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Three Thioesterases Are Involved in the Biosynthesis of Phosphinothricin Tripeptide in *Streptomyces viridochromogenes* Tü494[▽]

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Phosphinothricin tripeptide (PTT) is a peptide antibiotic produced by *Streptomyces viridochromogenes* Tü494, and it is synthesized by nonribosomal peptide synthetases. The PTT biosynthetic gene cluster contains three peptide synthetase genes: *phsA*, *phsB*, and *phsC*. Each of these peptide synthetases comprises only one module. In neither PhsB nor PhsC is a typical C-terminal thioesterase domain present. In contrast, a single thioesterase GXSXG motif has been identified in the N terminus of the first peptide synthetase, PhsA. In addition, two external thioesterase genes, *theA* and *theB*, are located within the PTT biosynthetic gene cluster. To analyze the thioesterase function as well as the assembly of the peptide synthetases within PTT biosynthesis, several mutants were generated and analyzed. A *phsA* deletion mutant (MphsA) was complemented with two different *phsA* constructs that were carrying mutations in the thioesterase motif. In one construct, the thioesterase motif comprising 45 amino acids of *phsA* were deleted. In the second construct, the conserved serine residue of the GXSXG motif was replaced by an alanine. In both cases, the complementation of MphsA did not restore PTT biosynthesis, revealing that the thioesterase motif in the N terminus of PhsA is required for PTT production. In contrast, TheA and TheB might have editing functions, as an interruption of the *theA* and *theB* genes led to reduced PTT production, whereas an overexpression of both genes in the wild type enhanced the PTT yield. The presence of an active single thioesterase motif in the N terminus of PhsA points to a novel mechanism of product release.

The antibiotic phosphinothricin tripeptide (PTT; also called bialaphos) is produced by *Streptomyces viridochromogenes* Tü494 and *Streptomyces hygroscopicus*, respectively (4, 20). It consists of two molecules, L-alanine and the unusual amino acid phosphinothricin (PT) (Fig. 1). The bioactive compound PT shows bactericidal, fungicidal, and herbicidal activity, as it is similar to the structure of glutamic acid and, thus, competitively inhibits glutamine synthetase.

A biosynthetic pathway for bialaphos was postulated previously (44) based on the analysis of blocked mutants. In addition, the biosynthesis of PTT has been investigated by genetic and biochemical means (6, 17, 35, 36, 50). The precursor peptide *N*-acetyl-demethylphosphinothricin (N-Ac-DMPT) tripeptide is synthesized in at least 13 steps from intermediates of the primary metabolism and assembled by nonribosomal peptide synthetases (NRPSs). During or after assembly, N-Ac-DMPT is converted to PT by methylation and deacylation (1, 12, 35, 50). Within the completely sequenced PTT cluster, 24 genes were identified that are involved in the biosynthesis of PTT (5, 36). The PTT biosynthetic gene cluster harbors three genes, *phsA*, *phsB*, and *phsC*, which encode NRPSs (Fig. 2A) (36).

NRPSs are involved in the biosynthesis of many pharmacologically important natural products, such as penicillin and vancomycin (44, 48). These peptides are synthesized via a thiotemplate mechanism (25, 39). NRPSs are characterized by their modular organization. Each module is responsible for the handling of one amino acid. Furthermore, each module is subdivided into various domains. The adenylation domain (A domain) is required for the specific recognition and activation of an amino acid to form an aminoacyladenylate. The activated amino acid is converted into a thioester attached to the 4′-phosphopantetheine (4′PP) arm of the peptide carrier protein (PCP or T domain). The elongation of the peptide chain takes place at the condensation domain (C domain), where the amino acids are covalently bound and transferred to the next module. After the final peptide chain length has been achieved, the thioesterase domain (TE domain), which normally is localized at the C terminus of the last amino acid-activating module, cleaves off the peptide chain from the enzyme complex (7, 43). This type of TE is a member of the so-called type I TEs (TEIs), which show high sequence similarity to the TEs of fatty acid synthases of vertebrates (25).

The modules are classified by their function and arrangement within the peptide synthesis. The first amino acid-activating module (initiation module) consists of only the A and PCP domains. In many bacterial systems, such initiation modules stand alone, whereas later modules are organized as multimodular enzyme units. These multimodular enzymes have a C-A-PCP arrangement and represent the elongation modules. The so-called termination module carries an additional TE domain at the C terminus of the last module (27). The nonribosomal biosynthesis of PTT differs from that of the known bacterial systems. Sequence analysis has revealed that all three

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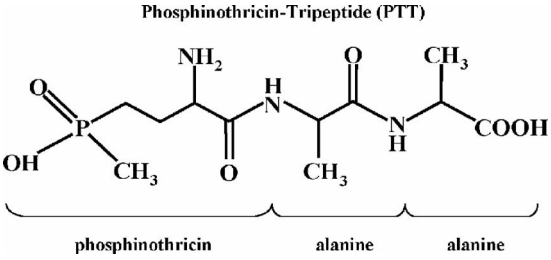

FIG. 1. Chemical structure of PTT and relevant compounds.

peptide synthetase genes encode only one peptide synthetase module. Furthermore, these genes are not juxtaposed in the biosynthetic gene cluster (35, 37). An in silico analysis, as well as biochemical and genetic experiments, proved that PhsA is responsible for the activation of the first amino acid, N-Ac-DMPT, the precursor of PT, whereas PhsB and PhsC each activate one alanine (37). PhsA consists of A and PCP domains; PhsB of PCP, C, and A domains; and PhsC of C, A, and PCP domains (Fig. 3A) (12, 37). Currently, it is not possible to determine which of the two alanylylation steps is catalyzed by PhsB and PhsC. The fact that the two enzymes cannot replace each other suggests a defined positioning for each protein in the PTT assembly line. However, in PTT synthetases, short communication-mediating domains (COM domains) that mediate interactions between peptide synthetases (14, 15) could not be identified (37).

Surprisingly, a typical TE domain at the C terminus of PhsB or PhsC is missing. However, a highly conserved TE GXSXG motif at amino acids (aa) 14 to 18 was localized at the N terminus of PhsA (Fig. 3A and B). Furthermore, the two genes *theA* and *theB* (formerly known as *the1* and *the2* [36]) were identified, and their gene products showed high similarity to type II TEs (TEII). TEIIs are autonomous, monomeric pro-

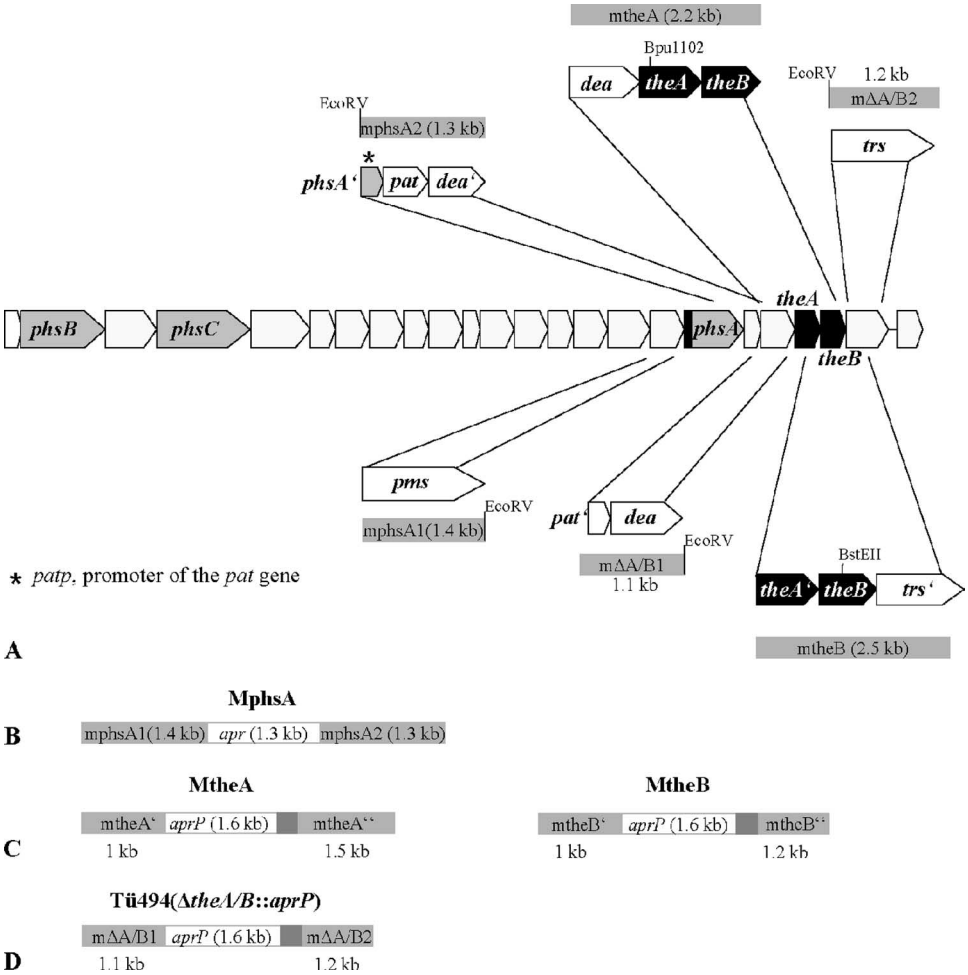

FIG. 2. Localization of the peptide synthetase genes *phsA*, *phsB*, and *phsC* and the TE genes *theA* and *theB* in the PTT biosynthetic gene cluster from *S. viridochromogenes* Tü494. (A) The 5′ end of *phsA* containing the sequence of the TE motif is highlighted. *pat*, phosphinothricin *N*-acetyltransferase gene; *pat′*, part of the *pat* gene; *dea*, deacylase gene; *dea′*, part of the *dea* gene; *trs*, putative transporter gene; *trs′*, part of the *trs* gene; and *phsA′*, part of the *phsA* gene. The PCR fragments mphsA1, mphsA2, mtheA, and mtheB, which were used for the construction of the mutants MphsA, MtheA, and MtheB, are schematically illustrated. (B) Replacement of 1.7 kb of the *phsA* gene with the apramycin cassette (*apr*), resulting in the mutant MphsA. (C) Insertion of the apramycin-*ermEp* cassette (*aprP*) within *theA* and *theB*, respectively, resulting in the mutants MtheA and MtheB. (D) Replacement of the *theA* and *theB* genes with the apramycin-*ermEp* cassette (*aprP*), resulting in the mutant Tü494(Δ*theA*/*theB*::*aprP*).

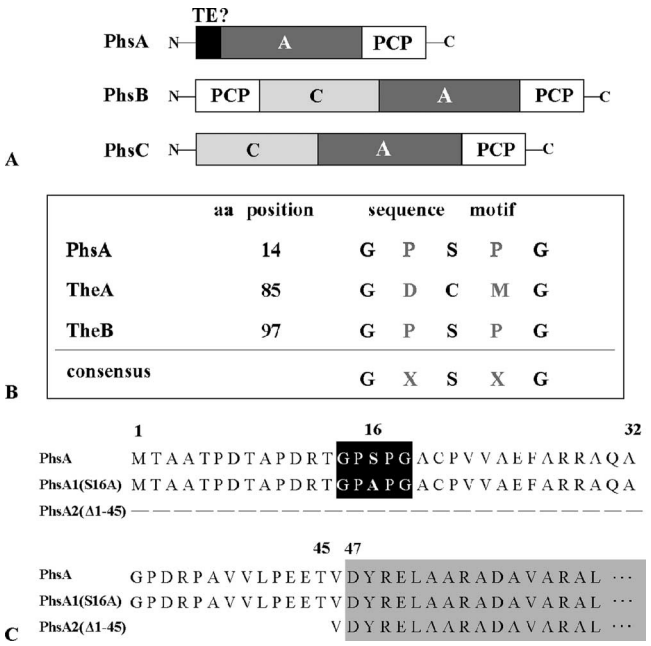

FIG. 3. (A) Domain organization of the peptide synthetases PhsA, PhsB, and PhsC. (B) Localization of the TE motif GXSXG in PhsA, TheA, and TheB. In TheA, the active serine site is replaced by a cysteine residue. (C) Schematic representation of the primary sequence of the native PhsA protein as well as the mutated PhsA variants PhsA1(S16A) and PhsA2(Δ1-45). The TE motifs in PhsA and PhsA1(S16A) in which the conserved serine residues were changed to alanines are highlighted by a black box. The beginning of the adenylation domain of PhsA at aa 47 is marked by a gray box.

teins, while TEIs typically are integrated in the last module of peptide synthetases. Within polyketide and nonribosomal polypeptide biosyntheses, it was shown that TEII enzymes have editing roles (16, 38).

The PTT-specific TheA and TheB proteins belong to the TE family of α/β hydrolases. Members of this family contain the characteristic serine-aspartate-histidine (Ser-Asp-His) catalytic triad (45). TheB possesses the active-site Ser and His residues as part of the highly conserved GXSXG and GXHF motifs, respectively. In TheA, the GXHF motif also was identified, but the active-site serine in the GXSXG motif, which normally is involved in the catalytic activity of TEs, is replaced by a cysteine (Fig. 3B). It has been shown that the exchange of the serine residue for a cysteine can lead to the loss of TE activity (46). However, it also has been described that the same substitution had no significant effect on TE activity (49). Furthermore, the presence of a conserved Asp residue in both proteins was not apparent. The absence of the His or Asp residues also was observed in some other TEs (23, 32). The essential catalytic residue is the active-site serine, whereas the His residue, stabilized by Asp, acts as a catalytic base to remove a proton from Ser, which attacks the acyl carrier protein-bound thioester substrate (45).

In this study, we describe experimental approaches to clarify the function of the three putative TEs within PTT biosynthesis. A primary model of product release from the PTT assembly system is postulated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains, cosmids, and plasmids used in this work are listed in Table 1. The morphological and physiological properties of wild-type *S. viridochromogenes* Tü494 and of the *phsA*, *theA*, and *theB* mutants were examined on yeast malt medium (YM) (35). Cultivation was carried out at 30°C; liquid cultures were incubated in 100 ml of medium in an orbital shaker (180 rpm) in 500-ml Erlenmeyer flasks with steel springs. Spores were isolated as previously described (19).

Amplification, cloning, restriction mapping, and in vitro manipulation of DNA. For the amplification of DNA fragments by PCR, the following reaction mixture was used: 0.5 μg of template DNA (see Table 3), 1.0 μg of each primer (Tables 2 and 3), 10 μl of 10× reaction buffer (containing 20 mM $MgCl_2$), 5% dimethylsulfoxide, 0.2 mM deoxynucleoside triphosphates, and 1 μl of polymerase (*Pwo* polymerase; Roche, Mannheim, Germany) or Herculase (Stratagene) (Table 3). After a denaturation step (5 min at 98°C), 25 cycles of amplification (90 s at 94°C, 90 s at the annealing temperature of the primer pair [Table 3], and 2 min at 72°C) were performed. Each resulting PCR fragment (Table 3) was sequenced to exclude any mutations during PCR. DNA isolation methods were used as previously described (19, 42). Restriction nucleases were purchased from various suppliers and used according to their instructions. The transformation of *Escherichia coli* was performed by the $CaCl_2$ method (31). *E. coli* XL1 Blue (8) was used for standard cloning experiments.

Biological assay for PTT production. PTT production was analyzed in a biological assay using *Bacillus subtilis* and *E. coli* XL1 Blue as test organisms. Equal amounts of the strains to be tested were cultivated in YM medium for 5 days. Fifty-milliliter samples of the cultures were centrifuged, the cell pellet was resuspended in 1 ml YM medium, and the resulting suspension was spread uniformly on the surface of a defined YM agar plate (45 ml per plate; diameter, 9 cm). After 5 days of incubation at 30°C, blocks of agar were cut out and applied to *B. subtilis* and *E. coli* test media (1, 34). The plates were incubated overnight at 37°C, and the antibiotic production was assayed by the area of the inhibition zone around the agar blocks.

Gene replacement mutagenesis of *phsA*. For the replacement of *phsA* (1.9 kb) with an apramycin resistance cassette, two *phsA* gene-flanking fragments, mphsA1 (1.4 kb) and mphsA2 (1.3 kb) (Fig. 2A; Table 3), were amplified by PCR using the primer pairs P1/P2 and P3/P4 (Tables 2 and 3), respectively. mphsA1 and mphsA2 carry a synthetic EcoRV restriction site at the 3′ end and 5′ end, respectively. It was considered important to maintain a region of 215 bp in front of the stop codon of *phsA*, which overlaps with the *pat* promoter, to ensure the transcription of the *pat* gene (Fig. 2A). The fragments mphsA1 and mphsA2 were cloned into pJOE890 (2), resulting in pSE1 and pSE2. mphsA1 then was isolated as an EcoRI fragment from pSE1 and cloned into the *E. coli* vector pK18 (29), resulting in pSE3. In the next step, mphsA2 was cloned downstream of mphsA1 as an EcoRV/EheI fragment into the EcoRV site of pSE3. Finally, the resulting plasmid pSE4 was digested with EcoRV, and the apramycin resistance cassette *apr*, isolated as an EcoRV/SmaI fragment from pEH13 (17), was inserted into the EcoRV site between mphsA1 and mphsA2, resulting in pSE5. The plasmid was transferred into *S. viridochromogenes* Tü494 by polyethylene glycol-mediated transformation of protoplasts as previously described (19, 42). By a double-crossover event, 1.7 kb of the chromosomal *phsA* gene was replaced with an apramycin resistance cassette. To exclude an integration of the whole plasmid into the genome by a single-crossover event, which would confer kanamycin resistance, the apramycin-resistant clones were tested for kanamycin sensitivity. The PTT production of the resulting mutant MphsA (Fig. 2B) was tested in a biological assay against *E. coli* XL1 blue.

Site-specific mutagenesis. In order to replace serine with alanine in the TE motif of PhsA, site-specific mutagenesis was performed using QuikChange site-directed mutagenesis kits (Stratagene Europe, Amsterdam, The Netherlands). The plasmid pDS1 (35), carrying the *phsA* gene was digested with PstI, and the resulting 622-bp PstI fragment (*phsA′*), which represents the 5′ end of *phsA* comprising the sequence for the TE motif, were cloned into pK18. The resulting plasmid pSE6 served as the template for PCR using the mutagenic primer pair P5/P6 (Tables 2 and 3). By introducing a base substitution, a new SfiI restriction site was generated, which facilitated the identification of plasmids in which site-specific mutagenesis was effected. Plasmids carrying the point-mutated 622-bp *phsA* fragment [*phsA1′*(S16A)] were named pSE6*. *phsA1′*(S16A) then was isolated as a 622-bp PstI fragment from pSE6*. pDS1 then was digested with PstI, and the native 622-bp fragment was exchanged for the point-mutated *phsA1′*(S16A) fragment in pDS1. The resulting plasmid, carrying the point-mutated *phsA* gene [*phsA1*(*S16A*)] (Fig. 3C), was designated pSE7.

Complementation of the mutant MphsA. For the complementation of MphsA, the native *phsA* and the point-mutated *phsA1*(*S16A*) genes, each carrying an

TABLE 1. Bacterial strains, plasmids, and cosmids used in this study

Bacterial strain, plasmid, or cosmid	Relevant genotype and/or phenotype	Source or reference
Strains		
S. viridochromogenes		
Tü494	PTT-producing wild type	4
MphsA	Replacement of 1.7 kb of *phsA*; *apr*; no PTT production	This study
MtheA	Gene interruption of *theA*; *apr*; reduced PTT production	This study
MtheB	Gene interruption of *theB*; *apr*; reduced PTT production	This study
Tü494(Δ*theA*/*theB*::*aprP*)	Replacement of *theA* and *theB*; *apr*; reduced PTT production	This study
E. coli		
XL1 Blue	*recA1 end A1 gyrA96 thi-1 hsdR17 supE44 relA1lac*[F′ *proAB lac1*^{q} *Z*Δ M*15* Tn*10*(*tet*^{r})]	8
Cosmids		
pPtcos2	Carries a part of the PTT biosynthetic gene cluster	1
Plasmids		
pK18/19	pUC derivative; *aphII*; *lacZ′* complementation system	29
pRSETB	T7 promoter expression system, 6× His tag, *bla*	Invitrogen
pJOE890	*bla*, two multiple cloning sites, two *ter* genes	2
pGEM-T-Easy	pUC-derivative, *lacZ′ bla*	Promega
pDRIVE	*lacZ′* complementation system, ampicillin and kanamycin resistance, multiple cloning site	Qiagen
pGM190	*Streptomyces-E. coli* shuttle vector, *aphII tsr*, inducible *tipA* promoter, medium copy number	G. Muth, personal communication
pWHM3	*Streptomyces-E. coli* shuttle vector, *trs bla*	47
pDS1	pUC18 derivative carrying the *phsA* gene	35
pDS3	pQE30 derivative carrying the mutated *phsA* gene *phsA2*(Δ*1-45*), in which the first 135 bp are deleted	34
pEH13	pUC21 derivative carrying the *aprP* resistance cassette	17
pEH15	pK18 derivative carrying constitutive *ermEp*	17
pSE1	pJOE890 derivative carrying mphsA1, the fragment upstream of *phsA*	This study
pSE2	pJOE890 derivative carrying mphsA2, the fragment downstream of *phsA*	This study
pSE3	pK18 derivative carrying mphsA1	This study
pSE4	pSE3 derivative carrying mphsA2 behind mphsA1	This study
pSE5	pSE4 derivative, carrying *aprP* inserted between mphsA1 and mphsA2	This study
pSE6	pK18 derivative carrying the 622-bp PstI fragment (*phsA′*) of the 5′ end of *phsA*	This study
pSE6*	pSE6 derivative carrying *phsA1′*(*S16A*)	This study
pSE7	pDS1 derivative carrying *phsA1*(*S16A*)	This study
pSE10	pGEM-T-Easy derivative carrying the fragment mtheA	This study
pSE11	pJOE980 derivative carrying the fragment mtheB	This study
pSE12	pK19 derivative carrying the fragment mtheA	This study
pSE13	pK18 derivative carrying the fragment mtheB	This study
pSE14	pSE12 derivative carrying *aprP* inserted into mtheA	This study
pSE15	pSE13 derivative carrying *aprP* inserted into mtheB	This study
pSE16	pGEM-T-Easy derivative carrying the *theA′* gene	This study
pSE17	pGEM-T-Easy derivative carrying the *theB′* gene	This study
pSE18	pRSETB derivative carrying the *theA′* gene	This study
pSE19	pRSETB derivative carrying the *theB′* gene	This study
pSE20	pEH15 derivative carrying the RBS of pRSETB followed by the *theA′* gene	This study
pSE21	pEH15 derivative carrying the RBS of pRSETB followed by the *theB′* gene	This study
pSE22	pWHM3 derivative carrying *ermEp theA′*	This study
pSE23	pWHM3 derivative carrying *ermEp theB′*	This study
pSE31	pK18 derivative carrying the mutated *phsA* gene *phsA2*(Δ1-45), in which the first 135 bp are deleted	This study
pSE32	pRSETB derivative carrying *phsA2*(Δ*1-45*)	This study
pSE34	pGM190 derivative carrying the mutated *phsA* gene *phsA2*(Δ*1-45*)	This study
pSE40	pDRIVE derivative carrying the *phsA* gene	This study
pSE41	pGM190 derivative carrying the native *phsA* gene	This study
pSE42	pDRIVE derivative carrying *phsA1*(*S16A*)	This study
pSE43	pGM190 derivative carrying the point-mutated *phsA1*(*S16A*) gene	This study
pSE44	pDRIVE derivative carrying the fragment mΔA/B1	This study
pSE45	pDRIVE derivative carrying the fragment mΔA/B2	This study
pSE46	pK18 derivative carrying mΔA/B1	This study
pSE47	pSE46 derivative carrying mΔA/B2 behind mΔA/B1	This study
pSE48	pSE47 derivative carrying *aprP* inserted between mΔA/B1 and mΔA/B2	This study

TABLE 2. Primer sequences

Primer	Sequence (5′–3′)	Significant property[a]
P1	ATGACCATCCACAACCCCGAGGAAC	
P2	AAGATATCTCAGCTCGCCTCTTCCTCGGTG	EcoRV restriction site
P3	AAGATATCAGGTGGGTGAGATCTG	EcoRV restriction site
P4	ATGCGGAGCAGGTCCTCGGTGTCCAG	
P5	ACCGGCCCGGCCCCGGGGGCCTGCCCGGTCGTCGCGGAG	Exchange T for G
P6	CTCCGCGACGACCGGGCAGGCCCCCGGCCGGGCCGGT	Exchange A for C
P7	ATCATATGACCGCAGCGACACCG	NdeI restriction site
P8	ATAAGCTTCTACGTCCCCTTCAGTTCG	HindIII restriction site
P9	AACTGGCACACCCGCAACGGCGATGTG	
P10	AGGTCCTTCGTGTGCGAGAACAGGTAG	
P11	ACCTCCTCTTCGGCGACTGCATGGGCG	
P12	AGGGTGGAAAGCCCTGCCATGATGTGG	
P13	AAAGATCTGTGACCGACTGGATCCAGAG	BglII restriction site
P14	AATAAGCTTTCACGCGGTGCTCCCCGGCTCCG	HindIII restriction site
P15	AAGGATCCGTGAGCGGGCGGGGCG	BamHI restriction site
P16	ATAAGCTTTCACTGCCACGTCCGGTC	HindIII restriction site
P17	TACACCCACCTGCTGAAGTCC	
P18	AAGATATCTCACCGCTCGCCCTCCTCGG	EcoRV restriction site
P19	AAGATATCGTGAGAGACACCGGCCCGGC	EcoRV restriction site
P20	TCACCGCGCACAGTGCCGCACG	

[a] The sequences from which the properties are derived are underlined in the primer sequence.

NdeI and HindIII restriction site at its 5′ and 3′ ends, respectively, were isolated by PCR using the primer pair P7/P8 (Tables 2 and 3). For the amplification of *phsA* and *phsA1*(*S16A*), genomic DNA of wild-type *S. viridochromogenes* and the plasmid pSE7, respectively, served as templates.

The amplified fragments were subcloned into the vector pDRIVE (Qiagen, Hilden, Germany), resulting in the plasmids pSE40 and pSE42. These plasmids were digested with NdeI/HindIII and subsequently cloned into the replicative medium-copy-number *Streptomyces-E. coli* shuttle vector pGM190 (G. Muth, personal communication) behind the *tipA* promoter, resulting in pSE41 and pSE43. Furthermore, the complementation plasmid pSE34, carrying a *phsA* fragment in which the first 135 bp were deleted, was generated. This mutated *phsA* fragment, *phsA2*(Δ*1-45*) (Fig. 3C) (originally called *phsA** [34]), was isolated as a BamHI/HindIII fragment from the plasmid pDS3 (34) and cloned into BamHI/HindIII-restricted pK18 (pSE31). Subsequently, *phsA2*(Δ*1-45*) was isolated from pSE31 as a EcoRI/HindIII fragment and was cloned into the vector pRSETB (Invitrogen, Karlsruhe, Germany), resulting in pSE32. This cloning step was required to obtain the appropriate restriction sites for the insertion of *phsA2*(Δ*1-45*) into the vector pGM190. In the final step, *phsA2*(Δ*1-45*) was isolated from pSE32 as an NdeI/HindIII fragment and cloned into the pGM190 vector, resulting in pSE34. The plasmids pSE34, pSE41, and pSE43 were used for the transformation of MphsA. Finally, PTT production in the complemented MphsA, MphsA(pSE34), MphsA(pSE41), and MphsA(pSE43) mutants was tested by a biological assay. As the complemented MphsA mutants were cultivated in YM medium with 10 μg/ml kanamycin, a kanamycin-resistant *E. coli* strain was used as a test organism for the biological assay.

Immunoblot analysis of PhsA1(S16A). For the detection of the gene product of *phsA1*(*S16A*), an immunoblot experiment was performed. *S. viridochromogenes* Tü494, MphsA, MphsA(pSE41), and MphsA(pSE43) cells were cultivated in YM medium for 5 days. The harvested cells were resuspended in lysis buffer and subsequently broken twice using a French press (at 10,000 lb/in^2). The protein lysates were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. The transfer of separated proteins to a nitrocellulose membrane (BioTraceNT; Pall Life Science, Dreieich, Germany) was achieved by the semi-dry method using the MilliBlot graphite electroblotter (Millipore, Bedford, MA). The immunoblotting analysis was performed by a standard technique using a 1:5,000 dilution of a primary PhsA antibody (35), alkaline phosphatase-conjugated goat anti-rabbit antibody (Bio-Rad Laboratories GmbH, München, Germany), and chloronaphtol as the reagent.

Gene interruption mutagenesis of *theA* and *theB* genes. For the generation of the *theA* and *theB* mutants MtheA and MtheB, the 2.5-kb mtheA and 2.2-kb mtheB fragments comprising the *theA* and *theB* genes, respectively (Fig. 2A; Table 3), were amplified using the primer pairs P9/P10 and P11/P12 (Tables 2 and 3). MtheA was cloned into the vector pGEM-T-Easy (Promega, Madison, WI), and mtheB was cloned into pJOE890, thereby generating the plasmids pSE10 and pSE11. Subsequently, mtheA and mtheB were isolated as EcoRI fragments and then inserted in the EcoRI-restricted pK19 (29) and pK18 vectors, respectively. The resulting plasmids pSE12 and pSE13 were digested with Bpu1102 and BstEII, respectively. The generated sticky ends were filled in with *Klenow* polymerase (Fermentas, St. Leon-Rot, Germany) to obtain blunt ends (pSE12* and pSE13*). Afterwards, the apramycin-*ermEp* resistance cassette (designated *aprP*) (17) was isolated from pEH13 as an EcoRV/StuI blunt end fragment and was inserted into pSE12* and pSE13*. As the genes *theA*, *theB*, and *trs* (Fig. 2A) are translationally coupled, the presence of the *ermE* promoter behind the *apr* gene in the mutants should avoid polar

TABLE 3. Amplified fragments and PCR conditions

Fragment[a]	Primer pair	Annealing temp (°C)	Polymerase	Template
mphsA1 (1.4 kb)	P1 and P2	64	*Pwo*	Genomic DNA of Tü494
mphsA2 (1.3 kb)	P2 and P3	60	Herculase	Genomic DNA of Tü494
phsA1′ (S16A) (622 bp)	P5 and P6	68	*Pwo*	pSE6
phsA/phsA1(S16A) (1.8 kb)	P7 and P8	58	*Pwo*	Genomic DNA of Tü494/pSE7
mtheA (2.5 kb)	P9 and P10	60	Herculase	Genomic DNA of Tü494
mtheB (2.2 kb)	P11 and P12	70	*Pwo*	Genomic DNA of Tü494
theA (0.75 kb)	P13 and P14	60	Herculase	pPtcos2
theB (0.78 kb)	P15 and P16	62	Herculase	pPtcos2
mΔA/B1 (1.1 kb)	P17 and P18	62	*Pwo*	Genomic DNA of Tü494
mΔA/B2 (1.2 kb)	P19 and P20	70	*Pwo*	Genomic DNA of Tü494

[a] The value given in parentheses is the expected size of the PCR fragment.

effects on the genes located downstream. The resulting plasmids pSE14 and pSE15 were used for the generation of the *theA* and *theB* mutants MtheA and MtheB (Fig. 2C) by gene replacement as described above. PTT production in MtheA and MtheB was tested by a biological assay against *B. subtilis*.

Complementation of MtheA and MtheB. For the complementation of MtheA and MtheB, the complementation plasmids pSE22 and pSE23 were constructed. Therefore, the *theA* and *theB* genes were amplified by PCR using the primer pairs P13/P14 and P15/P16, respectively (Tables 2 and 3). *theA* carries a BglII site and *theB* a BamHI site at their 5′ ends, and both have a HindIII site at the 3′ end. The obtained *theA′* (758 bp) and *theB′* (783 bp) fragments each were subcloned in the pGEM-T-Easy vector, resulting in pSE16 and pSE17. *theA′* was cloned as a BglII/HindIII fragment and *theB′* as a BamHI/HindIII fragment into the BglII/HindIII-restricted *E. coli* expression vector pRSETB. As the *theA* and *theB* genes were amplified without their own ribosome binding sites (RBS), these fragments were cloned behind an RBS sequence, which was supplied by the pRSETB vector. The resulting plasmids were named pSE18 and pSE19. Furthermore, *theA′* and *theB′* were isolated together with the RBS of pRSETB as XbaI/HindIII fragments and subcloned into pEH15 (17) behind the *ermE* promoter (*ermEp*), resulting in pSE20 and pSE21. Finally, the resulting *ermEp theA′* and *ermEp theB′* fragments were cloned as EcoRI and HindIII fragments, respectively, into the *Streptomyces-E. coli* shuttle vector pWHM3 (47). The constructed plasmids pSE22 and pSE23 were used for the transformation of MtheA, MtheB, and wild-type *S. viridochromogenes* Tü494. The PTT production of the supplemented strains Tü494(pSE22), Tü494(pSE23), MtheA(pSE22), and MtheB(pSE23) was analyzed using a biological assay against *B. subtilis*.

Generation of a *theA*/*theB* double mutant by gene replacement mutagenesis. For the replacement of *theA* and *theB* with the apramycin-*ermEp* resistance cassette (*aprP*), two of the TE gene-flanking fragments, mΔA/B1 (1.1 kb) and mΔA/B2 (1.2 kb) (Fig. 2A; Table 3), were amplified by PCR using the primer pairs P17/P18 and P19/P20 (Tables 2 and 3), respectively. mΔA/B1 and mΔA/B2 carry a synthetic EcoRV restriction site at their 3′ and 5′ ends, respectively. The fragments mΔA/B1 and mΔA/B2 were cloned into pJOE890, resulting in pSE44 and pSE45. mΔA/B1 was isolated as an EcoRI fragment from pSE44 and was cloned into the *E. coli* vector pK18, resulting in pSE46. In the next step, mΔA/B2 was cloned downstream of mΔA/B1 as an EcoRV/HindIII fragment into pSE46. Finally, the resulting plasmid pSE47 was digested with EcoRV, and the *aprP* resistance cassette, which was isolated as an EcoRV/StuI fragment from pEH13, was inserted into the EcoRV restriction site between mΔA/B1 and mΔA/B2. The resulting plasmid pSE48 was used for the generation of the *theA*/*theB* double mutant strain Tü494(Δ*theA*/*theB*::*aprP*) (Fig. 2D) by gene replacement as described above. PTT production in Tü494(Δ*theA*/*theB*::*aprP*) was tested by a biological assay against *B. subtilis*.

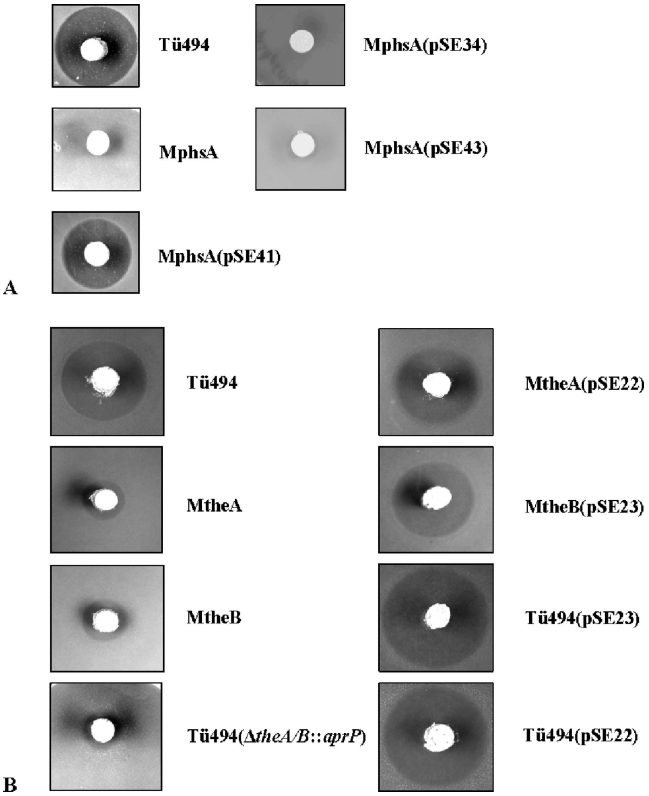

FIG. 4. (A) Analysis of PTT production of wild-type strain Tü494, the mutant MphsA, and the *phsA*-, *phsA2*(Δ*1-45*)-, and *phsA1*(*S16A*)-complemented mutants MphsA(pSE41), MphsA(pSE34), and MphsA (pSE43) in a biological assay. (B) Analysis of PTT production of wild-type strain Tü494, the mutants MtheA and MtheB with the native *theA* and *theB* genes, respectively, complemented mutants MtheA(pSE22) and MtheB(pSE23), the wild-type strain overexpressing *theA* and *theB* [Tü494(pSE22) and Tü494(pSE23), respectively], and the *theA*/*theB* double mutant Tü494(Δ*theA*/*theB*::*aprP*).

RESULTS

Analysis of the *phsA* mutant MphsA. In order to analyze the functionality of the TE motif at the N terminus of PhsA, first the *phsA* deletion mutant MphsA was generated. Therefore, two fragments upstream and downstream of *phsA* were amplified, resulting in the fragments mphsA1 and mphsA2 (see Materials and Methods) (Fig. 2A). Both fragments were cloned into the pK18 vector and then were separated by the insertion of an apramycin resistance cassette isolated from the vector pEH13 (as described in Materials and Methods). The resulting plasmid, pSE5, was used for the generation of the *phsA* mutant MphsA. After the transformation of wild-type *S. viridochromogenes* Tü494, two apramycin-resistant and kanamycin-sensitive clones were obtained, indicating the replacement of the *phsA* gene by a double-crossover event. For one of the two clones (MphsA) (Fig. 2B) isolated, the correct integration of the apramycin resistance cassette was verified by Southern hybridization (data not shown). The PTT production of MphsA was analyzed in a biological assay, and no inhibition zone was detected (Fig. 4A), which proved that *phsA* is required for PTT biosynthesis. To confirm that the loss of PTT production was caused by the deletion of *phsA* and not by polar effects, the mutant was complemented with the native *phsA* gene. The *phsA* gene was cloned into the *Streptomyces-E. coli* shuttle vector pGM190 behind the inducible *tipA* promoter, resulting in the plasmid pSE41. As the *tipA* promoter has basal activity, the experiment was performed without induction. PTT production was restored in MphsA after complementation (Fig. 4A), confirming that the loss of PTT production was due to the deletion of the *phsA* gene and not due to polar effects on downstream genes. However, the amount of PTT in the complemented *phsA* mutant was less than that in the wild type. A reason for the impaired PTT production might be that the natural balance between the three peptide synthetases, which is necessary for efficient antibiotic production, cannot be achieved by the nonspecific promoter and the high-copy-number plasmid pSE41 (3, 24).

Analysis of the functionality of the TE motif in PhsA. To analyze the functionality of the TE motif in the N terminus of PhsA, differently mutated PhsA derivatives were constructed.

First, PhsA2(Δ1-45) was generated, in which the first 45 aa were deleted (see Materials and Methods) (Fig. 3C). As the sequence for the TE GXSXG motif is located at aa 14 to 18, the TE motif also was affected by the deletion. Sequence analysis of PhsA by SMART (21, 33) revealed that the adenylation domain of PhsA starts at aa 47 (corresponding to bp 141).

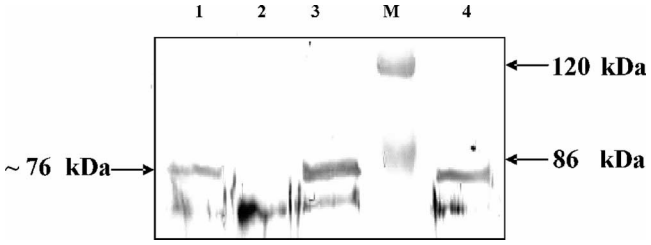

FIG. 5. Detection of PhsA in an immunoblotting experiment using antibodies against PhsA. Lane 1, Tü494; lane 2, MphsA; lane 3, MphsA(pSE41); lane M, prestained protein marker (Fermentas, St. Leon-Rot, Germany); and lane 4, MphsA(pSE43).

Therefore, the deletion of the first 45 aa should not affect the adenylation activity of PhsA. The *phsA2*(*Δ1-45*) gene was isolated from the plasmid pDS3 and cloned, as described in Materials and Methods, into the vector pGM190 behind the *tipA* promoter. The resulting plasmid, pSE34, then was used for the complementation of MphsA. In a biological test, it was shown that PhsA2(Δ1-45) cannot restore PTT production (Fig. 4A), indicating that the TE motif plays an important role during PTT biosynthesis.

To prove that the failure of PTT production in MphsA (pSE34) was exclusively due to the absence of the TE motif and not due to the deleted first 45 aa, PhsA1(S16A), in which the highly conserved serine residue is replaced by an alanine, was constructed (Fig. 3C). This should lead to the loss of TE activity (22). The replacement of serine in PhsA was performed by site-directed mutagenesis via PCR using primers in which the T of the serine-coding triplet TCC was changed to a G to generate an alanine-coding triplet. The mutated *phsA* gene, *phsA1*(*S16A*), was cloned behind the *tipA* promoter of pGM190 to give pSE43 (for details, see Materials and Methods). pSE43 then was used for the complementation of the *phsA* deletion mutant MphsA. MphsA(pSE43) also was not able to produce PTT (Fig. 4A).

To prove that the failure to synthesize PTT after complementation with the mutated *phsA* gene [*phsA1*(*S16A*)] was caused by a mutation and not by a lack of expression, an immunoblotting experiment using antibodies against PhsA was performed. For immunoblotting, the lysates of MphsA(pSE43) and, as controls, wild-type *S. viridochromogenes* Tü494 and the mutants MphsA and MphsA(pSE41) (carrying the native *phsA* gene) were used. The denatured PhsA (76 kDa) described previously (12) was detected in all samples except in the *phsA* mutant MphsA (Fig. 5), proving that the lack of PTT production in MphsA(pSE43) was due to the mutated TE motif in PhsA. These results confirm that the TE motif in the N terminus of PhsA is required for PTT production.

Analysis of the *theA* and *theB* mutants MtheA and MtheB. In silico analysis revealed the presence of two individual TE genes, *theA* and *theB*, in the PTT biosynthetic gene cluster (36) (Fig. 2A). To elucidate the function of these two external TEs, *theA* as well as *theB* mutants were generated by the insertion of the apramycin resistance cassette equipped with the constitutive *ermE* promoter (*aprP*). The presence of the *ermE* promoter downstream of the *apr* gene should prevent polar effects on the genes, which are located downstream of *theA* and *theB*, respectively, as shown for other *S. viridochromogenes* Tü494 mutants in which the *aprP* cassette was used for the mutagenesis of PTT biosynthetic genes (17, 36, 37). Two fragments of the PTT biosynthetic gene cluster, containing *theA* and *theB*, respectively, were amplified by PCR. The resulting fragments mtheA (2.5 kb) and mtheB (2.3 kb) (Fig. 2A) were cloned into pK19 and pK18 vectors, respectively (see Materials and Methods). In the next step, the *aprP* cassette was inserted into each of the two genes. The resulting plasmids pSE14 and pSE15 were used for the transformation of *S. viridochromogenes* and the subsequent isolation of mutants MtheA and MtheB, in which the native genes were replaced by the mutated ones as described above (Fig. 2C). The correct integration of the apramycin resistance cassette in the mutants was verified by Southern hybridization (data not shown). The bioassay revealed that antibiotic activity was reduced about 80% in MtheA and about 70% in MtheB compared to that of the wild type (Fig. 4B).

To confirm that the activity was a result of the mutation and not due to polar effects on the downstream genes *theA* and *theB*, the mutants were complemented with their native genes. The *theA* and *theB* genes were amplified by PCR and cloned into the *Streptomyces-E. coli* shuttle vector pWHM3. The resulting constructs pSE22 and pSE23 were used for the transformation of MtheA and MtheB, respectively. In both cases, the complementation led to the restoration of PTT production to nearly the wild-type level, which proved that the reduced PTT production in MtheA and MtheB was an effect of the interruption of *theA* and *theB* genes (Fig. 4B). These results also indicate that the TEs are not essential for PTT biosynthesis but are required for efficient PTT production. Therefore, an editing role for both TEs by removing aberrant intermediates or wrongly activated amino acids from the peptide synthetase complex can be suggested.

Effect of overexpression of *theA* and *theB* in wild-type *S. viridochromogenes* Tü494. If *theA* and *theB* have an editing role, the overexpression of both genes in *S. viridochromogenes* Tü494 should enhance antibiotic production. Therefore, the complementation plasmids pSE22 and pSE23 (see Materials and Methods) also were used to transform the wild type. The antibiotic production of the recombinant strains was assayed by measuring the inhibition zones against sensitive indicator bacteria (Fig. 4B). Tü494(pSE22) and Tü494(pSE23) each produced ~50% more PTT or more active PTT than the wild type. This result supports the postulation that both TEs exhibit an editing role.

Analysis of the *theA*/*theB* double mutant Tü494(*ΔtheA*/*theB*::*aprP*). To support the assumption that both TEIIs TheA and TheB have an editing role, a *theA*/*theB* double mutant was generated by the replacement of the *theA* and *theB* genes by *aprP*. As described above, the presence of the *ermE* promoter behind the *apr* gene should prevent polar effects on the genes, which are located downstream of *theA* and *theB*. Two fragments of the PTT biosynthetic gene cluster, which flank *theA* and *theB*, respectively, were amplified by PCR. The resulting fragments, mΔA/B1 (1.1 kb) and mΔA/B1 (1.2 kb) (Fig. 2A), were cloned into the vector pK18 and then were separated by the insertion of the a*prP* cassette. The resulting plasmid pSE48 was used for the generation of the *theA*/*theB* double mutant. After the transformation of wild-type *S. viridochromogenes* Tü494, two apramycin-resistant and kanamycin-sensitive clones were obtained, indicating the replacement of *theA* and

theB by a double-crossover event. For one of the two clones [Tü494(Δ*theA*/*theB*::*aprP*); Fig. 2D] isolated, the correct integration of the apramycin resistance cassette was verified by PCR (data not shown). The PTT production of Tü494(Δ*theA*/*theB*::*aprP*) was analyzed in a biological assay against *B. subtilis*. The bioassay revealed that the antibiotic activity in Tü494(Δ*theA*/*theB*::*aprP*) was reduced to 9% compared to that of the wild type (Fig. 4B). This result supports the assumption that both TE genes, *theA* and *theB*, have an editing role, as the mutant still is able to produce PTT.

DISCUSSION

PTT biosynthesis represents a special case of bacterial nonribosomal peptide synthesis. Three peptide synthetase genes, *phsA*, *phsB*, and *phsC*, are involved in the biosynthesis of PTT, with each encoding one peptide synthetase module. In addition, these three peptide synthetase genes are not clustered but are encoded at different positions of the biosynthetic gene cluster (35, 36). In this NRPS system, PhsA is the initiation module; however, it is not clear whether PhsB or PhsC represents the termination module. A typical TE domain in the C terminus of PhsB as well as in PhsC, which would allow classification as a final module, is missing. However, neither of these two enzymes can replace the other. Consequently, a defined positioning for PhsB and PhsC in the PTT assembly line is likely (37). Between the tyrocidine synthetases TycA, TycB, and TycC, so-called short COM domains are described that mediate the interaction between the peptide synthetases. The COM domains are classified into the accepter COM domain (COM^A) and the donor COM domain (COM^D). At the N terminus of the initiation module and at the C terminus of the termination module, a COM domain usually is lacking (14, 15, 26). However, in the PTT synthetases PhsA, PhsB, and PhsC, such COM domains are not apparent (37).

Instead of an integrative C-terminal TE domain in PhsB or PhsC, a highly conserved TE GXSXG motif in the N terminus of PhsA was identified. However, the region surrounding the motif has no overall homology to other TE domains. Usually the TE motif is part of an ~280-aa-long region that is located at the C terminus of the terminal amino acid-activating module (13). Additionally, the PTT biosynthetic gene cluster harbors the genes *theA* and *theB*, the gene products of which show high similarity to TEIIs. A TEII is assumed to have an editing role within the biosynthesis of polypeptides and polyketides (16, 38). To our knowledge, no other biosynthetic gene cluster has been described in which two TEII genes are located. In most cases, in addition to the integrated TEI, only one autonomous TE-encoding gene is part of the cluster. An example is the biosynthetic gene cluster of the lipopeptide antibiotic surfactin, which harbors the *srfA* TE gene coding for TEII (32). Based on these facts, two alternatives for the release of PTT from the peptide synthetase complex can be postulated. In the first model, it is assumed that the TE motif in PhsA is functional. In this case, the three peptide synthetases arrange in a manner such that the C terminus of PhsB or PhsC and the N terminus of PhsA can interact, and the tripeptide can be cleaved off from the peptide synthetase complex with the participation of the TE motif. Here, the two TEs TheA and TheB have editing roles within PTT biosynthesis (Fig. 6A). In the second model, it is assumed that the TE motif is not functional. In this case, one of the TEIIs of PTT biosynthesis is responsible for the release of the tripeptide from the peptide synthetase complex. The other TE would have editing roles in PTT biosynthesis (Fig. 6B). A similar system is described for product release in nangchangmycin biosynthesis. In that case, a TE domain is not included in either of the two type I polyketide synthases involved in the biosynthesis of the polyether nangchangmycin. Instead, polyether chain release is catalyzed by the gene product of *nanE*. The *nanE* gene is located within the biosynthetic gene cluster and encodes a monofunctional TE (23).

In order to investigate whether the TE motif in PhsA is active, first the nonpolar *phsA* deletion mutant MphsA was generated, which served as a recipient for the subsequent analysis of the mutated *phsA* genes. This mutant lost the ability to produce PTT. For the analysis of the TE motif, the first 45 aa of the N terminus of PhsA, which comprises the TE motif, were deleted. After the complementation of MphsA with this construct, no PTT production was detectable, indicating that the TE motif has a role in PTT biosynthesis. This result supports the first model of product release. Another interpretation of these results is that, because of the missing N terminus of PhsA, an interaction between the N terminus of PhsA and the C terminus of PhsB or PhsC is not possible. As mentioned above, the N and C termini of the peptide synthetase play an essential role for the selective interaction and communication between peptide synthetases mediated by COM domains (14, 15). Although no COM domains were identified in the PTT peptide synthetases (37), it cannot be excluded that there are specific sequences that exhibit the function of a COM domain and thus are responsible for the selective interaction of PhsA, PhsB, and PhsC. If the first model represents the NRPS assembly within PTT biosynthesis, the N terminus of the initiation module (PhsA) and the C terminus of the last module (PhsB/C) have to interact. Thus, the failure to produce PTT after complementation with PhsA2(Δ1-45) also might be explained by the deletion of a short COM domain-like region in the N terminus of PhsA. In this case, an interaction of PhsA and PhsC or PhsA and PhsB, which has to be postulated for the first model, would not be possible and consequently would lead to a failure of PTT production.

To determine whether the lack of PTT production was caused by the absence of the TE motif or of the N terminus of PhsA, a second variant PhsA [PhsA1(S16A)] was constructed in which only the catalytic active serine site of the TE GXSXG motif was changed to an alanine. This exchange usually leads to the loss of TE activity, as shown for the TEI of the L-α-aminoadipyl-L-cysteinyl-D-valine synthetase and the TEII Srf TE from the surfactin biosynthetic gene cluster (W. Kullow, H. von Döhren, J. Kennedy, and G. Turner, presented at Enzymology of Biosynthesis of Natural Products, Technische Universitat, Berlin, Germany, 1996) (22). After the complementation of the mutant MphsA with PhsA1(S16A), no PTT production was detectable in a biological assay. This indicates that the TE motif in the N terminus of PhsA is required for the release of PTT from the NRPS complex. However, it is not yet clear how the singular TE motif in PhsA, without the 280-aa TE region, which normally is important for activity, catalyzes product release. A sequence analysis of the first 45 aa in front of the adenylation domain in PhsA by using the bioinformatics

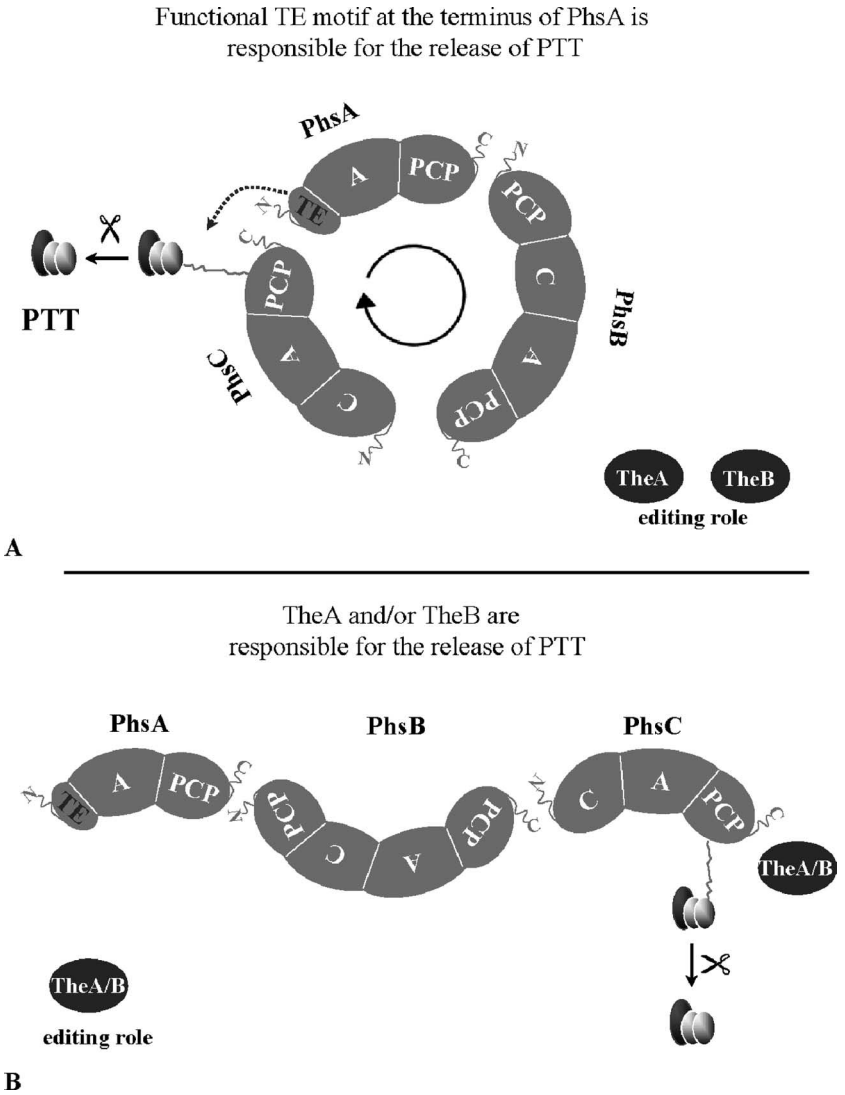

FIG. 6. Models for the interaction of PhsA, PhsB, and PhsC as well as TheA and TheB. (A) A fully functional TE motif is supposed to be present in PhsA. TheA and TheB have editing roles in PTT biosynthesis. (B) The TE motif in PhsA is not functional. One of the two TEs is involved in PTT release, and the other one has a corrective function. In both models, the order for the peptide synthetase assembly might be changed to PhsA, PhsC, and PhsB.

tool Hhpred (40) revealed that the TE motif comprising the 45-aa region in PhsA has no homology to the TE. Further, it is conceivable that this N-terminal region interacts with other PTT-specific proteins that are related to TE-like proteins. Therefore, the complete PTT biosynthetic gene cluster was screened for a TE-like gene (data not shown). Except for the two TEIIs, no TE-like protein was identified. To clarify if the single TE motif is sufficient for TE activity, a biochemical analysis will be performed with PhsA in further studies.

To exclude the possibility that the TheA and/or TheB protein is involved in the product release, the nonpolar mutants MtheA and MtheB were generated. In each case, the mutation led to reduced PTT production. On the other hand, the overexpression of TheA and TheB, respectively, in the wild type led to enhanced PTT production (50% increase). The results regarding TheA indicate that the identified motif (GXCXG), in which a cysteine instead of a serine is located in the catalytic center, has no detectable effect on TE activity. A similar exchange performed for the analysis of a thioesterase within fatty acid synthesis also showed no dramatic effect on TE activity (43).

Therefore, it can be concluded that TheA and TheB might have an editing role within PTT biosynthesis. This assumption is supported by the phenotype of the *theA*/*theB* double mutant Tü494(Δ*theA*/*theB*::*aprP*). The mutant still produces 9% of the amount of PTT produced by the wild type, but this amount is less than that of either the MtheA or MtheB mutant.

The functions of TEIIs have been analyzed in different assembly-line syntheses. In polyketide synthesis, it has been described that the function of TEII is to remove abnormal polyketides from the acyl carrier protein domain of the polyketide synthase (9, 16). Such molecular mistakes can appear during biosynthesis by different mechanisms. The aberrant polyketides carrying a wrong oxidation state and therefore

a chain elongation cannot proceed, and the polyketide biosynthesis fails. In this case, TEII cleaves off the aberrant polyketides from the polyketide synthase complex. Besides the removal of aberrant intermediate products from the biosynthetic machinery, a further model was described for the function of TEII in which TEII is involved in the regeneration of misprimed NRPS (38). Before the synthesis of the polypeptide can start, the inactive NRPS has to be activated by attaching the prosthetic group coenzyme A to the PCP domain. This reaction is catalyzed by the 4′PP-transferase. The 4′PP-transferase also is able to recognize acyl-coenzyme A as a substrate and to attach it to the inactivated PCP. In this case, the synthesis of the polypeptide is not possible, because the thiol group of the PCP is blocked by the acyl chain. Consequently, TEII cleaves off the acyl chain, and peptide synthesis can take place. As both the removal of aberrant intermediate products from the biosynthetic machinery and the regeneration of misprimed NRPSs strongly influence the antibiotic yield, editing by TEIIs represents a rate-limiting step in antibiotic biosynthesis (9, 16, 38).

Also in the case of PTT biosynthesis, TheA and TheB are important to maintain normal levels of the antibiotic. It is conceivable that incorrect amino acids assemble during peptide elongation because of a specific feature of the A domain in PhsB and PhsC. An in silico analysis of the specificity region (10, 30, 41) of PhsB and PhsC predicted that PhsB has a serine-activating domain and PhsC a proline-activating domain. A biochemical analysis of PhsB and PhsC, however, revealed that PhsB and PhsC each preferentially activate alanine. However, besides alanine, the specificity for short-chain amino acids such as serine or aminobutyric acid, but not for proline, as predicted, also was measured (37). Therefore, it may be possible that instead of alanine, other amino acids are linked to N-Ac-DMPT, resulting in altered PTT derivatives. This may lead to decreased PTT activity against bacteria, as the di-alanine residue is needed for the transport of PT into the bacterial cell (11). It seems likely that the functions of the two TEs TheA and TheB are the release of wrongly activated amino acids and the regeneration of misprimed NRPSs. This suggestion is supported by the results of this study, as the inactivation of the TE genes *theA* and *theB* led to reduced PTT production. Furthermore, the increase in antibiotic activity caused by the overexpression of *theA* and *theB* in *S. viridochromogenes* Tü494 may be attributed to an enhanced editing function by the release of aberrant PTT derivatives or of the regeneration of misprimed NRPSs. It has been shown that the accessory expression of TEII for *Saccharopolyspora erythraea* leads to an 80% increase in the production of the erythromycin aglycon 6-deoxyerythronolide B in cultures of recombinant *E. coli* (28). Further biochemical studies will be carried out to analyze the substrate specificity of TheA and TheB proteins.

Considering all of the results, the first model (Fig. 6A) seems to represent the mode of PTT biosynthesis. Here, the three peptide synthetases arrange in a manner such that the N-terminal TE motif can physically interact with the C-terminal PCP of PhsB or PhsC. Consequently, the peptide chain is cleaved off the peptide synthetase complex by the action of the N-terminal TE motif of PhsA. The peptide synthesis thereby starts and ends in PhsA, which would represent both the initiation and termination module. This kind of cyclic arrangement of NRPS was not previously described; therefore, this study introduces a new strategy for NRPS arrangement.

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