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Functional analysis of methyltransferases participating in streptothricin-related antibiotic biosynthesis

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Streptothricin (ST) and its related compounds produced by *Streptomyces* strains are broad-spectrum antibiotics that consist of carbamoylated D-gulosamine, amino-acid side chain, and streptolidine lactam moieties. BD-12, a streptothricin-related antibiotic, has a glycine-derived side chain and two *N*-methyl groups, whereas ST-F carrying the L-β-lysine side chain has no methyl group. In our previous studies, we identified and characterized the BD-12 and ST biosynthetic gene clusters. Here we report the functional analysis of two methyltransferase genes (*orf 6* and *orf 13*) in the BD-12 biosynthetic gene cluster. Combinatorial biosynthesis using these two methyltransferase genes and the ST biosynthetic gene cluster resulted in the production of three methylated forms of ST-F. Among them, *N*,*N*′-dimethyl-ST-F, a novel compound generated in the present study, showed bacteria-specific antibiotic activities, although ST-F exhibits antibiotic activities against both prokaryotes and eukaryotes. Our findings also demonstrated that the *orf 6* and *orf 13* genes are responsible for the *N*-methylations of the amide bonds in the streptolidine lactam and in the amino-acid side chain linkage, respectively, and that *N*-methyl modification of the streptolidine lactam confers resistance in part against an ST hydrolase, SttH.

[**Key words:** Methyltransferase; Biosynthesis; streptothricin; Antibiotics; BD-12]

Streptothricins (STs) produced by *Streptomyces* strains are broad-spectrum antibiotics. The first member of STs to be identified, ST-F (Fig. 1), was isolated from *Streptomyces lavendulae* in 1943 (1). ST-F consists of a carbamoylated D-gulosamine to which L-β-lysine (β-Lys) and the amide form of the unusual amino acid streptolidine (streptolidine lactam) are attached. After the discovery of ST-F, the analogue compounds with β-Lys oligopeptides [oligo(β-Lys)] have been identified (Fig. 1), and STs carrying the longer oligo(β-Lys) side chains show higher levels of antibacterial activity (2).

ST-F inhibits protein biosynthesis in prokaryotic cells (3). STs also strongly inhibit the growth of eukaryotes, such as yeasts (4–6), fungi (7), protozoa (8), insects (9), plants (10), and mammals (11–14), but are not currently used therapeutically because of their inherent toxicity, including nephrotoxicity (15–17). To reduce ST-F's toxicity against eukaryotes, substantial efforts with organic chemistry-based strategies have been made (18). Meanwhile, we reported an enzymatic approach to alter ST chemical structures using a microbial enzyme, SttH, which hydrolyzes the amide bond of streptolidine lactam (2), and the opening of the lactam ring changed the antimicrobial activity of ST-D from broad spectrum to bacteria-specific (2). In addition to these previous approaches, biosynthetic engineering with genetic modification could be a more attractive strategy for increasing the structural diversity of ST-group antibiotics.

We previously identified the ST-biosynthetic gene cluster (accession no. AB684619) in *Streptomyces rochei* NBRC12908 (Fig. 2), which produces ST-F, ST-E, ST-D, and ST-C (Fig. 1), and elucidated the oligo(β-Lys) side-chain biosynthetic mechanisms mediated by three unique nonribosomal peptide synthetases (NRPSs): Orf 5, Orf 18, and Orf 19 (19). Among them, Orf 19 is a key adenylation enzyme that mediates the activation and oligomerization reactions of the β-Lys monomer unit. Therefore, inactivation of the *orf 19* gene prevents the production of ST-E, ST-D, and ST-C.

Very recently, we further identified the BD-12 biosynthetic gene cluster (Fig. 2) from *Streptomyces luteocolor* NBRC 13826 (20). BD-12 (Fig. 1) is the ST analogue that possess a glycine-derived side chain rather than the β-Lys residue (21,22). We demonstrated that in BD-12 biosynthesis, the peptide bond between the amino sugar intermediate (streptothrisamine) and the glycine residue is catalyzed by a Fem-like enzyme (Orf 11) in a tRNA-dependent manner rather than by NRPSs (Fig. 3) (20). In addition, a new ST-related compound, glycylthricin (Figs. 1 and 3), was enzymatically produced by Orf 11 *in vitro*.

In peptide antibiotics, *N*-methylation is a potent chemical modification for regulating biological functions (23,24). A few ST group compounds, including BD-12, also have one or more *N*-methyl groups (Fig. 1): e.g., citromycin produced by *Streptomyces*

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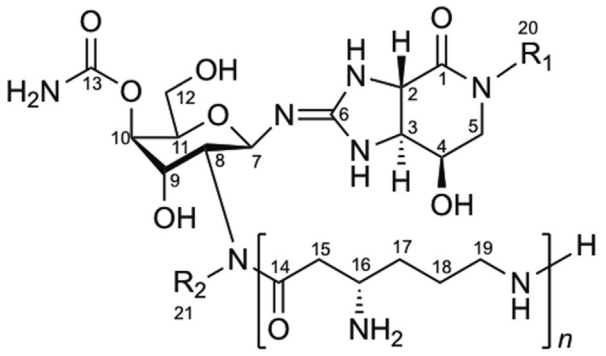
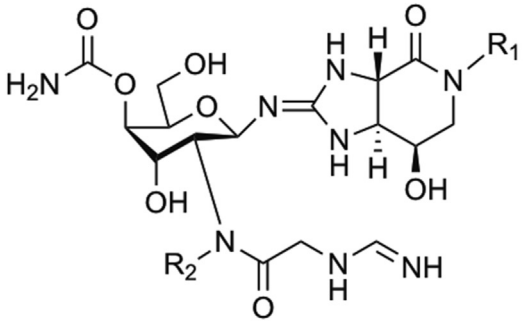
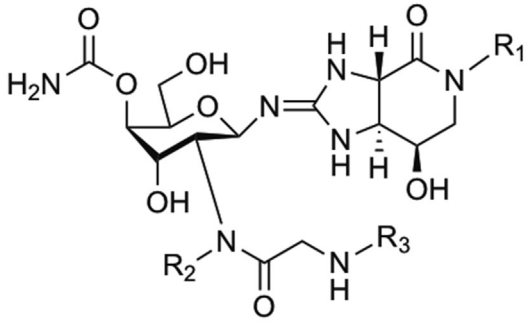


ST-F (R_1,R_2 = H, n = 1) ST-E (R_1,R_2 = H, n = 2)
ST-D (R_1,R_2 = H, n = 3) ST-C (R_1,R_2 = H, n = 4)
ST-B (R_1,R_2 = H, n = 5) ST-A (R_1,R_2 = H, n = 6)
ST-X (R_1,R_2 = H, n = 7)

N-methyl-ST-F (R_1 = CH_3, R_2 = H, n = 1)
N'-methyl-ST-F (R_1 = H, R_2 = CH_3, n = 1)
N,N'-dimethyl-ST-F (R_1, R_2 = CH_3, n = 1)
N-methyl-ST-D (R_1 = CH_3, R_2 = H, n = 3)



BD-12 (R_1 = CH_3, R_2 = CH_3)
citromycin (R_1 = H, R_2 = CH_3)



glycylthricin (R_1, R_2, R_3 = H)
glycynothricin (R_1, R_2 = CH_3, R_3 = H)
A-269A (R_1 = H, R_2, R_3 = CH_3)

FIG. 1. Chemical structures of STs and ST-group antibiotics.

olivoreticuli nov. var. MCRL-0358 (25), A-269A produced by *Streptomyces* sp. strain No. A-269 (26), and glycinothricin produced by *Streptomyces griseus* No. 979 (27). Citromycin shows half the antibiotic activity of BD-12 against prokaryotes but twice that of BD-12 against eukaryotic microorganisms. A-269A exhibits antibacterial activity against *Escherichia coli* despite its lower antibacterial activity against eukaryotes. *N*-Methyl modification should thus be a more attractive strategy for increasing the diversity of the ST chemical structure.

This report describes the functional analysis of two methyltransferase genes found in the BD-12 biosynthetic gene cluster and the combinatorial production of *N*-methylated ST compounds using the methyltransferase genes. We also demonstrate that the *N*-methylation of the streptolidine lactam moiety of ST confers resistance in part to hydrolysis by SttH.

MATERIALS AND METHODS

Chemicals STs (clonNAT, a mixture of ST-F, ST-E, ST-D, ST-C, and ST-B) were obtained from Werner BioAgents (Jena, Germany), and ST-F was purified as previously reported (19). All other chemicals used were of analytical grade.

Bacterial strains and plasmids *Streptomyces lividans* TK23 and *Streptomyces avermitilis* SUKA17 (28) were used as heterologous host strains for gene expression experiments. *Streptomyces* integrating vectors, pKU493A_aac(3)IV (ϕK38-1 integrating vector) and pKU1016 (ϕC31 integrating vector) (28), were used for the gene expression experiments. *E. coli* W3110, *Bacillus subtilis* NBRC13169, and *Saccharomyces cerevisiae* S288C (the same genetic background as strain CKY8) were used for minimum inhibitory concentration (MIC) studies.

Heterologous expression of methyltransferase genes (*orf 6* and *orf 13*) from the BD-12-biosynthetic gene cluster The ST biosynthetic gene cluster (30 kbp, Fig. 2) possessing an in-frame deletion of the *orf 19* gene (STΔorf 19), which was constructed in a previous study (19), was cloned into the integrating vector, pKU493A_aac(3)IV, to generate pKU493A-STΔorf 19 by a standard method. pKU493A-STΔorf 19 was then introduced into *S. avermitilis* SUKA17, and the resulting transformant was used as a heterologous host strain, which serves the methyltransferases (Orf 6 and Orf 13) with ST-F as a substrate.

The *orf 6* and *orf 13* genes from the BD-12 biosynthetic gene cluster were amplified by PCR using two sets of primers (5′-CGGTGCACCACGGGATCCCCTGTCCGTCCC-3′ (BD-12_ORF6_BamH_F) plus 5′-GCGGCGGTGCTCTAGACCGAGGGACACCCG-3′ (BD-12_ORF6_Xba_R) and 5′-CCTGGCCCGACTCCCTCTAGAGCTGCGCCA-3′ (BD-12_ORF13_Xba_F) plus 5′-TAGGCCGCCCCAAGCTTGCCCGCGTCGGGG-3′ (BD-12_ORF13_HindIII_R). The two amplified DNA fragments were ligated with pKU1016 to yield three expression vectors: pKU1016-orf 6, pKU1016-orf 13, and pKU1016-orf 6-orf 13. These vectors were respectively introduced into the *S. avermitilis* SUKA17 transformant harboring pKU493A-STΔorf 19, and the resulting transformants were cultured in AVM medium (pH 7.0) containing 6% (w/v) glucose, 0.2% (w/v) yeast extract (Difco Laboratories, Franklin Lakes, NJ, USA), 0.2% (w/v) $(NH_4)_2SO_4$, 0.5% (w/v) $CaCO_3$, 0.25% (w/v) NaCl, 0.05% (w/v) K_2HPO_4, 0.01% (w/v) $MgSO_4 \cdot 7H_2O$, 0.005% (w/v) $FeSO_4 \cdot 7H_2O$, 0.005% (w/v) $ZnSO_4 \cdot 7H_2O$, and 0.005% (w/v) $MnSO_4 \cdot 4H_2O$ for 6 days at 28°C. To detect *N*-methylated ST-F compounds, the supernatants of the culture broth were analyzed by high-performance liquid chromatography and electrospray ionization mass spectrometry (HPLC-ESI-MS, Esquire 4000; Bruker, Billerica, MA, USA).

Purification of *N,N'*-dimethyl-ST-F Culture broth (4000 ml) of the *S. avermitilis* SUKA17 transformant harboring pKU493A-ST_orf 19 and pKU1016-orf 6-orf 13 was centrifuged, and the supernatant obtained was mixed with 4000 ml of chloroform. After the mixture was shaken vigorously, the aqueous layer from centrifugation was mixed with 1600 ml of acetonitrile and 20.54 g of NH_4HCO_3, and then was centrifuged again. The resulting supernatant was loaded onto a Dowex 50W × 4 column (100–200 mesh; NH_4^+-form; 4.0 × 10 cm; Dow Chemical, Midland, MI, USA), and the column was washed with 400 ml of 30% (v/v) acetonitrile in water. The sample was eluted in a stepwise fashion with 400 ml of 0.25, 0.5, 1, and 1.5 M NaCl in 30% (v/v) acetonitrile. The *N,N'*-dimethyl-ST-F fractions eluted with 1 and 1.5 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 60 ml of water, and then was combined and applied to an activated-carbon column (2.0 × 10 cm) that had been equilibrated with water. The column was washed with water, and the *N,N'*-dimethyl-ST-F fraction was eluted with 50% acetone in 0.01 N HCl. After removal of the organic solvent, the aqueous layer 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 (Sunniest RP-AQUA; 5 μm; 250 × 20 mm; ChromaNik Technologies, Osaka, Japan) at 40°C at a flow rate of 20 ml min^{-1} and with a mobile phase composed of 15% (v/v) acetonitrile and 0.1% (v/v) *n*-heptafluorobutyric acid

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