	Ascomycota	*Aspergillus nidulans*	NRPS, PKS, lysine	67–69
PptA	CAK46165	Fungi	Ascomycota	*Aspergillus niger*	PKS and NRPS	70
PptA	AY607103	Fungi	Ascomycota	*Aspergillus fumigatus*	PKS and NRPS	71, 72
PptB	XP_746591	Fungi	Ascomycota	*Aspergillus fumigatus*	mitoFAS	73
Lys5	AAO26020	Fungi	Ascomycota	*Candida albicans*	Lysine	74
Ppt1	AER36018	Fungi	Ascomycota	*Cochliobolus sativus*	Lysine, NRPS, PKS	75
Ppt1	DQ028305	Fungi	Ascomycota	*Colletotrichum Graminicola*	Lysine, NRPS, PKS	
Ppt1, FfPpt1	HE614113	Fungi	Ascomycota	*Fusarium fujikuroi*	Lysine, NRPS, PKS	76
Integrated PPTases		Fungi	Ascomycota	*Part of PKS type I or FAS type I*	FAS	20
PptasePchr, Pc13g04050	XP_002558841	Fungi	Ascomycota	*Penicillium chrysogenum*	Lysine, NRPS, PKS	77
Lys5	CAA96866	Fungi	Ascomycota	*Saccharomyces cerevisiae*	Lysine	1, 78, 79
PPT2	Q12036	Fungi	Ascomycota	*Saccharomyces cerevisiae*	mitoFAS	80

PPTase	Protein accession no.	Sectio	Phylum	Species	Function/specificity	Citation
New8	G2TRL9	Fungi	Ascomycota	*Schizosaccharomyces pombe*	mitoFAS (putatively)	81
1314154/Lys7	Q10474	Fungi	Ascomycota	*Schizosaccharomyces pombe*	Lysine	1, 82
Ppt1	EHK16960	Fungi	Ascomycota	*Trichoderma virens*	Lysine, NRPS, PKS	83
CpPPT	AAW50594	Protista	Apicomplexa	*Cryptosporidium parvum*	FAS	84
DiAcpS	EAL69712	Protista	Mycetozoa	*Dictyostelium discoideum*		85
DiSfp	EAL64498	Protista	Mycetozoa	*Dictyostelium discoideum*		85
SupC	A4U8R1	Bacteria		*Aplysina aerophoba* symbiont	PKS	86, 87
LubD	F8S277	Bacteria		*Aplysina aerophoba* symbiont	NRPS	87, 88

Table 2

Overview of the *E. coli* synthases and PPTases. Phylogenetically *E. coli* is divided into several reference groups.[117] Using the nucleotide sequence of the PPTases and complete synthases, we used multigeneblast[118] to identify the presence of these genes in *E. coli* genomes ("Present in"). The designation "+++, ++ and +" correspond to decreasing activity of PPTase on synthase, whereas '−' corresponds to no activity and '?' to unknown.

Synthase		**PPTases**			
		AcpS	**EntD**	**AcpT**	**ClbA**
	Present in	**A, B1, B2, C, D, E, S**	**A, B1, B2, C, D, E, S**	**A, B1, B2, C, D, E, S**	**B2**
FAS	A, B1, B2, C, D, E, S	+++[3]	+[1]	+[1]	?
Enterobactin	A, B1, B2, C, D, E, S	−	+++[97]	?	++[115]
Yersiniabactin	B1, B2, D (A,E)	−	+[115]	?	++[115]
Colibactin	B2	−	−	?	+++[115]
O157	D,E	−	?	+[35]	?

Table 3

Overview of kinetic parameters for different PPTases and different carrier protein substrates.

PPTase	Target *apo*-CP	k_{cat}/K_m (μM^{-1} min^{-1})	Ref
EcAcpS	*E. coli* ACP	10–50	1, 11
EcAcpS	Gra ACP	6	11
EcAcpS	Fren ACP	1.6	11
EcAcpS	Tcm ACP	0.25	11
EcAcpS	Otc ACP	0.26	11
Sfp	*B. subtilis* SrfB1	80	28
Sfp	*B. subtilis* SrfB2	31	28
Sfp	*E. coli* EntB	4	28
Sfp	*S. cerevisiae* Lys2-PCP	>14	28
Sfp	*E. coli* ACP	1	28
PaPcpS	*P. aeruginosa* ACP (1.6–12.5 μM)	32.5	41
PaPcpS	*P. aeruginosa* ACP (23–234 μM)	2.6	41
PaPcpS	*B. subtilis* ACP (2.2–25 μM)	8.6	41
PaPcpS	*B. subtilis* ACP (25–206 μM)	2.9	41
PaPcpS	TycC3 PCP (0.9–12 μM)	0.5	41
PaPcpS	TycC3 PCP (25–150 μm)	0.04	41
PaPcpS	pchE ArCP (1–10 μM)	1.1	41
PaPcpS	pchE ArCP (21–155 μM)	0.13	41
EcAcpS	*B. subtilis* ACP (25–206μM)	1.8	41
EcAcpS	*B. subtilis* ACP (2.2–25 μM)	110	41
Sfp	*B. subtilis* ACP (25–206 μM)	1.2	41
Sfp	*B. subtilis* ACP (2.2–25 μM)	0.3	41
Sfp	TycC3 PCP (25–150 μM)	21.6	41
AASDHPPT	*H. sapiens* AcpP	3.6	25
AASDHPPT	*H. sapiens* mit ACP	1.0	25
AASDHPPT	*B. brevis* PCP	0.6	25
AASDHPPT	*B. subtilis* ACP	0.05	25
Lys5	Lys2-PCP	3	79
Svp	BlmI	2.8	56
Svp	Tcmm	28	56
Sfp	BlmI	1.9	56
Sfp	Tcmm	0.08	56
FdmW	FdmH	8.1	53
FdmW	Tcmm	0.6	53
Svp	FdmH	0.4	53
Svp	Tcmm	7.6	53
Sfp	YbbR13	0.091	257

PPTase	Target *apo*-CP	k_{cat}/K_m (μM^{-1} min^{-1})	Ref
Sfp	A1	0.00049	[257]
EcAcpS	YbbR13	0.0033	[257]
EcAcpS	A1	0.015	[257]
EcAcpS	*E. coli* ACP	50	[258]
EcAcpS	NodF	0.05	[258]
EcAcpS	Hybrid *E. coli* ACP-NodF	0.01	[258]

Table 4

List of currently solved structures

Organism	Type	Ligands	Publication Year	PDB code	Ref
M. tuberculosis	AcpS	*Apo*, 3′,5′ ADP	2009,2011	3NE1, 3H7Q, 3NE3, 4HC6	151
B. antracis	AcpS	2x 3′,5′ ADP	2012	3HYK	294
V. cholerae	AcpS	CoA	2012	3QMN	294
M. smegmatis	AcpS	*Apo*	To be published	3GWM	
S. aureus	AcpS	*Apo*, ACP	2012	4DXE	294
C. ammoniagenes	AcpS	*Apo*, CoA	2011	3NE9, 3NFD	151
B. subtilis	AcpS	*Apo*, CoA, ACP, Inhibitors (not deposited)	2000, 2005(not depositied)	1F7T, 1F7L, 1F80	271, 295
S. pneumoniae	AcpS	*Apo*, 3′,5′ ADP	2000	1FTE, 1FTF, 1FTH	296
S. coelicolor	AcpS	*Apo*, CoA, Acetyl-CoA, H110A/CoA, D111A/CoA	2011	2JCA, 2JBZ, 2WDO, 2WDS, 2WDY	126
P. yoelii	AcpS	3′,5′ ADP	To be published	2QG8	
B. subtilis	Sfp	CoA	1999	1QR0	297
H. sapiens	Sfp	*Apo*, CoA, hACP/CoA	2007	2YBD, 2C43, 2CG5	260
S. cerevisiae	Part of type I FAS	*Apo*, CoA	2009	2WAS, 2WAT	22