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tRNA-Dependent Aminoacylation of an Amino Sugar Intermediate in the Biosynthesis of a Streptothricin-Related Antibiotic

Chitose Maruyama,[a] Haruka Niikura,[a] Miho Izumikawa,[b] Junko Hashimoto,[b] Kazuo Shin-ya,[c] Mamoru Komatsu,[d] Haruo Ikeda,[d] Makoto Kuroda,[e] Tsuyoshi Sekizuka,[e] Jun Ishikawa,[e] Yoshimitsu Hamano[a]

Department of Bioscience, Fukui Prefectural University, Yoshida-Gun, Fukui, Japan[a]; Japan Biological Informatics Consortium (JBIC), Tokyo, Japan[b]; National Institute of Advanced Industrial Science and Technology, Tokyo, Japan[c]; Kitasato Institute for Life Sciences, Kitasato University, Sagamihara, Kanagawa, Japan[d]; National Institute of Infectious Diseases, Tokyo, Japan[e]

ABSTRACT

The antibiotic streptothricin (ST) possesses an amino sugar bound to an L-β-lysine (β-Lys) residue via a peptide bond. The peptide bond formation has been shown to be catalyzed by a nonribosomal peptide synthetase (NRPS) during ST biosynthesis. The focus of this study is the closely related ST analogue BD-12, which carries a glycine-derived side chain rather than a β-Lys residue. Here, in *Streptomyces luteocolor* NBRC13826, we describe our biosynthetic studies of BD-12, which revealed that the peptide bond between the amino sugar and the glycine residue is catalyzed by a Fem-like enzyme (Orf11) in a tRNA-dependent manner rather than by an NRPS. Although there have been several reports of peptide bond-forming tRNA-dependent enzymes, to our knowledge, Orf11 is the first enzyme that can accept an amino sugar as a substrate. Our findings clearly demonstrate that the structural diversity of the side chains of ST-type compounds in nature is generated in an unusual manner via two distinct peptide bond-forming mechanisms. Moreover, the identification and functional analysis of Orf11 resulted in not only the production of new ST-related compounds, but also the provision of new insights into the structure-activity relationship of the ST-related antibiotics.

IMPORTANCE

The antibiotic streptothricin (ST) possesses an amino sugar bound to an L-β-lysine (β-Lys) side chain via a peptide bond formed by a nonribosomal peptide synthetase (NRPS). BD-12, an analogue of ST, carries a glycine-derived side chain rather than β-Lys, and here, we describe the BD-12-biosynthetic gene cluster from *Streptomyces luteocolor* NBRC13826, which contains the *orf11* gene encoding a novel tRNA-dependent peptide bond-forming enzyme. The unique Fem-like enzyme (Orf11) accepts the amino sugar as a substrate and mediates the peptide formation between the amino sugar intermediate and glycine. Our studies demonstrate that the structural diversity of the side chains of ST-related compounds in nature is generated via two distinct peptide bond-forming mechanisms.

Streptothricins (STs) produced by *Streptomyces* strains are broad-spectrum antibiotics and are chemically characterized by the L-β-lysine (β-Lys) residue and its oligomeric side chains [oligo(β-Lys)]. Since the initial identification, in 1943, of ST-F with one β-Lys residue as the first member of the ST group of antibiotics (1), STs with an oligo(β-Lys) consisting of two to seven residues have been identified (Fig. 1). ST-F inhibits protein biosynthesis in prokaryotic cells (2), and STs carrying the longer oligo(β-Lys) side chains show higher levels of antibacterial activity. Moreover, STs strongly inhibit the growth of eukaryotes, such as yeasts (3–5), fungi (6), protozoa (7), insects (8), plants (9), and mammals (10–13). Although STs have been used effectively as selective agents for recombinant DNA work in some of these organisms, STs are not currently used therapeutically due to their inherent toxicity. In addition to the STs, it has been reported that *Streptomyces* strains produce ST analogues that possess a glycine-derived side chain rather than the β-Lys residue: BD-12 (14, 15), citromycin (16, 17), glycinothricin (18), A-269A (19), and A-269A′ (19) (Fig. 1). These analogues display potent antibacterial activities, although their molecular targets remain unclear. Also, like the STs, the ST analogues are not used clinically due to their toxicity.

We previously identified the ST-biosynthetic gene cluster (accession no. AB684619) in *Streptomyces rochei* NBRC 12908 (Fig. 2) and elucidated the biosynthetic mechanisms of the oligo(β-Lys) side chains (20). Nonribosomal peptide synthetases (NRPSs) are known to catalyze the assembly of a myriad of structurally complex peptide natural products (21). However, in the previous study, we identified three unique stand-alone NRPSs among the ST-biosynthetic enzymes and showed that they assembled the structurally simple peptide oligo(β-Lys) (Fig. 3A). The biosynthesis is initiated by adenylation of β-Lys in Orf5 (stand-alone adenylation [A] domain), and the resulting L-β-lysyl-*O*-AMP is loaded

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Address correspondence to Yoshimitsu Hamano, hamano@fpu.ac.jp.

C.M. and H.N. contributed equally to this research.

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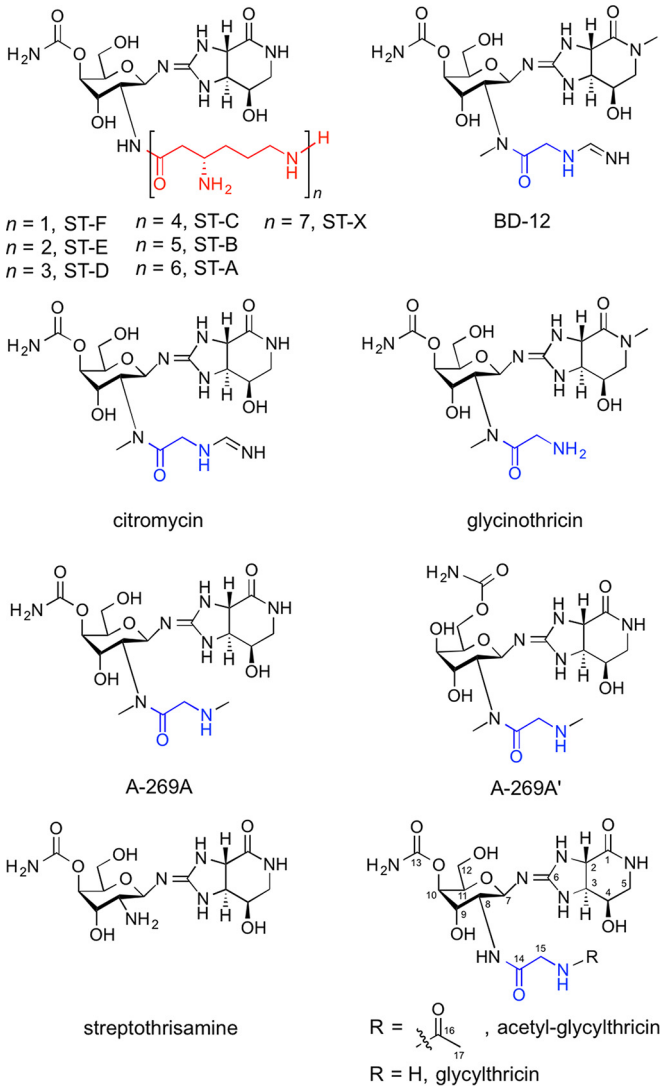

FIG 1 Chemical structures of STs and the ST-related antibiotics. The β-Lys and glycine residues are shown in red and blue, respectively.

onto the thiolation (T) domain of Orf18. β-Lys molecules adenylated by Orf19 (a second stand-alone A domain) are not directly loaded onto the T domain but are used as extending units for elongation of the oligo(β-Lys) chain on Orf18. Surprisingly, peptide bond formations for the elongation are iteratively catalyzed by Orf19 itself. The condensation (C) domain of Orf18 catalyzes the subsequent condensation reaction between the covalently bound oligo(β-Lys) (or β-Lys) and a freely diffusible amino sugar intermediate, streptothrisamine, ultimately releasing STs (20). Thus, the peptide bond between streptothrisamine and the amino acid side chain is formed by the NRPS machinery during ST biosynthesis.

In consideration of this, we hypothesized a similar NRPS pathway for glycine attachment in the biosynthesis of the ST analogues. Among the ST analogues, we focus here on BD-12 (Fig. 1), produced by *Streptomyces luteocolor* NBRC 13826, and describe the identification of its biosynthetic gene cluster. We demonstrate that, unexpectedly, the formation of a peptide bond between the streptothrisamine and the glycine residue is catalyzed, not by an NRPS, but by a Fem-like enzyme (Orf11) in a tRNA-dependent manner. Thus, the structural diversity of the side chains of the ST-type antibiotics occurring in nature is generated by two distinct peptide bond-forming mechanisms.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. luteocolor* NBRC 13826 was used as a BD-12 producer. *Streptomyces lividans* TK23 and *Streptomyces avermitilis* SUKA17 (22) were used as heterologous host strains for the gene expression experiments (Table 1). *Streptomyces* integrating vectors, pKU493A_aac(3)IV and pKU1016 (22), were used for the gene expression experiments (Table 1).

Draft genome sequencing of *S. luteocolor* NBRC 13826. Draft genome sequences of *S. luteocolor* NBRC 13826 were determined by using a MiSeq desktop sequencer (Illumina, San Diego, CA, USA). A genomic library with an insert size of 500 bp was constructed by using a Nextera XT DNA Sample Prep kit (Illumina). The sequencing run yielded 19,156,126 reads (accession no. DRA003165), with 300-bp paired-end sequences, providing over 600-fold genome coverage. The genome was assembled using a GS *de novo* Assembler (Newbler) version 2.7 (454 Life Sciences, Branford, CT, USA). The final assembly consisted of 262 scaffolds containing 9,298,666 bp, with a G+C content of 71.5% and an N_{50} scaffold size of 56,991 bp. Gene prediction was carried out with MetaGeneAnnotator (23) to identify the BD-12-biosynthetic gene cluster (accession no. LC122485).

Cloning of the BD-12-biosynthetic gene cluster. To clone the putative BD-12-biosynthetic gene cluster, the bacterial artificial chromosome (BAC) genomic library of *S. luteocolor* NBRC 13826 was prepared according to a method previously reported (24). Clones carrying the entire gene cluster for BD-12 biosynthesis were screened by PCR using two pairs of primers corresponding to the upstream and downstream regions of the gene cluster, ORF1_F plus ORF1_R and ORF26-ORF27_F plus ORF26-ORF27_R, respectively (Table 2). In the PCR screening, we found one of the BAC clones, pKU518BD12P2-C23 (inset size, 200,999 bp), which contained the putative BD-12-biosynthetic gene cluster (34 kbp) (Table 1 and Fig. 2).

The 34-kbp DNA fragment was further subcloned from pKU518BD12P2-C23 by an *in vivo* gene replacement mediated by λ-Red recombinase. Approximately 500-bp homologous regions upstream and downstream of the gene cluster were amplified by PCR with pKU518BD12P2-C23 as a template using two primer pairs, BD-12cluster_up_SpeI_F plus BD-12cluster_up_BsrGI_R and BD-12cluster_down_BsrGI_F plus BD-12cluster_down_NheI_R, respectively (Table 2). The two amplified fragments were ligated with pRED-sacB-aph (Table 1) (22). The resulting plasmid, BD12cluster-up-down/pRED, was linearized by PCR using the primer pair BD-12cluster_down_BsrGI_F and BD-12cluster_up_BsrGI_R (Table 2). The amplified fragment was mixed with the BAC clone, pKU518BD12P2-C23, that had been linearized by digestion with BsrGI. The mixture was introduced into *Escherichia coli* BW25113 carrying pKD46 (http://cgsc.biology.yale.edu/), in which λ-Red recombinase had been expressed, to generate a circular plasmid (pRED/BD-12cluster) (Table 1) that carried the 34-kbp fragment of the putative BD-12-biosynthetic gene cluster. After the digestion of pRED/BD-12cluster with SpeI and NheI, a 34-kbp DNA fragment was obtained and cloned into a *Streptomyces* integrating vector, pKU493A_aac(3)IV, to generate pKU493A-BD12 (Table 1).

Heterologous expression of the BD-12-biosynthetic gene cluster in *S. lividans* TK23 and *S. avermitilis* SUKA17. The constructed integration vector, pKU493-BD12, was introduced into two heterologous host strains, *S. lividans* TK23 and *S. avermitilis* SUKA17, by standard procedures (24, 25). The *S. lividans* TK23 transformant harboring pKU493-BD12 was cultured in S10.3 medium (20) for 2 days at 28°C. The *S. avermitilis* SUKA17 transformant harboring pKU493-BD12 was cultured in AVM medium containing 6% (wt/vol) glucose, 0.2% (wt/vol) yeast extract (Difco Laboratories, Franklin Lakes, NJ, USA), 0.2% (wt/vol)

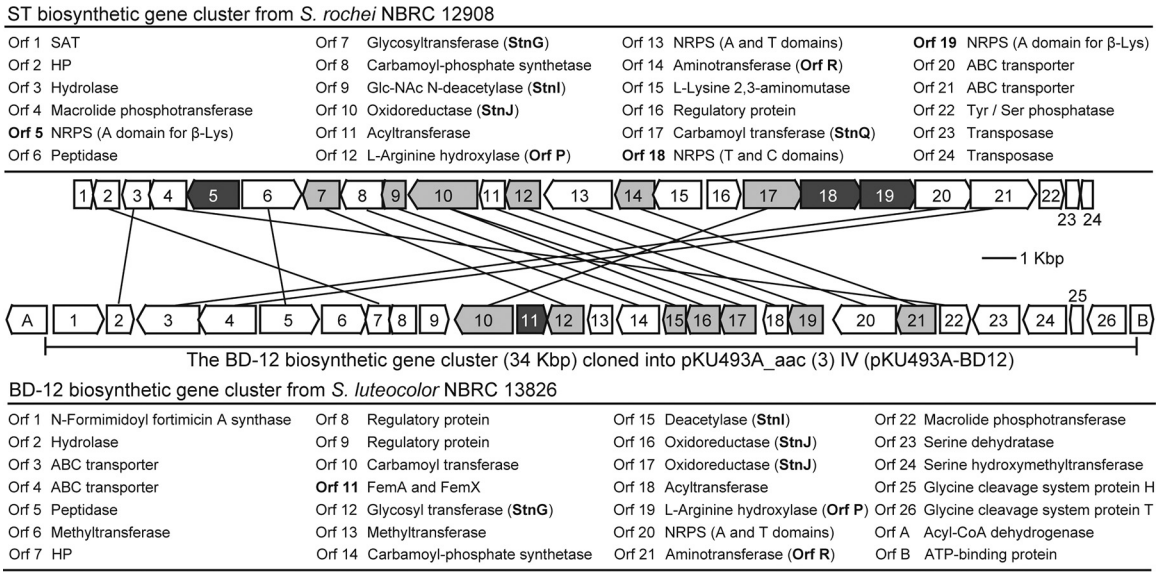

FIG 2 Gene organization of the ST- and BD-12-biosynthetic gene clusters. The ST-biosynthetic gene cluster (accession no. AB684619) from *S. rochei* NBRC 12908 (top) and the BD-12-biosynthetic gene cluster (accession no. LC122485) from *S. luteocolor* NBRC 13826 (bottom) are shown. The dark-gray-shaded genes are the genes responsible for the peptide bond formation between streptothrisamine and the amino acid residues. The light-gray-shaded genes are the genes responsible for streptothrisamine biosynthesis. The lines connect homologuous genes in the two biosynthetic gene clusters.

$(NH_4)SO_4$, 0.5% (wt/vol) $CaCO_3$, 0.2% (wt/vol) NaCl, 0.05% (wt/vol) K_2HPO_4, 0.01% (wt/vol) $MgSO_4 \cdot 7H_2O$, 0.005% (wt/vol) $FeSO_4 \cdot 7H_2O$, 0.005% (wt/vol) $ZnSO_4 \cdot 7H_2O$, and 0.005% (wt/vol) $MnSO_4 \cdot 4H_2O$ (pH 7.0) for 5 days at 28°C. To examine BD-12 productivity, the supernatants of the culture broth were analyzed by high-performance liquid chromatography and high-resolution electrospray ionization mass spectrometry analysis (HPLC-HR–ESI-MS) (LTQ/Orbitrap; Thermo Scientific, Waltham, MA, USA) or HPLC–ESI-MS (Esquire 4000; Bruker, Billerica, MA, USA).

Heterologous coexpression of the ST-biosynthetic gene cluster possessing in-frame deletions of the *orf18* gene and the *orf11* gene from the BD-12-biosynthetic gene cluster. The ST-biosynthetic gene cluster (30 kbp) possessing an in-frame deletion of the *orf18* gene (STΔorf18) of *S. rochei* NBRC12908, which was constructed in a previous study (20), was cloned into the pRED-sacB-aph vector using the same methods used for the construction of pRED/BD-12cluster. The STΔorf18 fragment was then cloned into the integrating vector, pKU493A_aac(3)IV, to generate pKU493A-STΔorf18 (Table 1). pKU493A-STΔorf18 was introduced into *S. avermitilis* SUKA17, and the resulting transformant was used for further experiments.

The *orf11* gene from the BD-12-biosynthetic gene cluster was amplified by PCR using one set of primers (orf 11_BamHI_F and orf 11_HindIII_R) (Table 2). The amplified DNA fragment was ligated into a *Streptomyces* integrating vector, pKU1016, to yield pKU1016-orf11 (Table 1). The pKU1016-orf11 vector was then introduced into the *S. avermitilis* SUKA17 transformant harboring pKU493A-STΔorf18, and the resulting transformant was cultured in AVM medium for 5 days at 28°C. The supernatant of the culture broth was analyzed by HPLC–ESI-MS.

Purification of acetyl-glycylthricin. The culture broth (1,000 ml) of the *S. avermitilis* SUKA17 transformant harboring pKU493A-STΔorf18

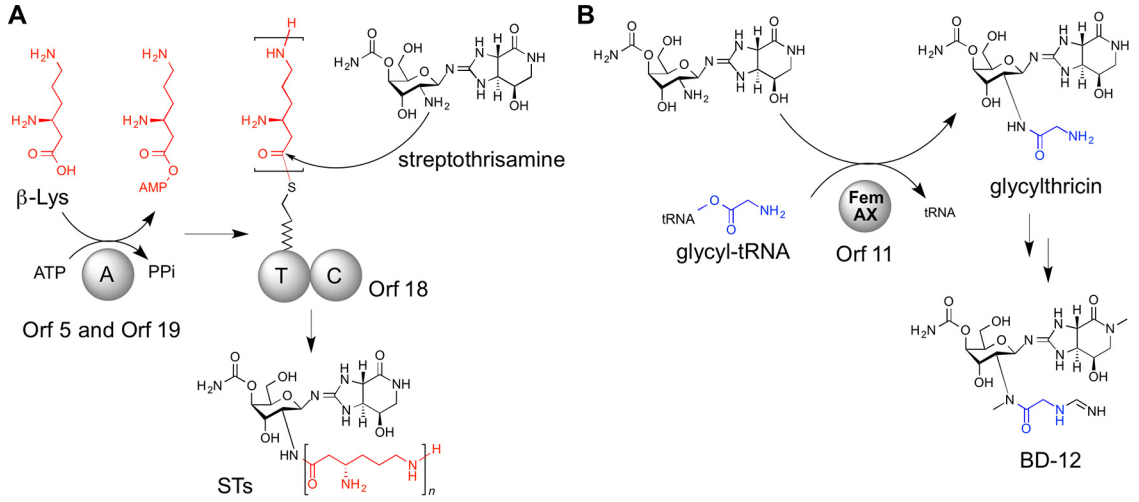

FIG 3 Peptide bond formation between amino sugars and amino acids in ST (A) and BD-12 (B) biosyntheses. The β-Lys and glycine residues are shown in red and blue, respectively.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference(s)
Streptomyces strains		
S. luteocolor NBRC 13826	BD-12 producer	14, 15
S. lividans TK23	Host strain for gene expression experiments	25
S. avermitilis SUKA17	Host strain for gene expression experiments	22
Plasmids		
pKU493A_aac(3)IV	Apm^r, *oriT* of RK2, pMB1 *ori*; ϕK38-1-integrating vector derived from pKU460	22
pKU1016	Neo^r (*aphII*), *oriT* of RK2, pMB1 *ori*; ϕC31-integrating vector derived from pKU460	22
pKU518	Amp^r Neo^r (*aphII*); ϕBT1-integrating BAC vector	24
pRED-sacB-aph	Neo^r (*aphII*) *sacB* Cm^r; cloning vector for *E. coli*	22
pKU518BD12P2-C23	pKU518 carrying the genomic DNA fragment (200,999 bp) that contains the BD-12-biosynthetic gene cluster (34 kbp) from *S. luteocolor* NBRC 13826	This study
pRED/BD-12cluster	pRED-sacB-aph carrying the BD-12-biosynthetic gene cluster (34 kbp) from *S. luteocolor* NBRC 13826	This study
pKU493A-BD12	pKU493A_aac(3)IV carrying the BD-12-biosynthetic gene cluster (34 kbp) from *S. luteocolor* NBRC 13826	This study
pKU493A-STΔorf18	pKU493A_aac(3)IV carrying the ST-biosynthetic gene cluster (30 kbp) with in-frame deletion of the *orf18* gene (STΔorf18) from *S. rochei* NBRC12908	This study
pKU1016-orf11	pKU1016 carrying the *orf11* gene from the BD-12-biosynthetic gene cluster	This study

[a] Apm^r, apramycin resistant; Neo^r, neomycin resistant; Cm^r, chloramphenicol resistant; Amp^r, ampicillin resistant.

and pKU1016-orf11 was centrifuged, and the supernatant obtained was mixed with 1,000 ml of chloroform. After vigorous shaking, the aqueous layer from the centrifugation was mixed with 200 ml acetonitrile and the mixture was filtered. The filtrate was loaded onto a Dowex 50W × 4 column (100 to 200 mesh; H^+ form; 4.0 by 10 cm; Dow Chemical, Midland, MI, USA), and the column was washed with 200 ml of 20% (vol/vol) acetonitrile in water. The sample was eluted in a stepwise fashion with 200 ml of 0.1, 0.15, 0.3, 0.45, and 0.6 M NaCl in 20% (vol/vol) acetonitrile. The acetyl-glycylthricin fractions eluted with 0.15, 0.3, and 0.45 M NaCl were combined. After removal of the organic solvent, the aqueous layer was lyophilized to give a white powder. This sample was dissolved in 100 ml of methanol, the insoluble materials were removed by centrifugation, and the methanol-soluble fraction was evaporated. The dried sample was dissolved in a small volume of water and fractionated by preparative HPLC using a reversed-phase column (Sunniest RP-AQUA; 5 μm; 250 by 10 mm; ChromaNik Technologies) at 40°C at a flow rate of 7 ml min^{-1} and with a mobile phase composed of 2% (vol/vol) acetonitrile and 0.1% (vol/vol) *n*-heptafluorobutyric acid (HFBA). Fractions were collected and monitored with a UV detector at 210 nm. The fraction containing acetyl-glycylthricin was lyophilized, dissolved in a small volume of 50% (vol/vol) acetonitrile in water, and fractionated by preparative HPLC using a hydrophilic-interaction liquid chromatography (HILIC) column (ZIC-HILIC; 5 μm; 250 by 10 mm; Merck, Kenilworth, NJ, USA) at 55°C at a flow rate of 8 ml min^{-1} and with a mobile phase composed of 77% (vol/vol) acetonitrile, 5 mM $HCOONH_4$, and 0.1% (vol/vol) formic acid (FA). Fractions were collected and monitored with a UV detector at 210 nm. The fraction containing acetyl-glycylthricin was lyophilized to give a white powder. This sample was dissolved in a small volume of water and fractionated by preparative HPLC using a reversed-phase column under the conditions described above. The fraction containing acetyl-glycylthricin was lyophilized and dissolved in a small volume of 50% (vol/vol) acetonitrile in water. To remove the residual HFBA from the sample, the solution was passed through a Varipure IPE column (Agilent Technologies, Santa Clara, CA, USA). The sample was lyophilized to obtain the purified acetyl-glycylthricin (approximately 1.5 mg), whose chemical structure was then determined by nuclear magnetic resonance (NMR) analysis.

Overexpression and purification of rOrf11 and rOrf1-SAT. The following two sets of PCR primers were designed and used to amplify the *orf11* gene from the BD-12-biosynthetic gene cluster and the *orf1* gene (named *orf1-SAT* in this study) (ST acetyltransferase [SAT]) from the ST-biosynthetic gene cluster: rORF11_BamHI_F and rORF11_HindIII_R for construction of N-terminally His_6-tagged Orf11 (rOrf11) and S.rochei_SAT_F and S.rochei_SAT_R for N-terminally His_6-tagged Orf1-SAT (rOrf1-SAT) (Table 1). The amplified fragments (*orf11*, 1.0 kbp; *orf1-SAT*, 0.7 kbp) were then ligated into pQE30 (Qiagen,

TABLE 2 Oligonucleotides used in this study

Oligonucleotide	Sequence
ORF 1_F	5′-GGGGGATCCTCGAAGACGGCCGACGCGCCAGGT-3′
ORF 1_R	5′-ACCAAGCTTTCAGTGCCACTCCCGG TGGACGTC-3′
ORF 26-ORF 27_F	5′-ATCTGTCATCGGAACCTGAGAGGTTCG-3′
ORF 26-ORF 27_R	5′-AAGAGCATTCCGGTGCCGCCGGATCCGT-3′
BD-12cluster_up_SpeI_F	5′-CCACAGACTAGTCCTTCCCTTCGGAGGGAATCTTGG-3′
BD-12cluster_up_BsrGI_R	5′-CACGAGTGTACATCGCCTGGCAGATCGGAGTGCAGG-3′
BD-12cluster_down_BsrGI_F	5′-CACGAGTGTACAACATCATTGCACGCGACTCTGTCC-3′
BD-12cluster_down_NheI_R	5′-CTAGCTAGCTGGGTGCCGCAGTCGTGCACGACGACG-3′
orf 11_BamHI_F	5′-GGGGGATCCGTCGATGTCCTCGATGCCCAG-3′
orf 11_HindIII_R	5′-ACCAAGCTTAAGCTCACCCCTGCGCAGCGC-3′
rORF 11_BamHI_F	5′-GGGGGATCCGGCCTGGAAGGGGCCGAAATTCGC-3′
rORF 11_HindIII_R	5′-ACCAAGCTTTCAGCGGCAGTAGACCGGATAAC T-3′
S.rochei_SAT_F	5′-GGGGGATCCACCACTCTTGACGACACGGCT-3′
S.rochei_SAT_R	5′-ACCAAGCTTTCAGGGGCAGGGCATGCTCAT-3′

Hilden, Germany). After confirmation of their sequences, the resulting plasmids, pQE30-ORF 11 and pQE30-ORF 1-SAT, were introduced into *E. coli* M15(pREP4) for expression as N-terminally His_6-tagged fusion proteins. The recombinant enzymes were purified by standard protocols with nickel-nitriloacetic acid (Ni-NTA) Sepharose (Qiagen).

Enzyme reactions with rOrf11. In the enzyme reaction, an *E. coli* T7 S30 Extract System for Circular DNA kit (Promega, Madison, WI, USA) was employed to supply aminoacyl-tRNA. A reaction mixture (200 μl) consisting of 50 mM Tris-HCl (pH 8.0), 1 mM streptothrisamine, 1 mM glycine (or 0.5 mM each proteinogenic amino acid to investigate the substrate specificity for aminoacyl-tRNAs), 20 μl S30 premix, 15 μl S30 extract, and 100 μg ml^{-1} rOrf11 was incubated at 37°C for 3 h. In addition, the enzyme reaction was carried out with or without RNase (10 μg ml^{-1}). The reaction mixture was mixed with 200 μl chloroform. After vigorous shaking, the aqueous layer from centrifugation was analyzed by HPLC–ESI-MS.

Enzyme reactions with rOrf1-SAT. A reaction mixture (200 μl) consisting of 100 mM Tris-HCl (pH 8.0), 1 mM ST-F, 1 mM acetyl-coenzyme A (CoA), and 100 μg ml^{-1} rOrf1-SAT was incubated at 30°C for 2 h. The reaction mixture was mixed with 200 μl chloroform. After vigorous shaking, the aqueous layer from centrifugation was analyzed by HPLC–ESI-MS. To confirm the acetylation of glycylthricin, 1 mM acetyl-CoA and 100 μg ml^{-1} rOrf1-SAT were added to the reaction mixture with rOrf11. After the incubation at 30°C for 2 h, the reaction mixture was analyzed by HPLC–ESI-MS.

Nucleotide sequence accession numbers. The sequence data for the draft genome and the BD-12- biosynthetic gene cluster of *S. luteocolor* NBRC 13826 were deposited in GenBank/DDBJ under Sequence Read Archive (SRA) accession number DRA003165 and GenBank accession number LC122485, respectively.

RESULTS

Identification of the BD-12-biosynthetic gene cluster from *S. luteocolor* NBRC13826. Recently, two research groups reported on genes responsible for biosynthesis of the streptolidine lactam and amino sugar moieties in ST (26, 27). The *orfP* and *orfR* genes from the ST producer *Streptomyces lavendulae* BCRC 12163 were found to participate in the early steps of the streptolidine lactam biosynthesis (26). The *stnG*, *stnI*, *stnJ*, and *stnQ* genes from the ST producer *Streptomyces* sp. strain TP-A0356 were involved in the amino sugar moiety of the ST biosynthesis (27). Their homologues also exist in the ST-biosynthetic gene cluster from *S. rochei* NBRC12908 (Fig. 2) (20). In the draft genome sequence of the BD-12 producer *S. luteocolor* NBRC13826 (accession no. DRA003165), we identified an ST-biosynthetic homologous gene cluster (34 kbp; accession no. LC122485) (Fig. 2). The gene cluster possessed the *orfP*, *orfR*, *stnG*, *stnI*, *stnJ*, and *stnQ* homologue genes. In addition to these genes, two methyltransferase genes (*orf6* and *orf13*), which could be responsible for the two N-methyl groups of BD-12, were found. This suggested that the ST-biosynthetic homologous gene cluster may encode the biosynthesis of BD-12. Surprisingly, NRPS genes homologous to *orf5* and *orf18*, which are present in the ST-biosynthetic gene cluster, were not found in this putative BD-12-biosynthetic gene cluster. This suggested that an NRPS-independent mechanism was responsible for the glycine attachment in the biosynthesis of BD-12.

Functional analysis of the BD-12-biosynthetic gene cluster by heterologous expression. To investigate the involvement of the gene cluster (34 kbp) from NBRC13826 in BD-12 biosynthesis, we introduced a *Streptomyces* integrating vector carrying the 34-kbp DNA fragment (pKU493A-BD12) (Fig. 2) into the BD-12 nonproducer *S. lividans* TK23. The resulting transformant was cultivated, and analysis of the culture broth by HPLC-HR–ESI-MS revealed that the transformant produced BD-12 (Fig. 4), while production of BD-12 was not observed in a transformant harboring an empty vector, pKU493A_aac(3)IV (Fig. 4). Introducing pKU493A-BD12 into the versatile genome-minimized host strain *S. avermitilis* SUKA17 (22) also revealed BD-12, although the productivity was significantly lower (see Fig. S1 in the supplemental material). These findings clearly demonstrate that the 34-kbp DNA fragment carries a complete set of genes responsible for BD-12 biosynthesis, including a certain gene(s) involved in the peptide bond formation between the glycine (or glycine derivative) and streptothrisamine.

Using HHpred, an online program for detecting the structural homology of hypothetical proteins (28), we searched for a candidate gene encoding a peptide-forming enzyme on the BD-12-biosynthetic gene cluster. We detected the structural homologues of the *orf11* gene product (Orf11), FemA (*Staphylococcus aureus*) (29) and FemX (*Weissella viridescens*) (30), although the amino acid sequence of Orf11 showed no significant similarity to those of FemA and FemX. Fem enzymes catalyze the aminoacyl-tRNA-dependent peptide bond formation between the peptidoglycan precursor and amino acids; FemA and FemX utilize Gly-$tRNA^{Gly}$ and Ala-$tRNA^{Ala}$, respectively. We thus expected that Orf11 could similarly utilize Gly-$tRNA^{Gly}$ as a substrate for the addition of glycine to streptothrisamine via a peptide bond (Fig. 3B).

Functional analysis of the *orf11* gene encoding the Fem-like enzyme. In order to directly assess the function of the *orf11* gene in the BD-12-biosynthetic gene cluster, it was coexpressed with a mutated ST-biosynthetic gene cluster carrying an in-frame deletion of the *orf18* gene (STΔorf18) because STΔorf18 produces streptothrisamine as the ST intermediate (20) and can provide Orf11 with streptothrisamine as a substrate. For this experiment, we constructed two integration vectors, pKU493A-STΔorf18 and pKU1016-orf11, which carried STΔorf18 and the *orf11* gene under the control of the constitutive *ermE* promoter, respectively. We introduced these two vectors into *S. avermitilis* SUKA17 and analyzed its culture broth by HPLC–ESI-MS. We anticipated that the resulting transformant would produce a new compound, glycylthricin (Fig. 1), with a molecular mass of 431.3 Da ($m/z = 432.3$ $[M + H]^+$). To our surprise, the transformant provided a compound with a molecular mass of 473.3 Da ($m/z = 474.3$ $[M + H]^+$) (Fig. 5A) rather than the expected compound, glycylthricin. This was considered to be an *orf11* gene-dependent compound, because a transformant harboring pKU493A-STΔorf18 and a pKU1016 empty vector produced no such compound (data not shown). The mass difference (42 Da) between the detected compound and our expected compound (glycylthricin) suggested that the detected compound was an acetylated derivative of glycylthricin. NMR analysis of the detected compound purified from the culture broth showed that it was indeed the acetylated form of glycylthricin (Table 3), and the compound was therefore designated acetyl-glycylthricin (Fig. 1).

***In vitro* analysis of Orf11.** To gain a better understanding of the enzymatic function and substrate of Orf11, we performed an enzyme reaction using N-terminally His_6-tagged Orf11 (rOrf11) produced as a homodimer in *E. coli* (Fig. 6). Streptothrisamine was incubated with rOrf11 and Gly-$tRNA^{Gly}$, and the reaction product was shown to be the expected compound, glycylthricin, based on HPLC–ESI-MS analysis (Fig. 5B). On the other hand, when RNase was added to the enzyme reaction, glycylthricin was not produced (Fig. 5C), demonstrating that Orf11 catalyzes the

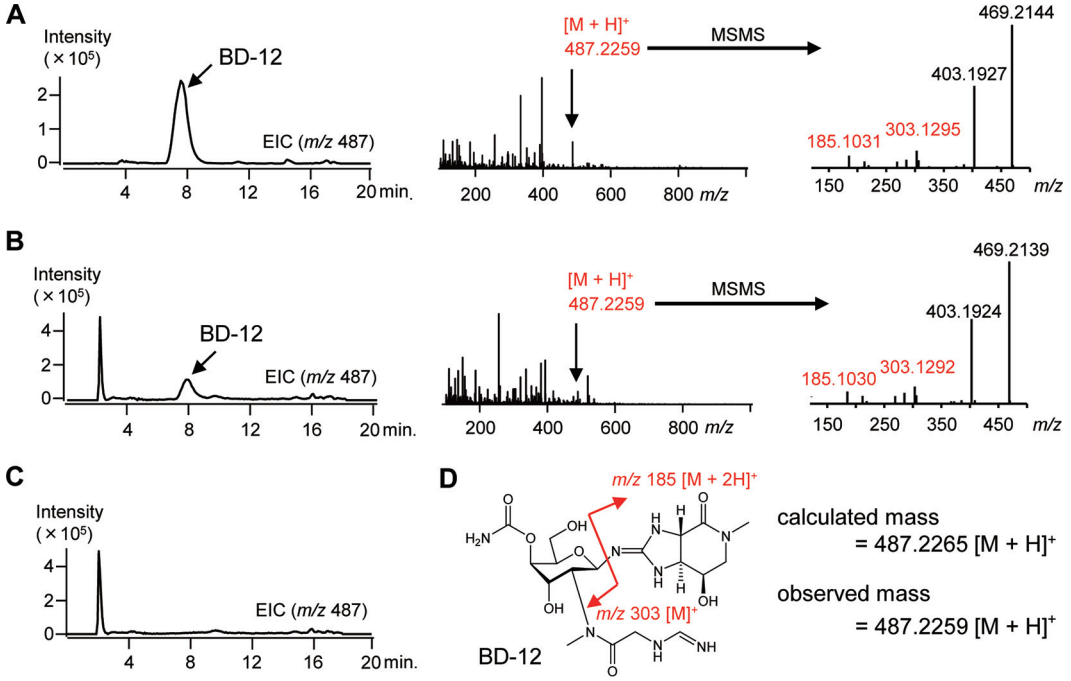

FIG 4 HPLC-HR–ESI-MS analysis of BD-12 produced by the *Streptomyces* strains. (A to C) *S. luteocolor* NBRC 13826 (BD-12 producer) (A), *S. lividans* TK23 harboring pKU493A-BD12 (B), and *S. lividans* TK23 harboring the empty vector [pKU493A_aac(3)IV] (C) were cultivated, and the culture broths were analyzed by HPLC-HR–ESI-MS using a reversed-phase column (Sunniest RP-AQUA; 3 μm; 100 by 2.0 mm; ChromaNik Technologies) at 30°C at a flow rate of 0.3 ml min^{-1} and with an initial gradient of 8% (vol/vol) acetonitrile in water to 16% (vol/vol) acetonitrile over 11 min, which was then ramped to 40% (vol/vol) acetonitrile over 5 min. Both acetonitrile and water contained 0.05% (vol/vol) HFBA and 0.05% (vol/vol) FA. Extracted ion chromatograms (EICs) for BD-12 (m/z = 487) are shown. (D) BD-12 chemical structure with the fragmentation pattern shown in red.

aminoacyl transfer reaction in a tRNA-dependent manner. Moreover, the investigation of substrate specificity suggested that rOrf11 can accept Ala-$tRNA^{Ala}$ as a substrate *in vitro*, although the productivity of the reaction product with a molecular mass of 445.2 Da (m/z = 446.2 $[M + H]^+$) was much lower than that of the reaction using Gly-$tRNA^{Gly}$ (Fig. 5D).

Identification of the acetyltransferase gene responsible for acetyl-glycylthricin production. Considering that Orf11 accepts Gly-$tRNA^{Gly}$ as a substrate, glycylthricin should be converted into acetyl-glycylthricin by an acetyltransferase in the coexpression experiment described above. In the ST-biosynthetic gene cluster from *S. rochei* NBRC12908, we identified the *orf1* gene as a putative ST self-resistance gene that encodes an ST acetyltransferase (SAT) (Fig. 2) (20). SATs in different ST-producing strains are well known to catalyze the acetyl-CoA-dependent acetylation at the β-amino group of the β-Lys moiety in ST-F, conferring ST resistance (31, 32). Although no reports about the substrate specificity of SATs have been published, the *orf1* gene product (Orf1-SAT) was considered to be a candidate for the enzyme that acetylates glycylthricin. To confirm the function of Orf1-SAT as an acetyltransferase, we first examined the acetylation of ST-F using N-terminally His_6-tagged Orf1-SAT (rOrf1-SAT) produced as a homotetramer in *E. coli* (see Fig. S2 in the supplemental material). HPLC–ESI-MS analysis of the reaction mixture confirmed the acetyl-CoA-dependent acetylation of ST-F (see Fig. S3 in the supplemental material). In addition, the *E. coli* strains expressing rOrf1-SAT showed resistance to ST-F (MIC, 250 μM), whereas a control strain not producing rOrf1-SAT was sensitive to ST-F (MIC, 8 μM), implying that the β-amino group of the β-Lys moiety in ST-F was acetylated by rOrf1-SAT. Next, glycylthricin was used as a substrate for rOrf1-SAT. Predictably, the enzyme reaction yielded a compound with a retention time corresponding to that of acetyl-glycylthricin (Fig. 5E). The tandem-MS (MS-MS) spectrum was also identical to that of acetyl-glycylthricin, confirming that rOrf1-SAT converted glycylthricin into acetyl-glycylthricin *in vitro*. These findings also demonstrated that glycylthricin produced in the *in vivo* coexpression experiment was indeed converted into acetyl-glycylthricin by the ST self-resistance gene in the ST-biosynthetic gene cluster.

Antibacterial activities of the new compounds generated in this study. As in the case of acetylated ST-F, we detected no antibiotic activity of acetyl-glycylthricin against *E. coli* W3110. However, we reliably detected the antibiotic activity of glycylthricin (Fig. 7).

DISCUSSION

Through these experiments, it was demonstrated that Orf11, the FemXA-like protein, transfers glycine from Gly-$tRNA^{Gly}$ to the amino group of streptothrisamine to produce glycylthricin (Fig. 3B). Glycylthricin is a possible intermediate in BD-12 biosynthesis, and three sequential reactions by two methyltransferases encoded by the *orf6* and *orf13* genes and an *N*-formimidoyl fortimicin A synthase homologue encoded by the *orf1* gene would produce BD-12. Glycylthricin, which was enzymatically synthesized by rOrf11, was a new ST-related compound. We were unable to confirm the chemical structure of glycylthricin by NMR analysis due to the small amount produced *in vitro*. However, the extensive analysis with rOrf1-SAT *in vitro* (Fig. 5B and E) clarified the chemical structure. Glycylthricin is closely related to glycino-

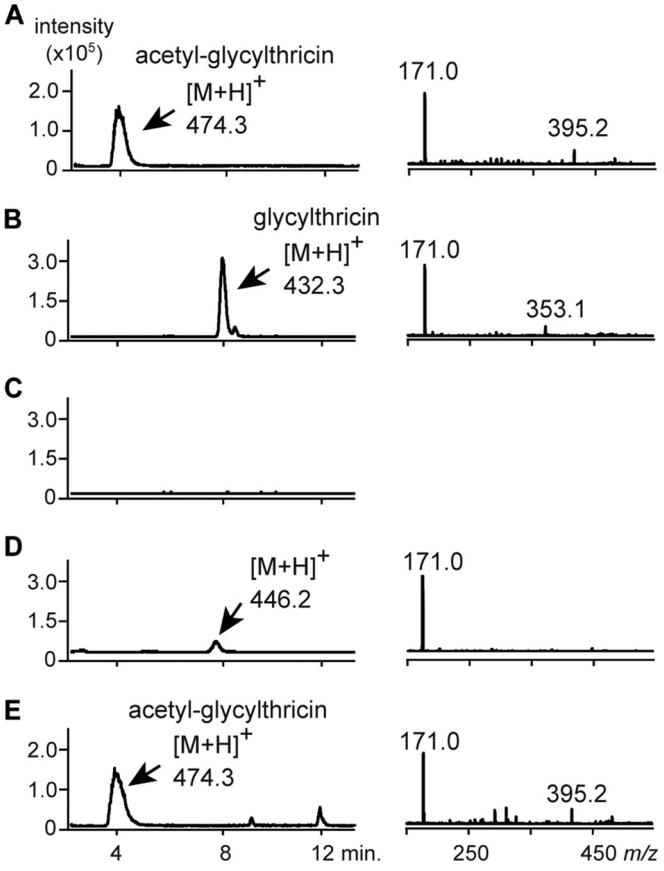

FIG 5 Functional analysis of Orf11 *in vivo* and *in vitro*. EICs and tandem mass spectrometry of the compounds are shown on the left and right, respectively. (A) The compound produced by the *S. avermitilis* SUKA17 strain harboring pKU493A-STΔorf18 and pKU1016-orf11 was analyzed by HPLC–ESI-MS. (B) rOrf11 was incubated with Gly-tRNA^{Gly} and streptothrisamine. (C) rOrf11 was incubated with RNase, Gly-tRNA^{Gly}, and streptothrisamine. (D) rOrf11 was incubated with Ala-tRNA^{Ala} and streptothrisamine. (E) The rOrf11 product (glycylthricin) was incubated with rOrf1-SAT and acetyl-CoA. These samples were analyzed by HPLC–ESI-MS using a reversed-phase column (Sunniest RP-AQUA; 3 μm; 100 by 2.0 mm) at 30°C at a flow rate of 0.3 ml min^{-1} and with a gradient of acetonitrile-water in 0.05% (vol/vol) HFBA and 0.05% (vol/vol) FA run over 15 min (3% [vol/vol] acetonitrile for 3 min, 3 to 15% [vol/vol] acetonitrile for 2 min, and 15% [vol/vol] for 10 min). EICs for acetyl-glycylthricin (m/z = 474.3) and glycylthricin (m/z = 432.3) are shown.

TABLE 3 Assignments of ^{13}C and ^{1}H NMR data for acetyl-glycylthricin[a]

Position	δ_C	δ_H (multiplicity; J in Hz)	^{1}H-^{1}H COSY	^{1}H-^{13}C HMBC
1	173.0			
2	56.3	4.31 (d, 14.1)	3	1, 4
3	52.2	4.35 (dd, 14.1, 2.2)	2	
4	62.1	4.62 (ddd, 5.6, 2.2, 1.2)	5	2
5	49.3	3.78 (dd, 14.4, 5.6)	4	1
		3.36 (dd, 14.4, 1.2)		1
6	164.2			
7	79.8	5.12 (d, 9.6)	8	6, 11
8	50.1	4.13 (dd, 9.6, 2.9)	7	10, 14
9	66.9	4.12 (t, 2.9)	10	8, 11
10	70.4	4.75 (dd, 3.5, 2.9)	9	8, 13
11	73.4	4.26 (dt, 6.5, 3.5)	12	7
12	60.5	3.70 (d, 6.5)	11	
13	158.2			
14	171.6			
15	42.6	3.92 (s)		14, 16
16	175.0			
17	21.8	2.06 (s)		16

[a] NMR assignment of acetyl-glycylthricin. ^{1}H and ^{13}C NMR spectra were recorded at 600 and 150 MHz, respectively, using the Varian NMR system 600 NB CL (Varian, Palo Alto, CA, USA). One- and two-dimensional experiments (double-quantum-filtered correlation spectroscopy [DQF-COSY] and constant-time heteronuclear multiple-bond connectivity [CT-HMBC]) were performed at ambient temperature, and the correlation positions are shown. The samples were dissolved in D_2O, and the solvent peak was used as an internal standard (δ_H, 4.80 ppm).

thricin (Fig. 1), which possesses two methyl groups and exhibits antibacterial activity. Because glycylthricin also showed antibacterial activity, the methyl groups of glycinothricin are unlikely to be essential for its antibacterial activity. More importantly, this also reveals that the ε-amino group and alkyl chain of the β-Lys residue in ST-F do not play an important role in the antibacterial activity of ST-F. Thus, the identification and functional analysis of Orf11 in the BD-12 biosynthesis study not only clarified the unique biosynthetic mechanism with aminoacyl-tRNA, but also provided important information regarding the structure-activity relationship of the ST-related antibiotics.

In the substrate specificity study of rOrf11, we demonstrated that the enzyme can accept Ala-tRNA^{Ala} in addition to Gly-tRNA^{Gly} (Fig. 5D). However, we did not detect Ala-tRNA^{Ala}-derived compounds in the coexpression experiment with the *orf11* gene and STΔorf18, probably due to the low productivities.

Although aminoacyl-tRNAs are normally dedicated to protein biosynthesis, a few microbial FemXA-like proteins have been reported to use aminoacyl-tRNAs and to catalyze peptide bond formation in natural-product biosynthesis. Ser-tRNA^{Ser} is used for the biosynthesis of valanimycin (33), while PacB transfers L-alanine from L-Ala-tRNA^{Ala} to the N terminus of a tetrapeptide intermediate during biosynthesis of pacidamycin (34). In the biosynthesis of dehydrophos, DphH and DphK both possess FemX domains at their C termini and utilize Leu-tRNA^{Leu} and Gly-tRNA^{Gly}, respectively, to form the peptide structure of dehydrophos (35). It was very recently demonstrated that, in the human pathogen *Pseudomonas aeruginosa*, tRNA-dependent aminoacy-

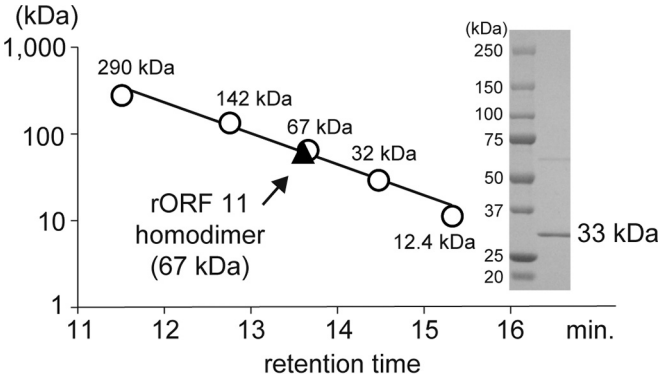

FIG 6 Enzymatic characterization of rOrf11. Purified rOrf11 was subjected to SDS-PAGE. Proteins were stained with CBB R-250. The relative molecular mass of rOrf11 was estimated by gel filtration chromatography (SunSec Diol; 4 mm; 300 by 4.6 mm; ChromaNik Technologies, Osaka, Japan). Glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), myokinase (32 kDa), and cytochrome *c* (12.4 kDa) were used as the standard molecular masses.

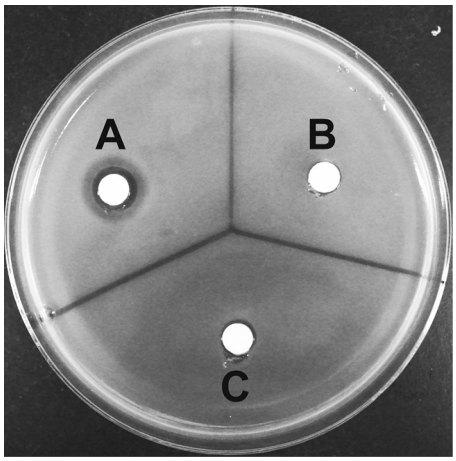

FIG 7 Antibacterial activities of glycylthricin and acetyl-glycylthricin. (A) rOrf11 was incubated with Gly-tRNA^{Gly} and streptothrisamine, producing glycylthricin. (B) rOrf11 was incubated with RNase, Gly-tRNA^{Gly}, and streptothrisamine, producing no compound (control). (C) The rOrf11 product (glycylthricin) was incubated with rOrf1-SAT and acetyl-CoA, producing acetyl-glycylthricin. The antibiotic activities of the reaction mixtures (50 μl) were investigated by paper disk assay using *E. coli* W3110.

lation of the polar head group of phosphatidylglycerol with alanine or lysine is catalyzed by FemX-like proteins to confer resistance to antibiotics (36). In addition, tRNA-dependent cyclodipeptide synthase (37) and tRNA-dependent lantibiotic dehydratase (NisB) (38) were reported, although they lack FemXA-like features. Although reports of amide bond-forming tRNA-dependent enzymes have been forthcoming, to our knowledge, Orf11 is the first characterized tRNA-dependent aminoacylating enzyme that can accept an amino sugar as a substrate.

In conclusion, we clarified that the tRNA-dependent peptide bond-forming enzyme Orf11 mediates the condensation reaction between streptothrisamine and glycine in the biosynthesis of BD-12, whereas the biosynthesis of ST-F employs NRPS machinery. Thus, the structural diversity of the amino acid side chains of the ST-type compounds occurring in nature is generated by two distinct peptide bond-forming mechanisms. STs and ST-related antibiotics are not currently used therapeutically, because they are toxic to both prokaryotes and eukaryotes. In order to engineer new ST-related compounds displaying bacterium-specific activities, genetic modification of these peptide bond-forming machineries would be advantageous.

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