Distribution and functional analysis of the phosphopantetheinyl transferase superfamily in *Actinomycetales* microorganisms

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Phosphopantetheinyl transferases (PPTases) are a superfamily of essential enzymes required for the synthetic processes of many compounds including fatty acid, polyketide, and nonribosomal peptide metabolites. These enzymes activate carrier proteins in specific biosynthetic pathways via the transfer of a phosphopantetheinyl moiety to a serine residue in the conserved motif of carrier proteins. Since many *Actinomycetales* microorganisms produce a number of polyketide and nonribosomal peptide metabolites, the distribution of PPTase genes was investigated in these microorganisms. PPTases were found in bacterial protein databases using a hidden Markov model search with the PF01648 (4′-phosphopantetheinyl transferase superfamily) model. *Actinomycetales* microorganisms harbor several genes encoding AcpS-type and Sfp-type PPTases in individual genomes, many of which were associated with the biosynthetic gene cluster for polyketide or nonribosomal peptide metabolites. The properties of these PPTases were evaluated in the heterologous expression system using the biosynthetic gene clusters and genes encoding PPTases found in the present study. Sfp-type PPTases were classified into two subgroups, and although the substrate specificities of the enzymes in one subgroup were wide, the catalytic activities of enzymes in the other subgroup were low. SAV_1784 of *Streptomyces avermitilis* possessed the most characteristic broad-range activity against several type I polyketide synthases and nonribosomal peptide synthetases.

phosphopantetheinyl transferase | posttranslational modification | type I polyketide synthases | nonribosomal peptide synthetases | heterologous expression

Microbial natural products have various structures and biological functions. Some of these products are used as clinically important drugs including antibacterial, antifungal, antitumor, antiviral, antinematode, and immunosuppressive agents (1). Macrocyclic polyketide compounds (macrolides or macrocyclic lactones) synthesized by type I (modular) polyketide synthases (PKSs) and peptide compounds synthesized by nonribosomal peptide synthetases (NRPSs) have diverse structures and various biological activities (2). The polyketide compounds erythromycin, amphotericin B, and avermectin are used in the treatment of bacterial, fungal, and nematode infectious diseases, respectively, while the peptide compounds β-lactam compound (cephamycin C) and actinomycin D are used as antibacterial and antitumor agents, respectively.

Phosphopantetheinyl transferases (PPTases) catalyze posttranslational modifications to carrier proteins in fatty acid synthases (FASs), PKSs, and NRPSs (3). Carrier proteins are either integrated into these synthases (type I) or are discrete subunits (type II) and, depending on the nature of the attached intermediate, are called acyl carrier proteins (ACPs) or peptidyl carrier proteins (PCPs), respectively (4–8). Each biosynthetic pathway may encode several carrier proteins, the number of which typically correlates with the length of the final product. Phosphopantetheinylation by PPTases occurs by the transfer of the 4′-phosphopantetheine prosthetic group from CoA to a conserved serine residue in the carrier proteins, converting the proteins from their inactive "apo" forms to their active "holo" forms (9). The 4′-phosphopantetheine arm on holo form carrier proteins has two functions for the elongation of the acyl or peptidyl chain on FAS, PKS, or NRPS. The free thiol group of the phosphopantetheine acts as a nucleophile for a covalent connection by a nucleophilic reaction for the intermediates to form an acyl or peptidyl thioester. The length and flexibility of this moiety assists in the relocation of intermediates between spatially distinct modules of the complex (10, 11).

PPTases have been extensively classified into three structural groups, which correlate with their substrate specificities and lengths (9, 12). The first group is "AcpS-type" PPTases. These PPTases are ∼115 amino acids in size, function as homotrimers, and are found in most microorganisms for specific modifications to discrete carrier proteins as an essential component of fatty acid synthesis (13–15). Some of these enzymes activate a wide range of substrates in vitro (carrier proteins of type I and II PKSs) (16–18). The second group is "Sfp-type" PPTases, which

Significance

***Actinomycetales* microorganisms are a rich source of secondary metabolites, and their genomes contain many biosynthetic gene clusters for metabolites including polyketide and peptide compounds synthesized by type I polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs). Genes encoding Sfp-type phosphopantetheinyl transferase (PPTase), which modifies the "apo" form to "holo" form carrier protein on type I PKSs and NRPSs, were widely distributed in *Actinomycetales* microorganisms, which is similar to the distribution of biosynthetic gene clusters for polyketide and peptide compounds synthesized by type I PKSs and NRPSs, respectively. Some actinomycete PPTases exhibited characteristic broad-range activities against several type I PKSs and NRPSs. These PPTases will be useful for the coexpression of several biosynthetic gene clusters including type I PKSs and NRPSs.**

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are classified by the prototype PPTase from *Bacillus subtilis*, Sfp. This PPTase is required for the activation of carrier proteins incorporated within the biosynthetic pathway that are responsible for the production of surfactin (11). These PPTases are monomeric (19) and approximately twice the size of AcpS-type enzymes (~230 amino acids), which suggests that they evolved by gene duplication from an AcpS ancestor (12). This family includes PPTase involved in cyanobacterial heterocyst differentiation (20). Sfp-type PPTases are considered to be optimized for carrier proteins of secondary metabolism, and carrier proteins on the multifunctional enzymes, type-I PKS and NRPS, are mainly activated by these PPTases (21). The third type of PPTase is incorporated as a domain at the C terminus of the α-subunit of fungal FASs, at which it catalyzes the in *cis* autophosphopantetheinylation of the carrier protein at the N terminus of the same polypeptide (22). PPTases integrated into the fungal PKS have been reported (23), but the reports of the integrated-type PPTases from bacterial origins were quite few. Type I PKSs carrying the PPTase domain at the C-terminal region had been found in *Saccharopolyspora erythraea* NRRL 2338 (SACE_2343) (24) and *Streptomyces avermitilis* MA-4680 (SAV_7361) (25); however, the products synthesized by these type I PKSs have not been identified.

Microorganisms possess multiple PPTases, such as AcpS-type and Sfp-type PPTases. The *B. subtilis* genome encoded AcpS and Sfp. The genome of *Escherichia coli* had three PPTases: AcpS (for FAS), EntD (for the synthesis of NRPS–siderophore enterobactin; ref. 26), and YhhU (uncharacterized PPTase; refs. 9 and 27). These enzymes act independently in distinct pathways and display contrasting specificities for carrier proteins. Sfp-type PPTases are proposed to have arisen via gene duplication (enzyme sizes, ~230 amino acids) and subsequent divergence from ancestral AcpS-like PPTases. Despite the absolute requirement for PPTases in a wide range of important and fundamental biosynthetic pathways, these PPTases have remained elusive due to their low sequence identities and lack of proximity to their respective biosynthetic clusters. *Actinomycetales* microorganisms are known as producers of a number of secondary metabolites, including polyketide and peptide compounds. Since the synthetic process of these polyketide and peptide compounds is involved in type I PKSs or NRPSs, the producing microorganisms will harbor specific PPTases for carrier proteins on type I PKSs or NRPSs.

Several AcpS-type PPTases have been characterized by the enzymatic conversion of the apo form to the holo form of carrier proteins in vitro (7, 12, 16, 18), and, in many cases, the characteristics of Sfp-type PPTases were also obtained by an in vitro reaction using an artificial small polypeptide containing a carrier protein domain from type I PKSs or NRPSs (11, 12, 24). The experimental characteristics of Sfp-type PPTases using native type I PKSs and NRPSs were not reported to date. We examined the characteristics of four Sfp-type PPTases of the industrial producer *S. avermitilis* MA-4680 using deletion mutants of these genes and a heterologous expression system based on *S. avermitilis* with foreign biosynthetic gene clusters for polyketide and peptide metabolites that are synthesized by type I PKSs or type NRPSs, respectively. (Phosphopantetheinylation was evaluated by the production of polyketide and peptide metabolites in vivo). We also examined the characteristics of exogenous actinomycete Sfp-type PPTases using the heterologous expression system.

Results

Distribution of Genes Encoding PPTases in *Actinomycetales* Microorganisms. Seventeen *Streptomyces* genomes and nine related *Actinomycetales* microorganism genomes were analyzed in the search for PPTases by a hidden Markov model search using PF01648 (4′-phosphopantetheinyl transferase superfamily). Most of the *Actinomycetales* genomes contained at least one pair of genes encoding AcpS-type and Sfp-type PPTases. *S. flavogriseus* ATCC 33331 and *Streptomyces* sp. SirexAA-E also had a pair of AcpS-type and Sfp-type PPTases (*SI Appendix*, Table S1). However, other actinomycete genomes contained several AcpS-type and Sfp-type PPTase genes. PPTases in *S. venezuelae* ATCC 10712 and *S. collinus* Tü365 were widely distributed, and the microorganisms harbored at least 8 and 11 Sfp-type PPTases, respectively. A phylogenetic analysis of amino acid sequence alignment revealed two major groups as AcpS-type and Sfp-type PPTases (Fig. 1). Sfp-type PPTases were further classified into two subgroups indicated in the Sfp-type PPTases of *Cyanobacteria* and *Proteobacteria* (28). Sfp-type PPTases from *Actinomycetales* microorganisms also belong to these two groups, which have the conserved motifs [ILV]G[ILV]D[AILV][EQ]…33~41-aa…WxxKEAxxK (WxxKEAxxK subtype) and [ILV]G[ILV]D[AILV]E…33~46-aa…FxxKESx[FY]K (FxxKESxxK subtype), respectively (*SI Appendix*, Fig. S1 *A* and *B*, *II*). The alignment of the amino acid sequences of FxxKESxxK subtype PPTases indicated that these PPTases have more conserved motifs upstream of the two conserved motifs described above. Two other conserved motifs are Rx[AC]AxxA..17~18-aa..Px[FW]Pxxxx[AG][AS]x[ST]H (*SI Appendix*, Fig. S1 *B*, *I*). The second motifs were found in *Actinomycetales* microorganisms, *E. coli* K-12 substr. MG1655, and *Pseudomonas putida* KT 2440 (also *P. aeruginosa* PAO1), whereas the corresponding PPTases were not found in *Cyanobacteria*. WxxKEAxxK subtype PPTases were more widely distributed, and the total number of this subtype of PPTases was approximately twice that of FxxKESxxK subtype PPTases (Fig. 1). Although PPTases incorporated as a domain at the C terminus of type I PKS had only been reported in erythromycin-producing *Sa. erythraea* NRRL 2338 and avermectin-producing *S. avermitilis* MA-4680 (24), an additional six PPTases were found as a domain at the C terminus of undefined type I PKSs from *Actinosynnema mirum* DSM 43827 (Amir_2494), *Amycolatopsis mediterranei* U32 (AMED_4605), *S. violaceusniger* Tü 4113 (Strvi_6300), *S. collinus* Tü 365 (B446_06810), *S. olivichromogenes* (accession no. WP_079065395), and *S. mirabilis* (accession no. WP_075031111). The products

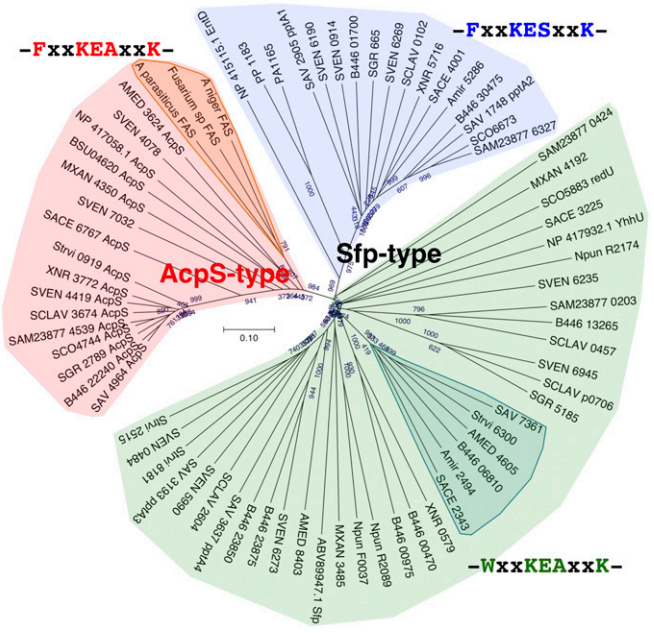

Fig. 1. Phylogenetic analysis of presumptive PPTases from bacterial databases. AcpS-type, FxxKESxxK subtype, and WxxKEAxxK subtype enzymes in Sfp-type PPTases are indicated in orange, blue, and green zones, respectively. PPTases incorporated as a domain at the C-terminal region of fungal fatty acid synthases and of actinomycete type I polyketide synthases are in deep orange and green zones, respectively. The distribution of PPTases in eight actinomycete microorganisms is indicated as individual colors. Locus tags or accession numbers are described in *Materials and Methods*. SCO5883 and SCO6673 of *S. coelicolor* A3 (2) were quite similar to SLIV_09190 (98% identity and 99% similarity) and SLIV_05145 (99% identity and 99% similarity) of *S. lividans* TK24, respectively.

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generated by these type I PKSs were unidentified; however, this type of PPTase incorporated into type I PKS may be widely distributed in *Actinomycetales* microorganisms. PPTases incorporated as a domain at the C terminus of fungal FASs were classified as AcpS-type PPTases, whereas PPTases on the C terminus region of the type I PKSs of *Actinomycetales* microorganisms were classified as Sfp-type PPTases (Fig. 1).

Characteristics of Four Sfp-Type PPTases in *S. avermitilis*. The anthelmintic macrocyclic lactone, avermectin, producer *S. avermitilis* (25) harbors one AcpS-type, four Sfp-type, and one PKS integrated-type PPTases (*SI Appendix*, Table S1). The four Sfp-type PPTases were classified into the above FxxKESxxK subtype (SAV_1748, PptA2 and SAV_2905, PptA1) and WxxKEAxxK subtype (SAV_3193, PptA3 and SAV_3637, PptA4), respectively. Three genes encoding PptA1, PptA3, and PptA4 were located in the region flanking the gene cluster including type I PKS (oligomycin) and NRPS genes (nrps1 and nrps2; the products were not identified by these NRPSs), respectively (avermitilis.ls.kitasato-u.ac.jp/gview/). The three genes, *pptA1*, *pptA2*, and *pptA4*, form an operon with a gene encoding phosphoesterase (possibly 3′,5′-ADP phosphatase; the reaction with the apo-carrier protein and CoA by PPTase generates a holo-carrier protein and 3′,5′-ADP. This phosphoesterase will catalyze the generation of 5′-AMP from 3′,5′-ADP). *S. avermitilis* produces three lactone-type polyketide compounds: filipins, avermectins, and oligomycin, which are synthesized by type I PKSs (29). Our initial experiments indicated that the phosphopantetheinyl transfer of apo-carrier proteins on filipin and avermectin PKSs was controlled by PptA1 and PptA2 because the single-deletion mutants, *pptA1* and *pptA2*, produced filipins and avermectins, whereas double-deletion mutants completely failed to produce either of these polyketide compounds (*SI Appendix*, Fig. S2).

Genetically engineered *S. avermitilis* SUKA series are suitable for the heterologous expression of biosynthetic gene clusters for polyketides and peptide compounds that are synthesized by type I PKSs and NRPSs (29–31). Phosphopantetheinylation by the four PPTases of *S. avermitilis* were examined in the production of actinomycin X_2 (NRPS), cephamycin C (NRPS), chloramphenicol (NRPS), indigoidine (NRPS), lactacystin (NRPS/PKS hybrid), pikromycin (type I PKS), and nemadectin (type I PKS). We constructed series of *pptA*-deletion mutants (single, double, triple, and quadruple deletions) in genetically engineered *S. avermitilis* SUKA38 carrying the hygromycin B resistance gene (*hph* from *S. hygroscopicus* ATCC 27438). All exogenous biosynthetic gene clusters were expressed on the linear plasmid SAP1 of *S. avermitilis*. Since the SAP1 plasmid possesses the transfer function, it was easy to introduce into each deletion mutant by simple mating between SUKA37 carrying the erythromycin resistance gene (*ermE* of *Sa. erythraea* NRRL 2338)/SAP1 vector containing the biosynthetic gene cluster and SUKA38 carrying hygromycin B resistance gene (*SI Appendix*, Fig. S3).

The production of the deep blue pigment, indigoidine, was completely terminated in the mutants lacking *pptA2* (Fig. 2, *IV*; Δ2, Δ1Δ2, Δ2Δ3, Δ2Δ4, Δ1Δ2Δ3, Δ1Δ2Δ4, Δ2Δ3Δ4, and Δ1Δ2Δ3Δ4). The production of actinomycin X_2 and chloramphenicol was markedly reduced in *pptA2*-deletion mutants, and the productivities of both compounds were completely terminated in mutants lacking the *pptA1* and *pptA2* genes (Fig. 2, *I*; Δ1Δ2, Δ1Δ2Δ3, Δ1Δ2Δ4, and Δ1Δ2Δ3Δ4). Another NRPS compound, cephamycin C, was normally produced in the single-deletion mutants of *pptA1* or *pptA2*, whereas production was completely terminated in double-deletion mutants of *pptA1* and *pptA2* (Fig. 2, *II*; Δ1Δ2, Δ1Δ2Δ3, Δ1Δ2Δ4, and Δ1Δ2Δ3Δ4). The productivities of the NRPS/PKS hybrid compound, lactacystin, and macrocyclic lactone compounds, pikromycin and nemadectin, were not affected in the single-deletion mutants, but were completely terminated in the mutants lacking *pptA1* and *pptA2* (Fig. 2, *V*–*VII*; Δ1Δ2, Δ1Δ2Δ3, Δ1Δ2Δ4, and Δ1Δ2Δ3Δ4). Thus, PptA1 and PptA2 both have the ability to phosphopantetheinylate several exogenous type I PKSs and NRPSs. PptA2 appears to be preferable

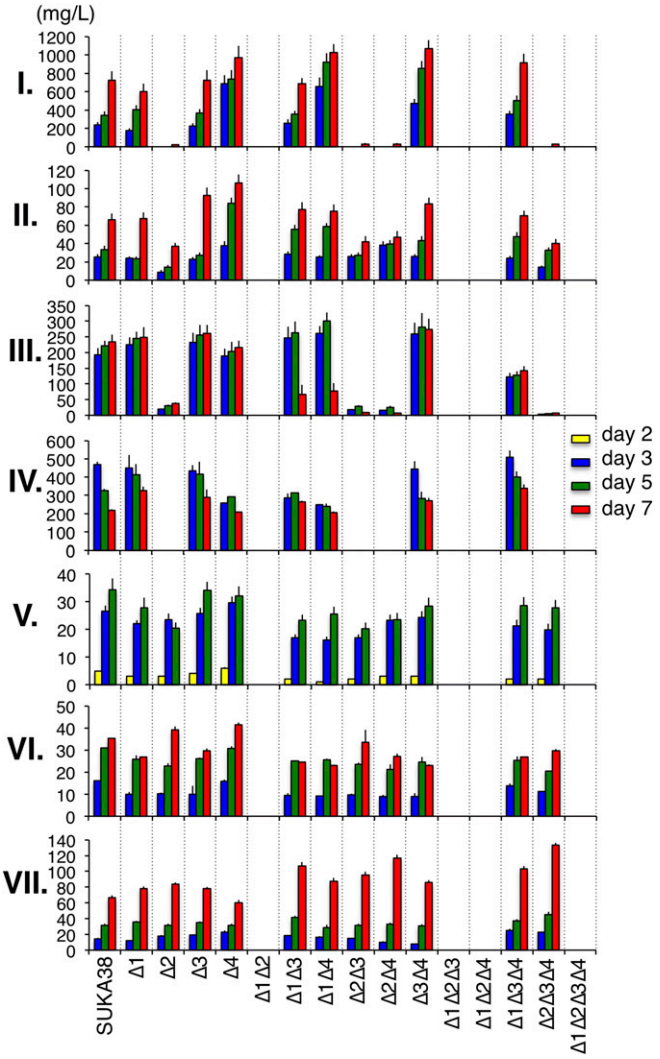

Fig. 2. Productivities of polyketide and peptide metabolites [(*I*) actinomycin X_2, (*II*) cephamycin C, (*III*) chloramphenicol, (*IV*) indigoidine, (*V*) lactacystin, (*VI*) pikromycin, and (*VII*) nemadectin] in various *pptA*-deletion mutants of *S. avermitilis*. Each metabolite was examined in engineered *S. avermitilis* SUKA38, Δ1 (*ΔpptA1*), Δ2 (*ΔpptA2*), Δ3 (*ΔpptA3*), Δ4 (*ΔpptA4*), Δ1Δ2 (*ΔpptA1 ΔpptA2*), Δ1Δ3 (*ΔpptA1 ΔpptA3*), Δ1Δ4 (*ΔpptA1 ΔpptA4*), Δ2Δ3 (*ΔpptA2 ΔpptA3*), Δ2Δ4 (*ΔpptA2 ΔpptA4*), Δ3Δ4 (*ΔpptA3 ΔpptA4*), Δ1Δ2Δ3 (*ΔpptA1 ΔpptA2 ΔpptA3*), Δ1Δ2Δ4 (*ΔpptA1 ΔpptA2 ΔpptA4*), Δ1Δ3Δ4 (*ΔpptA1 ΔpptA3 ΔpptA4*), Δ2Δ3Δ4 (*ΔpptA2 ΔpptA3 ΔpptA4*), and Δ1Δ2Δ3Δ4 (*ΔpptA1 ΔpptA2 ΔpptA3 ΔpptA4*) carrying the SAP1 vector containing the entire biosynthetic gene cluster. Productivity was calculated by at least three independent datasets, and vertical bars indicate SDs.

for modifying NRPSs later than type I PKSs. No metabolites were detected in mutants lacking at least two genes, *pptA1* and *pptA2*. The quadruple *pptA*-deletion mutants carrying the biosynthetic gene cluster for chloramphenicol-deleted *cmlL* (*cmlL* encodes a Sfp-type PPTase) did not produce any antimicrobial substances (*SI Appendix*, Fig. S4; the mutants carrying the entire biosynthetic gene cluster produced chloramphenicol). The mutants accumulated a compound with *m/z* 165.0673 (*SI Appendix*, Fig. S5) that was identical to 2-amino-3-(4-aminophenyl)propanal by an authentic sample prepared from *p*-aminophenylalanine by organic synthesis. The NRPS, CmlP, for chloramphenicol biosynthesis consists of adenylation, PCP, and reductase domains (32). An aldehyde product may be generated from an important intermediate, *p*-aminophenylalanine, by the reduction of the carboxylic acid residue.

Since compounds synthesized through aminoglycoside biosynthesis, ribosomal peptide synthesis, or the type II or III PKS pathway do not require phosphopantetheinylation by Sfp-type PPTases, the production of these compounds will not be affected in mutants lacking all genes encoding Sfp-type PPTases. The production of streptomycin (aminoglycoside; *SI Appendix*, Fig. S6), asukamycin (synthesized by type II PKS; *SI Appendix*, Fig. S7), phenolic lipids (synthesized by type III PKS; *SI Appendix*, Fig. S8), and thiostrepton (RiPPs: ribosomally synthesized and posttranslationally modified peptides; *SI Appendix*, Fig. S9) was not affected in quadruple *pptA*-deletion mutants because these biosynthetic processes do not require posttranslational modifications by Sfp-type PPTases. Discrete apo-carrier proteins in type II and III PKS systems would be modified by AcpS-type PPTase such as FAS system.

Evaluation of PPTases in *Actinomycetales* Microorganisms by a Heterologous Expression System Using Quadruple *pptA*-Deletion Mutants of *S. avermitilis*. The quadruple *pptA*-deletion mutants of *S. avermitilis* lack all genes encoding Sfp-type PPTases. Therefore, they are suitable for the evaluation of exogenous PPTase in a combination of quadruple *pptA*-deletion mutants and biosynthetic gene clusters for polyketide and peptide compounds synthesized by type I PKSs and NRPSs. Sfp of *B. subtilis* subsp. *subtilis* str. NCIB 3610 (accession no. ABV89947) was used for phosphopantetheinylation in the *E. coli* expression system, and its GC content of coding region is 46.67 GC mol%. A synthetic gene (62.96 GC mol%) encoding Sfp of *B. subtilis* subsp. *subtilis* NCIB 3610, the nucleotide sequence of which was optimized for *S. avermitilis* codon usage, was prepared (accession no. LC341265) for efficient translation in *S. avermitilis* and used as a positive control of the exogenous *pptA* gene. The expression level of each gene encoding exogenous PPTase by a native promoter was unknown. The expression levels of extrinsic PPTase gene were compared using its own promoter and a promoter optimized for expression in the production phase of *S. avermitilis*, using quadruple *pptA*-deletion mutants of *S. avermitilis* SUKA38 carrying the SAP1 vector containing entire biosynthetic gene clusters for cephamycin C and chloramphenicol (*SI Appendix*, Fig. S10). The productivities of the peptide compounds, cephamycin C and chloramphenicol, in quadruple *pptA*-deletion mutants were efficient under the control of the *pptA* gene by an alternative promoter, $P_{sav2794}$ (a promoter of the gene encoding secreted metalloprotease, SAV_2794; ref. 33). Chloramphenicol productivity under the expression of the gene encoding SGR_665 (*S. griseus* IFO 13350) or SCLAV_0102 (*S. clavuligerus* ATCC 27064) was enhanced more by $P_{sav2794}$ than the promoter of each *pptA* gene (*SI Appendix*, Fig. S10). Since the expression of the exogenous *pptA* gene using its own promoter was not suitable in *S. avermitilis*, all exogenous *pptA* genes were controlled under the optimized promoter $P_{sav2794}$. Exogenous PPTases (two subtypes of Sfp-type PPTases) of *Actinomycetales* microorganisms were chosen from peptide compound (generated by NRPS) producer *S. clavuligerus* ATCC 27064 (cephamycin C) and *S. venezuelae* ATCC 10712 (chloramphenicol), polyketide compound (generated by type I PKS) producer *Sa. erythraea* NRRL 2338 (erythromycin), and peptide/polyketide compounds (NRPS or type I PKS) nonproducer *S. griseus* IFO 13350 (streptomycin), *S. lividans* TK24 and *S. albus* J1074. Two PPTases from *Pseudomonas aeruginosa* PAO1 and *P. putida* KT 2440 were used as *Proteobacteria* PPTases.

The production of actinomycin X_2 in quadruple *pptA*-deletion mutants of *S. avermitilis* SUKA38 was fully restored by SAV_1748 (PptA2), SLIV_05145, XNR_5716, SCLAV_0102, SCLAV_2604, SVEN_0484, SGR_665, and Sfp. SAV_3193 (PptA3) did not have the ability to modify actinomycin NRPSs in the series of *pptA*-deletion mutants of *S. avermitilis*, whereas SAV_3637 (PptA4) had the ability to modify actinomycin NRPSs under the control of the $P_{sav2794}$ promoter. Furthermore, PPTases from both *Pseudomonas* strains exhibited the ability to modify actinomycin NRPSs (Fig. 3, *I*). Cephamycin C productivity was restored in quadruple-deletion

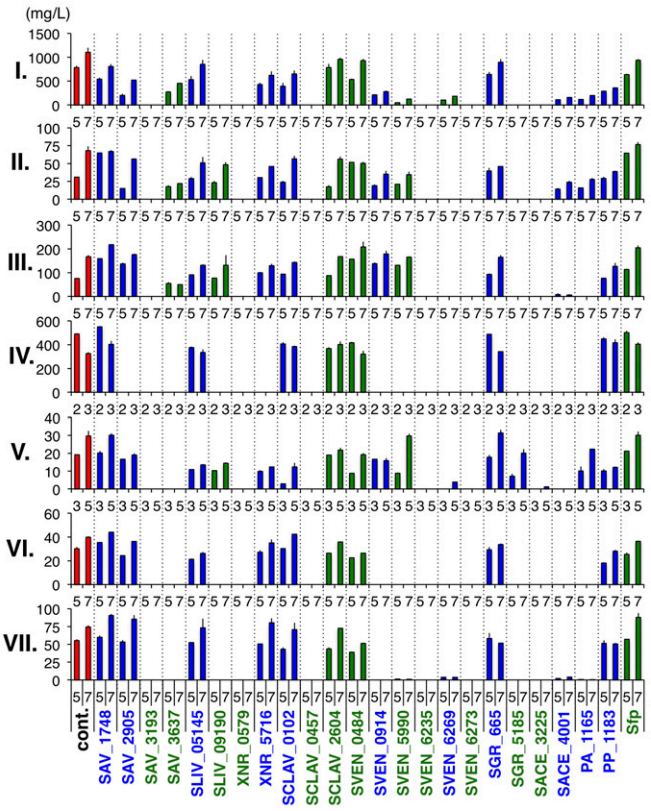

Fig. 3. Restoration of productivities of polyketide and peptide compounds synthesized by type I PKSs and/or NRPSs in *S. avermitilis* SUKA38 quadruple *pptA*-deletion mutants carrying the SAP1 vector containing the biosynthetic gene cluster by the introduction of the *pptA* gene controlled by an alternative promoter. Productivity was calculated by at least three independent datasets at 5 and 7 d incubation [indigoidine (*IV*), 2 and 3 d; lactacystin (*V*), 3 and 5 d], and vertical bars indicate SDs. *S. avermitilis* SUKA38 carrying the SAP1 vector containing the biosynthetic gene cluster was used as the control (cont.). PPTases written in blue and green are classified into FxxxKESxxK subtype and WxxxKEAxxK subtype PPTases, respectively. The quantitative analysis of metabolites (*I*, actinomycin X_2; *II*, cephamycin C; *III*, chloramphenicol; *IV*, indigoidine; *V*, lactacystin; *VI*, pikromycin; and *VII*, nemadectin) in the culture was described in *SI Appendix, SI Materials and Methods*.

mutants by SAV_2905 (PptA1), SAV_1748 (PptA2), SLIV_05145, SLIV_09190, XNR_5716, SACE_4001, SCLAV_0102, SCLAV_2604, SVEN_0914, SVEN_5990, SVEN_0484, SGR_665, and Sfp. PptA4 in *S. avermitilis* also had the ability to modify AcvS (cephamycin C NRPS; Fig. 3, *II*). Three genes encoding SLIV_09190, SVEN_0914, and SVEN_0484 were located in the biosynthetic gene clusters for undecylprodigiosin, chloramphenicol, and an unknown NRPS-PKS hybrid, respectively. Both *Pseudomonas* PPTases also modified AcvS (Fig. 3, *II*). The production of chloramphenicol in the quadruple-deletion mutants was fully restored by SAV_2905 (PptA1), SAV_1748 (PptA2), SCLAV_0102, SCLAV_2604, SVEN_0914, SVEN_5990, SVEN_0484, SGR_655, and Sfp. Chloramphenicol productivity was partially restored by SLIV_05145, SLIV_09190, and XNR_5716 (Fig. 3, *III*). The restoration of chloramphenicol production in quadruple *pptA*-deletion mutants was not completed by PptA3. A gene encoding SVEN_0914 was located in the gene cluster for the chloramphenicol biosynthesis of *S. venezuelae* ATCC 10740; however, SVEN_0484 and SVEN_5990 efficiently modified chloramphenicol NRPS (CmlP). The PPTase of *P. putida* KT 2440 modified CmlP, whereas that of *P. aeruginosa* PAO1 did not (Fig. 3, *III*). Indigoidine was synthesized by one NRPS enzyme, LbpA. Indigoidine productivity in quadruple-deletion mutants was restored by SAV_1748 (PptA2), SLIV_05145,

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XNR_5716, SCLAV_0102, SCLAV_2604, SVEN_0484, SGR_665, and Sfp. The *Pseudomonas* PPTase, PP_1183 restored indigoidine productivity, whereas PA_1165 did not (Fig. 3, *IV*). The total number of PPTases that modified indigoidine NRPS was less than that for modifications to other NRPSs.

Lactacystin was generated by type I PKS and NRPS, a process in which three carrier proteins (two in type I PKS and one in NRPS) were phosphopantetheinylated by Sfp-type PPTase. The productivity of lactacystin in quadruple *pptA*-deletion mutants was fully restored by SAV_1748 (PptA2), SVEN_5990, SGR_665, and Sfp. Lactacystin productivity was partially restored by SAV_2905 (PptA1), SLIV_05145, SLVI_09190, XNR_5716, SCLAV_0102, SCLAV_2604, SVEN_6269, SVEN_0914, SVEN_0484, and SGR_5185. Two *Pseudomonas* Sfp-type PPTases also restored lactacystin production (Fig. 3, *V*). The structure of the antibacterial 14-membered macrolide pikromycin is similar to that of erythromycin. Pikromycin productivity in quadruple-deletion mutants was restored by SAV_2905 (PptA1), SAV_1748 (PptA2), XNR_5716, SCLAV_0102, SCLAV_2604, SVEN_0484, SGR_665, PP_1183, and Sfp (Fig. 3, *VI*). Neither PPTases of erythromycin-producing *Sa. erythraea* NRRL 2338 had the ability to convert the apo form to the holo form of pikromycin PKSs. *Sa. erythraea* NRRL 2338 possesses two genes encoding discrete Sfp-type PPTases, and previous studies reported that SACE_4001 plays a role in modifications to erythromycin apo form PKSs (24). The productivity of nemadectin in quadruple *pptA*-deletion mutants was restored by SAV_2905 (PptA1), SAV_1748 (PptA2), SLIV_05145, XNR_5716, SCLAV_0102, SCLAV_2604, SVEN_0484, SGR_665, PP_1183, and Sfp. Restoration by SACE_4001, SVEN_6269, SVEN_5990, and SGR_5185 was weak. Although SACE_4001 has the ability to modify erythromycin apo form type I PKSs, SACE_4001 does not recognize other types of I PKSs (pikromcyin and nemadectin) exhibiting similar macrocyclic lactone biosynthesis as the substrates (Fig. 3, *VII*).

Discussion

Several Sfp-type PPTases were found not only in *Cyanobacteria* and *Proteobacteria*, but also in *Actinomycetales* microorganisms. Furthermore, the Sfp-type PPTases of *Actinomycetales* microorganisms were classified into two consensus groups: the WxxKEAxxK and FxxKESxxK subtypes. These PPTases have two conserved motifs [ILV]G[ILV]D[AILV][EQ] and WxxKEAxxK or FxxKESx[FY]K, which have been reported in PPTases in *Eubacteria* (28). These findings suggest that cyanobacterial and actinomycete PPTases have both evolved from the same ancestor. The existence of the two subtype groups described above in Sfp-type PPTases indicates that the evolution of FxxKESxxK subtype PPTases differs from that of WxxKEAxxK subtype PPTases. A phylogenetic analysis of aligned sequences revealed that PPTase domains on the C terminus of fungal FASs were classified into the clade of AcpS-type PPTases, whereas the domains on the C terminus of type I PKSs in *Actinomycetales* microorganisms were classified into the clade of Sfp-type PPTases. These results indicate that AcpS-type PPTases coevolved with FASs and Sfp-type PPTases with type I PKSs. Many *Actinomycetales* microorganisms produce a number of secondary metabolites including polyketide and peptide compounds synthesized by type I PKSs and NRPSs, respectively. Sfp-type PPTases were widely and diversely distributed in their genomes. Thus, most *Actinomycetales* microorganisms harbor one AcpS-type and at least two Sfp-type PPTases (WxxKEAxxK subtype and FxxKESxxK subtype PPTases).

A functional analysis of four Sfp-type PPTases in engineered *S. avermitilis* using a combination of *pptA*-deletion mutants and exogenous biosynthetic gene clusters revealed that two out of four genes were mainly expressed, and these two gene products were used for posttranslational modifications to this microorganism. Furthermore, two PPTases, SAV_1748 (PptA2) and SAV_2905 (PptA1), exhibited the ability to phosphopantetheinylate not only type I PKSs involved in the synthesis of endogenous metabolites (filipins, avermectins, and oligomycin), but also type I PKSs and NRPSs involved in the biosynthesis of exogenous polyketide and peptide compounds, respectively. SAV_1748 (PptA2) modifies type I PKSs and NRPSs. However, although SAV_2905 (PptA1; the gene located on the left side of the biosynthetic gene cluster for oligomycin) may also modify type I PKSs and NRPSs, modifications to type I PKSs were preferred. Genes encoding Sfp-type PPTases, except for SAV_1748 (PptA2), are located on the side flanking the biosynthetic gene cluster for type I PKS or NRPS. The results from multiple *pptA*-deletion mutants of *S. avermitilis* SUKA38 carrying entire biosynthetic gene cluster (Fig. 2) showed that two PPTases, SAV_3193 and SAV_3637, did not possess the ability to modify the apo form of type I PKSs or NRPSs; however, when a gene encoding SAV_3637 was expressed by the alternative promoter, $P_{sav2794}$, the production of actinomycin X_2, cephamycin C, and chloramphenicol was observed, but not in SAV_3193 (Fig. 3). These results indicated that a gene encoding SAV_3637 was in a cryptic state in *S. avermitilis*, and SAV_3193 did not possess the ability to modify the type I PKSs or NRPSs examined.

Acyl or peptidyl chain elongation during polyketide or peptide synthesis is accomplished on the phosphopantetheinyl moiety of holo form carrier proteins (ACP or PCP) on type I PKSs or NRPSs. Accordingly, deletions of all genes encoding Sfp-type PPTases in the genome resulted in the failed production of these metabolites and/or their intermediates because the acyl or peptidyl chain elongation reaction was not processed. No metabolites or their intermediates were detected in the culture extract of the quadruple-deletion mutants carrying the entire biosynthetic gene cluster for actinomycin X_2, indigoidine, cephamycin C, lactacystin, pikromycin, or nemadectin. However, transconjugants carrying the biosynthetic gene cluster for chloramphenicol produced a shunt product, 2-amino-3-(4-aminophenyl)propanal. In quardruple *pptA*-deletion mutants, an important intermediate, *p*-aminophenylalanine, may be converted to the adenylated derivative on the apo form CmlP. A carbonyl group of the ester on the adenylated amino acid may be reduced by the reduction domain at the C terminus of apo form CmlP.

Sfp-type PPTases were distributed more in *Actinomycetales* microorganisms than in those of *Eubacteria*. Since *Actinomycetales* microorganisms produce a number of polyketide and peptide compounds synthesized by type I PKSs and NRPSs, respectively, Sfp-type PPTases may also have evolved and been distributed in these microorganisms. Among actinomycete Sfp-type PPTases, catalytic activities against type I PKSs and NRPSs were found for many FxxKESxxK subtype PPTases, but not for some WxxKEAxxK subtype PPTases. Since these PPTases contain a conserved motif in their polypeptides, biosynthesis genes, involving other type I PKS and NRPS genes, should be investigated.

Some biosynthetic gene clusters for polyketide and peptide compounds contained a gene encoding Sfp-type PPTase. Three out of four PPTases in *S. avermitilis* were located in the gene clusters described above. A gene encoding SVEN_0914 (CmlL) was also located in the biosynthetic gene cluster for chloramphenicol. The activity of SVEN_0914 was reconfirmed by heterologous expression using the entire gene cluster and its derivative containing the in-frame deletion of *cmlL* (*SI Appendix*, Fig. S4). The *cmlL*-deletion mutants of chloramphenicol-producing *S. venezuelae* ATCC 10712 still produced chloramphenicol, but at lower productivity (32). Other PPTases of *S. venezuelae*, SVEN_0484 and SVEN_5990 (JadM; ref. 34), exhibited the catalytic activity to modify CmlP. SVEN_0484 was particularly modified by CmlP rather than SVEN_0914 (CmlL). *cmlL*-deletion mutants of *S. venezuelae* still produced chloramphenicol because the apo form of CmlP was still converted to its holo form by SVEN_0484 and SVEN_5990. The genes encoding SVEN_0484 and SVEN_5990 were located in the unknown NRPS-PKS hybrid cluster and the biosynthetic gene cluster for jadomycin, respectively.

A previous study reported that the AcpS-type PPTase (SCO4744) of *S. coelicolor* A3 (2) appeared to be extremely promiscuous in its

substrate specificity, accepting protein substrates from type I (Rat) and type II [ACP of *E. coli* and *S. coelicolor* A3 (2)] FASs as well as type I (norsoloric acid synthase) and type II (ACP of oxytetracycline PKS) PKSs (18). However, these ACPs of type I FASs and PKSs were small artificial polypeptides; therefore, it remains unclear whether the enzyme catalyzes modifications to native giant type I FASs or PKSs. AcpS-type PPTase, SAV_4964 (86% identity and 91% similarity to SCO4744), of *S. avermitilis* MA-4680 did not catalyze modifications to carrier proteins on the type I PKSs and NRPSs examined because *S. avermitilis* SUKA38 quadruple *pptA*-deletion mutants carrying the biosynthetic gene cluster including type I PKS or NRPS genes never produced polyketide or peptide metabolites. AcpS-type PPTases may modify the artificial apo form carrier proteins of type I PKSs and NRPSs prepared by a recombinant technique; however, their carrier proteins on native enzymes will not be modified. The carrier proteins on native type I PKSs and NRPSs may be exclusively modified by Sfp-type PPTases. Based on the results obtained for several PPTase activities under this heterologous expression system, not only SAV_1748 (PptA2) but also Sfp (*B. subtilis*) possessed the most characteristic broad-range activity against the several type I PKSs and NRPS examined. Although their modification activities were slightly weaker, SLIV_05145, SCLAV_2604, and SGR_665 also exhibited relatively broad-range activity. The expression of these genes encoding Sfp-type PPTases in the heterologous host may be useful for the preparation of the holo form of several type I PKSs and NRPSs.

Materials and Methods

Locus tags or accession numbers in figures are as follows: A_niger FAS, *Aspergillus niger* phosphopantetheine-protein transferase domain protein (accession no. OWW29013); A_parasiticus FAS, *A. parasiticus* FAS alpha subunit *aflA* (accession no. Q8TGA2); ABV89947.1_Sfp, *B. subtilis* subsp. *subtilis* str. NCIB 3610 (accession no. ABV89947); AMED, *A. mediterranei* U32; Amir, *A. mirum* DSM 43827; B446, *S. collinus* Tü 365; BSU, *B. subtilis* subsp. *subtilis* str. 168; Fusarium_sp FAS, *Fusarium* sp. NRRL 25184 FAS alpha subunit (accession no. AMD39096); MXAN, *Myxococcus xanthus* DK 1622; NP, *E. coli* str. K-12 substr. MG1655; Npun, *Nostoc punctiforme* PCC 73102; PA, *P. aeruginosa* PAO1; PP, *P. putida* KT 2440; SACE, *Sa. erythraea* NRRL 2338; SAM23877, *S. ambofaciens* ATCC 23877; SAV, *S. avermitilis* MA-4680; SCAB, *S. scabiei* 87.22; SCLAV, *S. clavuligerus* ATCC 27064; SCO, *S. coelicolor* A3 (2); SGR, *S. griseus* IFO 13350; Strop, *Salinispora tropica* CNB-440; Strvi, *S. violaceusniger* Tü 4113; SVEN, *S. venezuelae* ATCC 10712; and XNR, *S. albus* J1074.

Bacterial strains, growth conditions, bioinformatics, the construction of *pptA*-deletion mutants, the introduction of biosynthetic gene clusters for secondary metabolites, and analytical conditions for secondary metabolites are described in *SI Appendix, SI Materials and Methods*.

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