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Cloning and Heterologous Expression of the Thioviridamide Biosynthesis Gene Cluster from *Streptomyces olivoviridis*

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Thioviridamide is a unique peptide antibiotic containing five thioamide bonds from *Streptomyces olivoviridis*. Draft genome sequencing revealed a gene (the *tvaA* gene) encoding the thioviridamide precursor peptide. The thioviridamide biosynthesis gene cluster was identified by heterologous production of thioviridamide in *Streptomyces lividans*.

Thioviridamide is an *N*-acylated undecapeptide antibiotic which induces apoptosis selectively in E1A-transformed cells (1). The most unique structural feature of thioviridamide is the presence of five thioamide bonds formed between the amino acids (Fig. 1) (2). Only five thioamide compounds have been found in natural sources. Among them, thioviridamide, apo-methanobactin, and closthioamide are of bacterial origin, isolated from *Streptomyces olivoviridis*, *Methylosinus trichosporium*, and *Clostridium cellulolyticum*, respectively (3). Apo-methanobactin is a copper chelator containing two thioamide-bearing oxazolone rings and belongs to the ribosomally synthesized and posttranslationally modified peptide (RiPP) family. Genomic analysis of *M. trichosporium* has identified a gene encoding the apo-methanobactin precursor peptide containing a LCGSCYPCSCM sequence (underlining indicates the thioamide precursor). It is presumed that the sulfur atoms in the two thioamide groups could be supplied from the adjacent cysteine residues (the precursors of the oxazolone rings) (4). Closthioamide is a polythioamide antibiotic isolated from a strictly anaerobic bacterium (5). Although the closthioamide producer genome has been sequenced, its biosynthesis genes have remained elusive (6). In this study, we identified the thioviridamide biosynthesis gene cluster of *S. olivoviridis* NA05001 and demonstrated heterologous production of thioviridamide in *Streptomyces lividans* TK23.

Although most peptide antibiotics are biosynthesized by nonribosomal peptide synthetases (NRPSs) (7), thioviridamide contains an *S*-(2-aminovinyl)cysteine (AviCys) residue, which has been found in the linaridin family of RiPPs (8). Epidermin (9), microbisporicin (10), and cypemycin (11) are known to be AviCys-containing linaridins. AviCys is formed by cyclization between a serine/cysteine-derived dehydroalanine and a C-terminal cysteine via oxidative decarboxylation (11, 12). Thus, thioviridamide is presumed to be biosynthesized by posttranslational modification of a ribosomal precursor peptide containing a VMAAAA SIALHC or VMAAAACIALHC sequence.

Genomic DNA isolated from *S. olivoviridis* NA05001 was sequenced using an Illumina Hiseq 2000 platform at TaKaRa Bio Inc. (Otsu, Japan) after purification with a Genomic-tip 20/G kit (Qiagen). The sequence reads were assembled using the Edena assembler (version 3) (13) into 205 contigs totaling 7,712,087 bp. A sequence search identified the *tvaA* gene, encoding 75 amino acids containing VMAAAASIALHC at the C-terminal end (Fig. 1). FramePlot analysis (14) of 7.5 kbp from the 5′ end and 23 kbp from the 3′ end of the *tvaA* gene showed the presence of a putative thioviridamide biosynthesis gene cluster (Fig. 2).

The cluster contained 10 genes arranged as an operon (*tvaC*-

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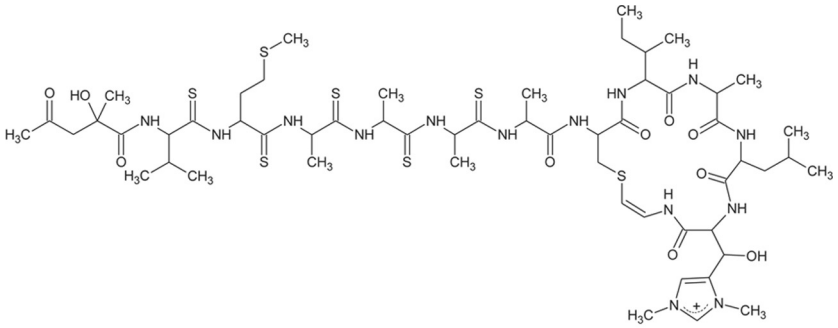

MTEKTQITDVQAFEDLVAKVQEMDGPAQASSTVAALAGLDAAELQNFLEE
KSGISPDEEAQGSVMAAAASIALHC

FIG 1 Structure of thioviridamide (top) and amino acid sequence of TvaA (bottom).

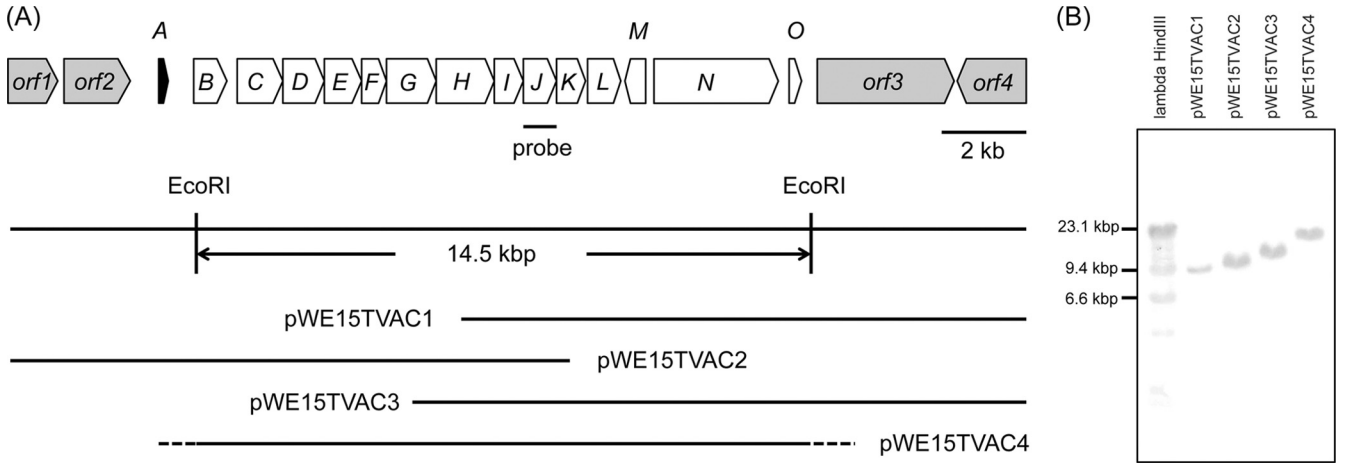

FIG 2 Thioviridamide biosynthesis gene cluster from *Streptomyces olivoviridis* NA05001. (A) The *tva* gene cluster from *Streptomyces olivoviridis* NA05001 and four overlapping cosmids, pWE15TVAC1 to pWE15TVAC4. (B) Southern analysis of the EcoRI-digested cosmids.

tvaL) downstream of a putative regulatory gene (the *tvaB* gene). Two presumably cotranscribed genes (*orf1* and *orf2*) upstream of the *tvaA* gene were excluded from the cluster based on high homology to a DNA polymerase and a protease, respectively (Table 1). A putative DNA polymerase gene (*orf4*) was also excluded, although assignments of four genes between the *tvaL* and *orf4* genes were unclear. To identify genes required for thioviridamide biosynthesis and the biosynthesis gene cluster, the candidate genes were cloned and expressed in *Streptomyces lividans* to produce thioviridamide.

A cosmid library was constructed in pWE15 (Agilent Technologies) from Sau3AI-digested genomic DNA of *S. olivoviridis* NA05001. Cosmid clones containing the *tva* gene cluster were detected by colony hybridization using a 0.8-kbp *tvaJ* gene fragment. The probe was labeled with an AlkPhos direct labeling kit (GE Healthcare) after the fragment was amplified and cloned from the genomic DNA with primers containing additional HindIII sites (5′-TAAAGCTTTCACGCGTCATCAGCCGGCCCGA-3′ and 5′-TCAAGCTTATGACGGCTGCGCGGAAGGGATT-3′). The cosmid clones were digested with EcoRI and subsequently analyzed by Southern hybridization using the same probe. A cosmid clone (pWE15TVAC4) contained a 14.5-kbp fragment from the *tvaB* gene to the *tvaO* gene (Fig. 2). A 2.0-kbp *tvaA* gene fragment containing the terminal EcoRI site was amplified from *S. olivoviridis* NA05001 genomic DNA by KOD-Plus (Toyobo) DNA polymerase using primers with an additional XbaI site (5′-CCTTCAGACGGAATTCATCGGCGAACGGC-3′ and 5′-GAGGGCGTCTAGAGAGCACCCCCGGAAAC-3′). The fragment was digested with EcoRI/XbaI and cloned into pBluescript II SK to construct pBS-TVA1. A 14.5-kbp EcoRI fragment of pWE15TVAC4 was ligated into EcoRI-digested pBS-TVA1. Resulting clones were sequenced to select one with the insert in the correct orientation. The plasmid was digested with XbaI/HindIII and ligated into XbaI/HindIII sites of the *Escherichia coli*/*Streptomyces* shuttle vector pWHM3 (15) to construct pWHM3-TVA. The expression plasmid passaged through *E. coli* JM110 was introduced into *S. lividans* TK23.

TABLE 1 ORFs in the thioviridamide biosynthetic gene cluster[a]

Protein	Size (aa)	Homologous protein (origin)	Identity/ similarity (%)	GenBank or NCBI accession no.	Deduced function
Orf1	401	DNA polymerase III subunit delta′ (*S. davawensis*)	90/93	CCK28488	
Orf2	529	Protease (*S. avermitilis*)	83/87	NP_825801	
TvaA	75	ABC transporter (*Penicillium griseofulvum*)	46/62	ACR02669	Precursor peptide
TvaB	275	SARP family regulator (*S. lavendulae*)	35/55	BAG74713	Regulator
TvaC	377	Phosphotransferase family protein (*Nostoc* sp.)	29/45	YP_007077015	Unknown
TvaD	328	Hypothetical protein (*Calothrix* sp.)	25/41	YP_007137464	Unknown
TvaE	308	Aminoglycoside phosphotransferase (*Frankia* sp.)	48/65	EHI90509.1	Unknown
TvaF	197	Phosphopantothenoylcysteine decarboxylase (*Exiguobacterium* sp.)	39/60	YP_002887224	Oxidative decarboxylase
TvaG	407	Type 12 methyltransferase (*Cyanothece* sp.)	43/61	YP_002481771	Methyltransferase
TvaH	452	Methanogenesis marker protein 1 (*Methanolinea tarda*)	36/53	EHF09819.1	Unknown
TvaI	218	TfuA-like core domain-containing protein (*M. tarda*)	44/63	EHF09820.1	Regulator
TvaJ	280	Phytanoyl-CoA dioxygenase (*Roseobacter litoralis*)	29/44	YP_004691882	Oxygenase
TvaK	220	Papain family cysteine protease (*Tannerella forsythia*)	27/39	YP_005014309	Protease
TvaL	282	Integral membrane protein (*S. pristinaespiralis*)	31/49	EDY64257.2	Unknown
TvaM	160	Histidine kinase (*S. somaliensis*)	55/66	WP_010468209.1	Regulator
TvaN	1,003	Large transcriptional regulator (*S. pristinaespiralis*)	67/75	EDY63194.2	Regulator
TvaO	94	Hypothetical protein (*S. viridochromogenes*)	54/68	ELS50220.1	Unknown
Orf3	1,089	Transcriptional regulator (*S. avermitilis*)	63/69	NP_825812	
Orf4	561	DNA polymerase I (*S. griseoflavus*)	87/92	EFL40026.1	

[a] *S.*, *Streptomyces*; SARP, *Streptomyces* antibiotic regulatory protein; CoA, coenzyme A.

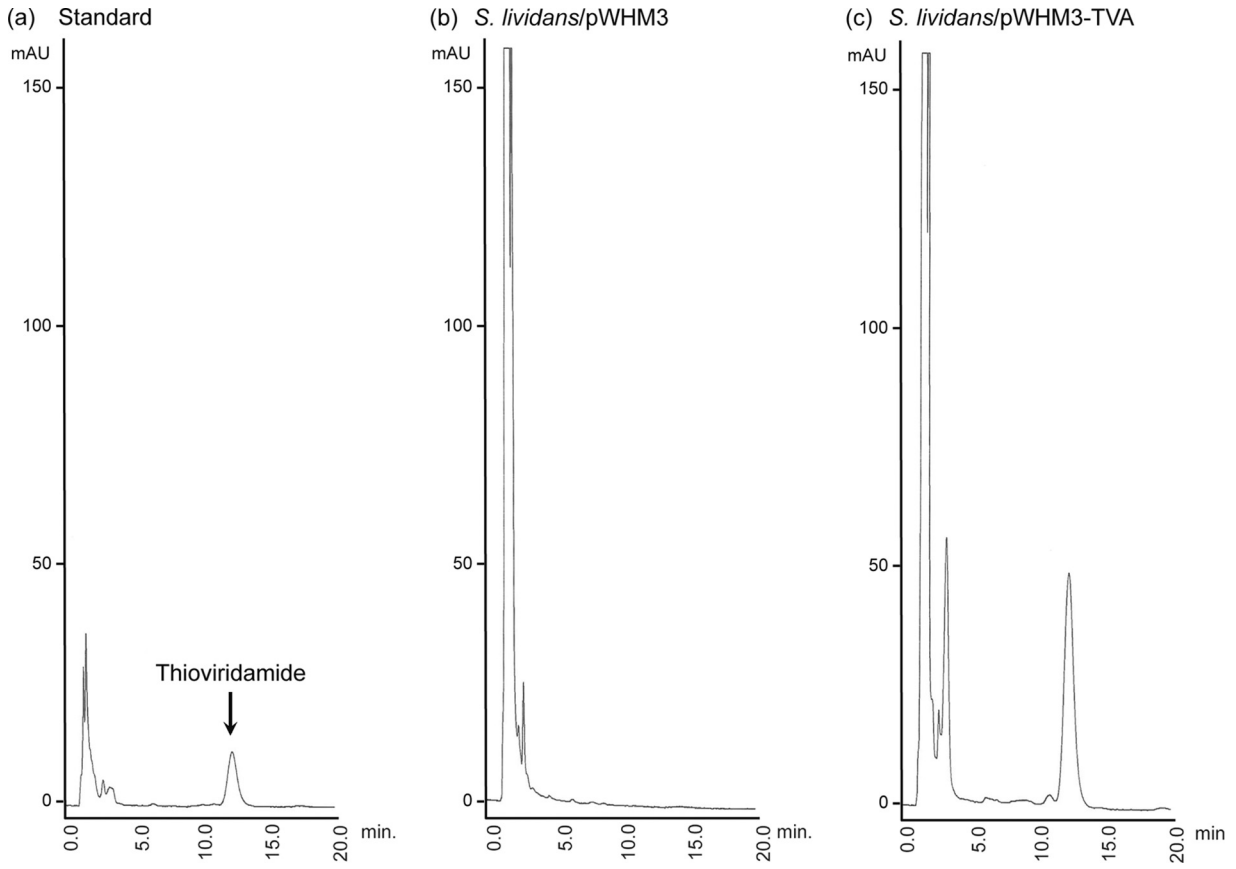

FIG 3 HPLC analysis of the mycelial extract of *S. lividans* expressing the *tva* gene cluster. HPLC profiles of (a) standard thioviridamide and the culture extract of *S. lividans* carrying (b) a control plasmid (pWHM3) or (c) a *tva* gene cluster-containing plasmid (pWHM3-TVA).

The transformant was cultivated in Erlenmeyer flasks containing a medium consisting of 2.5% glucose, 1.5% soybean meal, 0.2% dry yeast, 0.4% $CaCO_3$, and 10 μg/ml thiostrepton (pH 6.2) on a rotary shaker at 27°C for 4 days. The mycelial acetone extract was evaporated and then extracted with ethyl acetate (EtOAc) at pH 3. The extract was analyzed by high-pressure liquid chromatography (HPLC) using a YMC-Pack R-ODS-7 column with 80% MeOH–0.2% H_3PO_4 (2 ml/min). A UV absorption peak for thioviridamide at 274 nm was detected at a retention time of 12.4 min (Fig. 3). The production of thioviridamide was confirmed by the ^{1}H nuclear magnetic resonance (NMR) spectrum of the purified sample (see Fig. S2 in the supplemental material), thereby showing that the *tva* gene cluster (*tvaA* to *tvaO*) is responsible for thioviridamide production.

Among the *tva* gene products, a putative decarboxylase, TvaF, exhibits homology to EpiD (25% identity, 49% similarity) (9), MibD (39% identity, 57% similarity) (10), and CypD (27% identity, 49% similarity) (11), which are involved in AviCys formation. TvaK is a putative cysteine protease that likely cleaves the leader peptide from TvaA. Thioviridamide contains a unique amino acid, β-hydroxy-N^1,N^3-dimethylhistidinium, which has not been isolated from other organisms. Nevertheless, N^1-methylhistidine, N^3-methylhistidine, and β-hydroxyhistidine are commonly found in natural sources and known to be produced by posttranslational modification enzymes, including histidine protein methyltransferase Hpm1 (16), carnosine *N*-methyltransferase (17), and the bifunctional lysine-specific demethylase and histidyl-hydroxylase, NO66 (18). A putative methyltransferase, TvaG, and a putative dioxygenase, TvaJ, are presumably required for the formation of the β-hydroxy-N^1,N^3-dimethylhistidinium residue. These proteins, however, reveal no homology to known histidine-modifying enzymes. TvaB, TvaM, and TvaN are categorized as regulatory proteins based on sequence similarities. TvaI is also assignable as a regulator, because a homologous protein, TfuA, can regulate the production of trifolitoxin, a RiPP family antibiotic (19).

The remaining *tva* gene products might be involved in self-resistance, N-terminal acylation, or thioamide formation. TvaC and TvaE share similarity to aminoglycoside phosphotransferase. Since this enzyme is known to be responsible for resistance to aminoglycoside antibiotics, TvaC and/or TvaE are predicted to confer self-resistance. TvaL is a putative membrane protein with four transmembrane helices and might function as a transporter, because topologically related proteins are found in several RiPP biosynthesis clusters (20, 21). N-acylated RiPPs reported are few and include polytheonamides and epilancins. The N-terminal 5-dimethyl-2-oxohexanoate of polytheonamides A and B and the N-terminal lactate of epilancin 15X have been reported to be derived from threonine and serine, respectively (21, 22). The *N*-acyl group of thioviridamide appears to be serine-derived from the precursor peptide sequence. Although posttranslational modification genes in the polytheonamide and epilancin producers have been identified, no related gene is found in the *tva* gene cluster.

The *tva* gene products share no significant similarity to any proteins of the closthioamide producer, *C. cellulolyticum* H10,

suggesting that the two thioamide producers adopt different mechanisms for thioamide formation. TvaH is a possible candidate for a thioamide-forming enzyme and shows homology to a YcaO domain of GodD (24% identity, 40% similarity) in the biosynthesis gene cluster of goadsporin, a RiPP containing thiazole and oxazole rings (23). A YcaO domain-containing protein, BalhD, has been shown to activate the amide backbone of the precursor peptide using ATP in the biosynthesis of RiPPs (24). The activated amide carbon is proposed to react with the SH group of a cysteine residue as a possible mechanism of thiazole formation in azole-containing RiPPs. TvaH might catalyze the conversion of amide bonds into thioamide bonds in the presence of a sulfur donor. Our heterologous expression system is expected to be useful for identification of the gene functions.

In conclusion, this study has identified the biosynthesis gene cluster of thioviridamide from *S. olivoviridis* NA05001 and demonstrated heterologous production of thioviridamide in *S. lividans* TK23. Thioviridamide is confirmed to be derived from a ribosomally synthesized prepeptide.

Nucleotide sequence accession number. The sequence of the thioviridamide biosynthesis gene cluster was deposited in DDBJ under accession number AB819757.

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