Isolation and Characterization of the Gene Cluster for Biosynthesis of the Thiopeptide Antibiotic TP-1161†‡

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Received 17 June 2010/Accepted 4 September 2010

Recently, we isolated a new thiopeptide antibiotic, TP-1161, from the fermentation broth of a marine actinomycete typed as a member of the genus Nocardiopsis. Here we report the identification, isolation, and analysis of the TP-1161 biosynthetic gene cluster from this species. The gene cluster was identified by mining a draft genome sequence using the predicted structural peptide sequence of TP-1161. Functional assignment of a ~16-kb genomic region revealed 13 open reading frames proposed to constitute the TP-1161 biosynthetic locus. While the typical core set of thiopeptide modification enzymes contains one cyclodehydratase/dehydrogenase pair, paralogous genes predicted to encode additional cyclodehydratases and dehydrogenases were identified. Although attempts at heterologous expression of the TP-1161 gene cluster in Streptomyces coelicolor failed, its identity was confirmed through the targeted gene inactivation in the original host.

Thiazolyl peptides (commonly called thiopeptides) are a group of macrocyclic peptide antibiotics that are produced by various bacteria, including members of the Actinomycetales order (mainly soil-derived Streptomyces spp.) and Bacillus spp. (2). Thiopeptide antibiotics are potent inhibitors of protein synthesis in Gram-positive bacteria, including methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococcus faecium (VRE) (2). Molecular targets include the L11 binding region of the 23S rRNA and bacterial elongation factor Tu (EF-Tu) (2). Apart from translation inhibition, interference with transcription via targeting DNA-dependent RNA polymerase has also been reported for one thiopeptide antibiotic (12). Self-resistance to thiopeptides can be conferred through the specific methylation of the 23S rRNA or mutations in genes encoding the target molecules, including the 23S rRNA, ribosomal protein L11, or EF-Tu (2).

Despite excellent in vitro properties, structure-inherent low solubility causing low bioavailability (4) has hampered the development of thiopeptide antibiotics for clinical use. In addition to the potent antibacterial activity, thiopeptides have been shown to possess antimalarial activity (18, 24) and anticancer activity (3). Increasing knowledge about thiopeptide biosynthesis (16) and their biological activities might provide the basis for pharmacological exploitation of this interesting class of antibiotics.

Recently, we isolated a new thiopeptide antibiotic, TP-1161, from the fermentation broth of a marine Nocardiopsis species (9). Structure elucidation of TP-1161 (Fig. 1) classified the compound as a series d thiopeptide, which comprise the majority of known thiopeptides, with members such as the thio- cillins, thiomuracins, and GE2270A (2). A 2,3,6-trisubstituted pyridine domain central to a single peptide macrocycle, which typically carries multiple thiazole and oxazole heterocycles, is characteristic of the series d thiopeptides. The TP-1161 molecule shares a distinctive oxazole-thiazole-pyridine domain with a number of closely related thiopeptides of the series d, including the A10255 factors, berninamycin, and sulfomycin (2, 9).

Elucidation of the biosynthetic origin of several thiopeptides in the beginning of 2009 (14, 17, 29) revealed that these antibiotics are synthesized from chromosomally encoded precursor peptides that contain the amino acids constituting the backbone of the final thiopeptide framework at their C terminus. The ribosomally synthesized precursor peptide is transformed in a series of posttranslational enzymatic modifications into the final macrocyclic structure featuring multiple heterocycles and dehydrated amino acids. The core modifications, including heterocyclization, dehydrogenation, and dehydration of amino acid residues, seem to be catalyzed by a set of five enzymes most of which have distant homologs in biosynthetic pathways of other tailored ribosomal peptides, such as lantibiotics and cyanobactins (16). Here, we report identification, cloning, and analysis of the gene cluster governing biosynthesis of thiopeptide TP-1161 and propose the biosynthetic pathway for this antibiotic.

MATERIALS AND METHODS

General methods. DNA isolation and manipulations were carried out according to standard methods for Escherichia coli (26) and Streptomyces (15). Restriction enzymes, DNA ligase, and other materials for recombinant DNA procedures were purchased from standard commercial sources and used as provided. Isolation of DNA fragments from agarose gels and purification of PCR products were performed using QIAquick Gel extraction and PCR purification kits (Qiagen). Promega’s Wizard Plus SV miniprep DNA purification system or the NucleoBond Xtra plasmid DNA purification kit (Macherey-Nagel, Düren, Germany) were used for isolation of plasmids and cosmids. The Qiagen DNeasy blood and tissue kit was used for isolation of genomic DNA from Nocardiopsis and Streptomyces strains. Large-scale genomic DNA isolation for library construction and genome sequencing of Nocardiopsis sp. strain TFS65-07 was performed using the Kirby mix procedure (15). PCRs were performed using the Expand high-fidelity PCR system (Roche Applied Science). Southern blot anal-
sp. strain TFS65-07. Genomic shotgun sequencing of strains thereof were maintained on MS or ISP2 agar. Using the Chrysalis 36-cycle v2.0 sequencing kit. TFS65-07 was performed by Fasteris SA (Switzerland) using the Illumina se- 

E. coli intergeneric conjugation from Operon (Ebersberg, Germany). (DIG)-labeled probes prepared using the PCR DIG probe synthesis kit or DIG 

yses were carried out using positively charged nylon membranes and digoxigenin (DIG)-labeled probes prepared using the PCR DIG probe synthesis kit or DIG High Prime DNA labeling and detection starter kit II (Roche Applied Science). DNA sequencing from cosmids and plasmids was performed by Eurofins MWG Operon (Ebersbusch, Germany).

Bacterial strains, plasmids, cosmids, and culture conditions. All bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. Nocardiopsis sp. strain TFS65-07 was newly isolated from fjord sedi-

Nocardiopsis sp. strain TFS65-07 was maintained on M9 medium (0.5 g/liter glucose, 15 g/liter soy meal, 5 g/liter corn steep liquor, 2 g/liter CaCO3, and 0.1 g/liter MgCl2) plus 15 g/liter artificial seawater to 1 liter, pH 7.8). M9 medium was used for growth and sporulation of Nocardiopsis sp. strain TFS65-07, while recombinant TFS65-07 strains were selected for and maintained on 0.75% ISP2 agar supplemented with 50 µg/ml kanamycin. Liquid ISP2 medium was used as seed medium and fermentation of Nocardiopsis sp. strain TFS65-07 and recombinant strains thereof, while TP-1161 production was performed in PM4 medium (15 g/liter glucose, 15 g/liter soy meal, 5 g/liter corn steep liquor, 2 g/liter CaCO3, 1× artificial seawater to 1 liter, pH 7.8). X. coeruleus M512 and recombinant strains thereof were maintained on MS or ISP2 agar.

Genome sequencing and de novo genome sequence assembly for Nocardiopsis sp. strain TFS65-07. Genomic shotgun sequencing of Nocardiopsis sp. strain TFS65-07 was performed by Fasteris SA (Switzerland) using the Illumina se-

Identification and bioinformatics analysis of the thiopeptide antibiotic TP- 1161 biosynthetic gene cluster. The predicted amino acid sequence of the un- 
modeled structural peptide constituting the backbone of thiopeptide antibiotic TP-1161 was deduced from the final, posttranslationally modified molecule (Fig. 1) and used for finding the Nocardiopsis sp. strain TFS65-07 draft genome. Manual annotation of genes surrounding the TP-1161 precursor peptide gene was done by identifying protein-coding DNA sequences using the FramePlot 4.0 Beta program (http://nocardia.nib.go.jp/p4k) and analyzing predicted protein sequences using the protein BLAST (Basic Local Alignment Search Tool) (Table 1).

Construction of Nocardiopsis sp. strain TFS65-07 cosmid library and screening for TP-1161 biosynthetic gene cluster. A genomic cosmid library of Nocardiopsis sp. strain TFS65-07 was constructed using the Stratagene SuperCos1 cosmid vector kit according to the manufacturer's instructions. Genomic DNA of Nocardiopsis sp. strain TFS65-07 was prepared using the Kirby mix procedure (15), partially digested with MboI and ligated into the XbaI-, BamHI-, and calf intestinal alkaline phosphatase (CIP)-treated SuperCos1 vector. E. coli XL1-Blue MR served as the host for constructing the genomic library. The library consisting of 3,072 clones was screened by colony hybridization by the method of Jørgensen et al. (13). A 385-bp fragment comprising the putative TP-1161 precursor peptide gene (146 bp) plus surrounding regions was ampliﬁed by PCR from genomic DNA of strain TFS65-07. The fragment was cloned into pDrive (Qiagen) and the target sequence was conﬁrmed by DNA sequencing. The fragment was then labeled using the PCR DIG probe synthesis kit (Roche Applied Science) and used to screen the library for cosmids containing the putative TP-1161 biosynthetic gene cluster. Three cosmids clones were selected for further analysis, and end sequencing of the corresponding cosmids suggested the presence of the putative TP-1161 biosynthetic gene cluster in all three of the clones. Cosmid 8-B9 was used for further studies.

Gene inactivation experiments in Nocardiopsis sp. strain TFS65-07. For ge- 

Nitrogen-resistance of strain TFS65-07, a gene transfer system in the form of an intergeneric conjugation using E. coli S17.1 as the donor was established. The pK15mob-based (27) vector pKES (see Table S1 in the supplemental material) was used for all gene inactivation experiments in strain TFS65-07. To construct precursors of in-frame gene fragments, the gene (146 bp) plus surrounding regions was ampliﬁed by PCR using the primers (Table S1 in the supplemental material). The obtained PCR products were cloned into pDrive (Qiagen) and confirmed by DNA sequencing using standard M13 

Construction of Nocardiopsis sp. strain TFS65-07 in-frame deletion. In-frame deletion of ptp in cosmid 8-B9 was created by A-Red-mediated recombination as described previously (7, 11) yielding cosmid 8-B9Δptp. The DELtpap_fwd and DELtpap_rev primers (DEL stands for dele-

Fig. 1. Molecular structure of the thiopeptide antibiotic TP- 1161.
<table>
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<th>Gene</th>
<th>Size of protein (no. of amino acids)</th>
<th>Protein homolog</th>
<th>Source of protein homolog</th>
<th>% identity/% similarity</th>
<th>Proposed function</th>
<th>Homolog(s) in another cluster(s)</th>
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* NCBI accession numbers are given in square brackets. LSU, large subunit; SSU, small subunit.
* Percent identity and percent similarity to the most homologous protein according to a BLAST search.
* Homologs in clusters for thiopeptide and other tailored peptide clusters. NCBI accession numbers are given in square brackets.
Methylation-deficient SuperCos1 backbone of cosmid 8-B9, generating 8-B9_int. The DraI-BsaI mycin-resistant recombinants. The M512 chromosome was confirmed by Southern blot analysis of kanamycin. 7096 ENGELHARDT ET AL. APPL. ENVIRON. MICROBIOL.

C-terminal structural peptides are separated by a dash. Conserved residues are displayed in boldface type. Berninamycin (BnmA), GE2270A (TpdA GE), and thiostrepton (TsrA) are shown. Putative thiopeptide precursors are indicated by an asterisk. (The cyclothiazomycin (CltA), nosiheptide (NosM), siomycin (SioH), thiocillin (TclE-H), TP-1161 (TpaA), an unknown thiopeptide (TpaX*), thiromycins (TpdA TM), GE2270A (TpdA GE), and thiostrepton (TsrA) are shown. Putative thiopeptide precursors are indicated by an asterisk. The C-terminal structural peptides are separated by a dash. Conserved residues are displayed in boldface type.

FIG. 2. (A) Alignment of two thiopeptide precursor peptides, TpaA and TpaX, encoded by the genomic locus for TP-1161 biosynthesis (the structural peptide of TP-1161 and suggested structural peptide for unknown thiopeptide are underlined). Residues in TpaX that are identical to those in TpaA are indicated by periods. Residues in TpaX that are different from those in TpaA are shown. (B) Conserved residues in leader peptides of 11 thiopeptide precursors. The leader peptides in thiopeptide precursors berninamycin (BmnA*), an unknown thiopeptide (BmnX*), cychothiazomycin (CltA), nosiheptide (NosM), siomycin (SioH), thiocillin (TclE-H), TP-1161 (TpaA), an unknown thiopeptide (TpaX*), thiromycins (TpdA TM), GE2270A (TpdA GE), and thiostrepton (TsrA) are shown. Putative thiopeptide precursors are indicated by an asterisk. The C-terminal structural peptides are separated by a dash. Conserved residues are displayed in boldface type.

was then subcloned into pDrive, digested with EcoRI, and ligated into the corresponding restriction site of pKE5. The obtained construct, pKE27, was introduced into strain TFS65-07 by intergeneric conjugation as described above. Kanamycin-resistant exconjugants where the vector had inserted via a single crossover event were confirmed by Southern blot analysis. To allow for a second crossover event and excision of the vector, confirmed single-crossover mutants were subjected to two rounds of sporulation under nonselective growth conditions. Via replica plating, the Km^+ colonies were identified and colony PCR using the Ver_fwd and Ver_rev primers (see Table S1 in the supplemental material) followed by Southern blot analysis were used to identify the tpa deletion mutants.

Analysis of Nocardiosis sp. strain TFS65-07 mutants for production of TP-1161 and its congeners. Mutant strains of Nocardiosis sp. strain TFS65-07 obtained from insertional mutagenesis and gene deletion experiments were grown on 0.75% ISP2 agar (Difco) (supplemented when necessary with 50 μg/ml kanamycin) until sporulated. Spores were used to inoculate 25 ml of seed medium in 500-ml baffled flasks. Seed cultures were incubated for 5 to 6 days at 25°C and 225 rpm until dense growth was observed. Two milliliters of this seed culture was used to inoculate 100 ml of PM4 fermentation medium in 500-ml baffled flasks. Both seed and fermentation cultures contained glass beads (3-mm diameter) to avoid excessive formation of mycelial pellets. Fermentation cultures were incubated at 25°C and 225 rpm for 7 to 10 days. One to 5 ml of fermentation cultures were used for extraction with the same volume of dimethyl sulfoxide (DMSO). After centrifugation (2,600 g, 10 min) and stored at −20°C. For detection of production of TP-1161 and its congeners, the LC-DAD-TOF analyses (analyses where a liquid chromatograph [LC] was connected to a diode array detector [DAD] and time of flight [TOF] apparatus) were performed as described previously (9).

Attempted heterologous expression of TP-1161 biosynthetic gene cluster in S. coelicolor M512. For heterologous expression of the TP-1161 biosynthetic gene cluster, cosmids 8-B9 was modified using λ-red-mediated recombination introducing the inte gene (int) and the attachment site (attP) of phage ΦC31 into the cosmid backbone by the method of Eustaquio et al. (10). The DraI-BsaI fragment with inte cassette of pD787 was used to replace the bla gene in the S. coelicolor M512 chromosome. To isolate the biosynthetic gene cluster for thiopeptide antibi-obotic TP-1161, the predicted TP-1161 precursor peptide sequence was used to mine the draft genome sequence obtained with the genome mining tool (TOP) apparatus) were performed as described previously (9).

Analysis of S. coelicolor M512 recombinants for the TP-1161 production. To analyze the S. coelicolor M512 recombinants for the TP-1161 production, a range of fermentation media and cultivation conditions were used. Cultivations were performed in microwell plates as described previously (9) using 0.3× F134 (22), PM4, and seven additional media composed of components of either PM4 or 0.3× F134 medium (see Table S2 in the supplemental material). The wild-type TP-1161 producer Nocardiosis sp. strain TFS65-07, the parental S. coelicolor M512 strain, and S. coelicolor M145 were used as control strains. Seed cultures of all strains were prepared in 250-ml baffled flasks containing 50 ml of 2× YT medium (15) which was inoculated from frozen cell suspensions. Cultivations were performed at 30°C and 225 rpm until dense growth could be observed. If necessary, the mycelium of seed cultures was harvested, fragmented, mechanically, and reinculcated in fresh medium several times during cultivation to obtain homogeneous inoculums. The obtained seed cultures were used to inoculate the fermentation medium (3% in 800 μl fermentation medium). Fermentations were performed at 800 rpm and 85% humidity for 6 days at 30°C (S. coelicolor strains) or 14 days at 25°C (strain TFS65-07). Entire fermentation cultures were freeze-dried and extracted with DMSO (400 μl), and the extracts were analyzed for the presence of TP-1161 by LC-DAD-TOF as described previously (9).

Nucleotide sequence accession number. The sequence of the gene cluster surrounding the TP-1161 precursor peptide gene was submitted to GenBank and can be retrieved under accession number HM467197.

RESULTS AND DISCUSSION

Genome mining for the TP-1161 biosynthetic gene cluster. To isolate the biosynthetic gene cluster for thiopeptide antibiotic TP-1161, the predicted TP-1161 precursor peptide sequence was used to mine the draft genome sequence obtained for the producer strain, Nocardiosis sp. strain TFS65-07. A 147-bp-long open reading frame encoding the putative 48-amino-acid aa TP-1161 precursor peptide (designated TpaA) was identified. The tpaA product consisted of the 33-aa N-terminal leader peptide followed by the 15-aa structural peptide (Fig. 2A). The structural peptide sequence was found to be in agreement with the posttranslationally modified TP-1161 backbone except for the C-terminal threonine (Fig. 1), which was present in the structural peptide, but not in the mature TP-1161 molecule.

While no overall sequence homology could be detected between leader sequences of different thiopeptides, the leader sequences were found to be rich in acidic amino acids, and it was suggested that glutamic and aspartic acid residues might play important roles in substrate recognition by posttranslation modification enzymes (32). An alignment of 11 (puta-
Bioinformatics analysis of the TP-1161 gene cluster and functional confirmation of the TP-1161 biosynthetic locus. Bioinformatics analysis of the genomic region surrounding \( tpaA \) revealed 12 downstream open reading frames (ORFs) (designated \( tpaB \) to \( tpaL \)) proposed to encode enzymes for posttranslational modification of TpaA and self-resistance (Fig. 3 and Table 1). Enzymes mediating heterocyclization and dehydroalanine and dehydrobutyrine groups in thiopeptides are usually present as a set of moderately conserved proteins, with relatively low similarity values (typically around 50%) between homologs (16). Many of these proteins also have homologs in biosynthetic pathways for other tailored ribosomal peptides, including the lantibiotics (30), cyanobactins (8), goadsporin (23), and microcin B17 (31).

To verify that the assigned genomic region was indeed governing the biosynthesis of thiopeptide antibiotic TP-1161, two genes (\( tpaC \) and \( tpaH \)) encoding putative cyclodehydratases were chosen as targets for gene disruption experiments. Inactivation was accomplished through gene disruption by introducing suicide plasmids carrying internal fragments of the respective genes into \( Nocardiopsis \) sp. strain TFS65-07 via intergeneric conjugation. As expected, the inactivation of the two proposed cyclodehydratase-encoding genes resulted in complete loss of TP-1161 production, thus verifying the involvement of the proteins encoded by the genes in the biosynthesis of TP-1161 (Fig. 4). Complementation of \( tpaC \) and \( tpaH \) mutants was not attempted, since we did not possess replicating vectors for this species or suitable selective markers except for apramycin, which has already been used in the gene disruption constructs. From the organization of the genes, we can safely assume that at least in the case of \( tpaH \), the observed loss of TP-1161 production was due to a polar effect.

Uncommon paralogs for conserved thiopeptide modification enzymes. The characteristic thiazole and (methyl)oxazole heterocycles appear in thiopeptides due to the cyclodehydration of serine, cysteine, and threonine residues, where the side chains of these amino acids are cyclized against the preceding carbonyl groups, yielding dehydroheterocycles. Subsequent oxidation can convert the resulting thiazoline and oxazoline groups to their aromatic forms (25). Cyclodehydratases and dehydrogenases commonly appear as a set of two genes in thiopeptide clusters encoding relatively conserved enzymes (16). However, functional assignment of the genes constituting the proposed biosynthetic locus for thiopeptide antibiotic TP-1161 indicated the presence of three predicted cyclodehydratases (\( tpaC \), \( tpaD \), and \( tpaH \)) (Table 1). The two enzymes, TpaE and TpaF, showed homology to the flavin mononucleotide (FMN)-dependent McbC-like oxidoreductases, which suggests that they may be possible candidate enzymes mediating thiazoline/oxazoline oxidation and thus yielding the thiazole and (methyl)oxazole groups present in TP-1161 (Fig. 1). Paralogous genes encoding additional cyclodehydratases and dehydrogenases have so far only been described for the GE2270A biosynthetic locus, where the paralogs are assumed to be involved in modification of the side chain attached to the pyridine-6-position introducing the C-terminal oxazoline (21). The proposed TP-1161 biosynthetic gene cluster contains a total of five genes encoding enzymes which can be associated with polyazole formation through heterocyclization (\( tpaC \), \( tpaD \), and \( tpaH \)) of serine, cysteine, and threonine residues in TpaA followed by oxidation (\( tpaE \) and \( tpaF \)) (Fig. 3 and 5A).

Dehydrations of serine and threonine residues yielding dehydroalanine and dehydrobutyryl groups in thiopeptides are mediated by enzymes resembling LanB-type lantibiotic dehydratases, and typically there are at least two homologs present in thiopeptide clusters (16). TpaK and TpaL share 34% similarity with lantionine biosynthesis proteins (Table 1) and are therefore predicted to be involved in amino acid dehydration and formation of the four dehydroalanine groups and single dehydrobutyryl group present in TP-1161 (Fig. 5A).

The proposed TP-1161 cluster contains a second precursor peptide gene. Annotation of the predicted TP-1161 biosynthetic gene cluster also identified a second putative thiopeptide precursor gene (\( tpaX \)) located about 12 kb downstream of \( tpaA \) (Fig. 3). Both peptides encoded by these two genes have the same length (48 aa) and share 70% identity on amino acid level (Fig. 2A). However, attempts to detect the mature product of \( tpaX \) in \( Nocardiopsis \) sp. strain TFS65-07 TFS65-07 fermentation extracts have been unsuccessful. Multiple copies of thiopeptide precursor genes have been reported, for example, for the thiocillins, where the thiocillin biosynthetic gene cluster contains four identical copies of precursor peptides genes (4). The functions of these tandem genes are not clear, but Acker et al. demonstrated that only one plasmid-based gene copy introduced into a mutant with all four genes for precursor peptide deleted was sufficient to restore thiocillin biosynthesis.
FIG. 4. Analysis of the fermentation extracts of the TP-1161 wild-type producer *Nocardiopsis* sp. strain TFS65-07 and TP-1161 production gene disruption mutants. (A and B) TOF MS spectrum (A) and LC-DAD isoplot (B) of wild-type strain TFS65-07 extract; (C and D) LC-DAD isoplots of *tpaC* and *tpaH* disruption mutants. amu, atomic mass units; mAU, milli absorbance units.
Also, the proposed berninamycin cluster in the genome of Propionibacterium acnes KPA171202, which was identified in a bioinformatics search for thiazolyl peptide-encoding genes by Wieland Brown et al. (29), contains two genes encoding distinct putative precursor peptides. One of these genes is predicted to encode a 47-aa precursor peptide with the 15-aa berninamycin structural peptide (SCTTTSVSTSSSSSS) at its N terminus (29), whereas the second 58-residue precursor peptide (SCVETSVSSSSTSSS) differs in four positions from the structural peptide of berninamycin (the four different amino acids are underlined). However, a mature product for the second precursor has not been reported so far.

**Proposed pathway for the macrocyclization of TP-1161 precursor.** The exact mechanisms of macrocyclization and formation of the central 6-membered nitrogen-containing heterocycle in thiopeptides have not been experimentally verified yet. The currently promoted hypothesis involves cycloaddition of three precursor amino acids, identified by feeding studies as two serines and the carboxyl group of one cysteine residue (19, 20), yielding a dehydropiperidine intermediate (16). According to a mechanism proposed by Bycroft and Gowland on the constitution of the pyridine ring in micrococcins P₁ and P₂, cyclization of these three residues requires dehydration of the two serine residues (5). The pyridine ring of series d thiopeptides could then be obtained via dehydration of the resulting dehydropyridine moiety (16).

On the basis of the conducted homology searches, TpaH is proposed to represent a cyclohydratase involved in the intramolecular cyclization of TP-1161 precursor (Table 1). In addition to the characteristic YcaO family domain (residues 235 to 579), a signature of a putative serine peptidase domain (residues 235 to 579) was found at the TpaH C terminus, indicating a possible function of this protein in both macrocyclization and leader peptide cleavage. TpaH-mediated cyclodehydration of the N-terminal cysteine residue could provide the carboxyl group necessary for the formation of the dehydropyridine intermediate. Dehydration of two serine residues by a putative dehydratase TpaK and cycloaddition of the resulting dehydroalanine groups with the cysteine-derived carboxyl are presumably coupled to the elimination of the leader peptide and could yield the dehydropiperidine intermediate.

While the insertional inactivation of *tpaH* proved the involvement of TpaH in TP-1161 biosynthesis, the results of this experiment did not allow any evaluation of the above-mentioned hypothesis on macrocyclization and leader peptide cleavage, since the knockout mutant did not produce any metabolites that could be linked to the TP-1161 biosynthetic gene cluster.
TP-1161 putative mode of action and host resistance. In addition to the genes encoding enzymes for posttranslational modification, the TP-1161 cluster also contained a predicted 23S rRNA methyltransferase (Tpai) with high sequence homology (66% identity) to the methyltransferase of *Streptomyces* *auratus* conferring resistance to the thioppeptide antibiotic thiostrepton (28). Thus, Tpai most likely provides for the self-resistance to TP-1161 in the producer *Nocardiosis* sp. strain TFS65-07.

The core biosynthetic genes (*tpaA* to *tpaL*) are flanked by various genes encoding 50S and 30S ribosomal proteins (Fig. 3 and Table 1). Notable here is the presence of the 50S ribosomal protein L11 (*tpaL*) in close proximity to the biosynthetic genes, which suggests that L11 may be a molecular target for TP-1161 action. Sequence analysis of the *tpaL*-encoded L11 protein revealed mutations in both conserved residues located within the putative thioppeptide (thiostrepton) binding site (see Fig. S1 in the supplemental material), further strengthening this hypothesis, since mutations of these key amino acids in the N-terminal domain of L11 have previously been shown to confer thiostrepton resistance (6). Other genes encoding potential thioppeptide targets were also found in the immediate surroundings of the cluster, including the bacterial elongation factor Tu (EF-Tu, encoded by *tpa11*). Producers of the EF-Tu-targeting thioppeptides, such as the thiomuracin and GE2270A, avoid self-intoxication through nucleotide exchanges in their EF-Tu-encoding *tuf* genes causing replacement of glycine 275 (*E. coli* numbering) (21). However, no such mutations could be identified in the *tpa11* gene, thus leaving open the question of whether EF-Tu is a target for TP-1161.

Modification of the TP-1161 C-terminal tail. The termini of most thioppeptide tails are represented by carboxamide or carboxyl groups. TP-1161, however, possesses an unusual terminal aminoacetone moiety which, according to the structural peptide sequence, is derived from threonine. Tpaj exhibits sequence similarity with iron-containing alcohol dehydrogenases, in particular with 4-hydroxybutyrate dehydrogenases, and could be a possible candidate for tailoring the TP-1161 terminus. 4-Hydroxybutyrate dehydrogenases catalyse oxidation of the 4-hydroxybutanoic acid to succinate semialdehyde. Structural similarity between 4-hydroxybutanoic acid and threonine indicates a possible involvement of Tpaj in oxidation of the terminal threonine residue. Oxidation of threonine followed by spontaneous decarboxylation and tautomerization of the resulting keto-to-enol form could then yield aminoacetone (Fig. 5B). To investigate this hypothesis, an in-frame deletion mutant of *tpap* was constructed and analyzed for the production of the presumed TP-1161 derivative with C-terminal threonine. However, fermentation extract analyses proved a complete loss of TP-1161 production, and no derivatives related to TP-1161 could be detected by a thorough mass spectrometry (MS) scan (data not shown).

Cloning and heterologous expression of the TP-1161 gene cluster. To establish a versatile platform for genetic manipulation and functional analysis of the TP-1161 maturation machinery, the TP-1161 cluster was isolated from a genomic cosmid library of *Nocardiosis* sp. strain TFS65-07, and heterologous expression of the cluster in *S. coelicolor* M512 was attempted. The cosmid library of strain TFS65-07 was probed with a PCR-amplified genomic fragment comprising the TP-1161 precursor peptide gene and surrounding regions. End sequencing of cosmid 8-B9 identified in such screen confirmed the presence of the entire proposed TP-1161 biosynthetic gene cluster plus surrounding regions (Fig. 3) on this cosmid, which was used for further work.

For site-specific integration of the TP-1161 gene cluster into the chromosome of *S. coelicolor* M512, the SuperCos1 backbone of cosmid 8-B9 was modified by λ-Red-mediated recombination introducing the integrase gene (int) and the attachment site (attP) of phage FC31. Site-specific integration mutants produced TP-1161 under conditions tested (data not shown). To analyze whether this result was due to a general lack of expression of genes in the TP-1161 cluster, both the parental and recombinant strains of M512 were tested for growth on agar medium supplemented with thiostrepton. While the parental M512 strain was found to be sensitive to thiostrepton, the recombinant strains were resistant to thiostrepton, indicating that expression of the *tpaP*-encoded 23S rRNA methyltransferase conferred this phenotype. The reasons for the lack of TP-1161 production in the recombinants are not known, and gene expression studies in both original and heterologous hosts designed to address this question are under way.

**ACKNOWLEDGMENTS**

We are grateful to Michael Kemmler for suggestions on the mechanism of aminoacetone group formation, to Mervyn Bibb for kindly providing *S. coelicolor* M512, and to Håvard Sletta for help with design of the fermentation experiments.

This work was supported by the Norwegian University of Science and Technology and the Research Council of Norway.

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