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Functional manipulations of the tetramycin positive regulatory gene *ttmRIV* to enhance the production of tetramycin A and nystatin A1 in *Streptomyces ahygroscopicus*

Hao Cui[1] · Xianpu Ni[1] · Wei Shao[1] · Jian Su[1] · Jiaqi Su[1] · Jun Ren[1] · Huanzhang Xia[1]

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Abstract A putative regulatory gene *ttmRIV* located in the tetramycin biosynthetic gene cluster was found in *Streptomyces ahygroscopicus*. In-frame deletion of *ttmRIV* led to abolishment of tetramycin and significant enhancement of nystatin A1, whose production reached 2.1-fold of the H42 parental strain. Gene complementation by an integrative plasmid carrying *ttmRIV* restored tetramycin biosynthesis revealed that *ttmRIV* was indispensable to tetramycin biosynthesis. Gene expression analysis of the H42 strain and its mutant strain Δ*ttmRIV* via reverse transcriptase-PCR of the tetramycin gene cluster demonstrated that the expression levels of most biosynthetic genes were reduced in Δ*ttmRIV*. Results of electrophoretic mobility shift assays showed that TtmRIV bound the putative promoters of several genes in the tetramycin pathway. Thus, TtmRIV is a pathway-specific positive regulator activating the transcription of the tetramycin gene cluster in *S. ahygroscopicus*. Providing an additional copy of *ttmRIV* under the control of the *ermEp** promoter in the H42 strain boosted tetramycin A production to 3.3-fold.

Keywords PAS-LuxR · Regulator · *Streptomyces* · Tetramycin · Nystatin A1

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✉ Huanzhang Xia
xiahz612@sina.com

[1] School of Life Science and Biopharmaceutics, Shenyang Pharmaceutical Univerisity, Shenhe District, Wenhua Road 103, 110016 Shenyang, Liaoning, People's Republic of China

Introduction

Streptomyces are gram-positive soil actinomycetes that can produce various bioactive products, including antibiotics, insecticides, antitumor agents, and immunosuppressants, during their secondary metabolism. The secondary metabolism of *Streptomyces* is a complex process modulated by pleiotropic and pathway-specific regulators.

The pathway-specific regulators encoded by regulatory genes in the secondary metabolite biosynthetic gene cluster may serve as the most basic factors in the regulatory network. *Streptomyces* antibiotic regulatory proteins (SARPs) comprise the first identified pathway-specific regulator family. Several regulators containing the OmpR-like winged DNA-binding domain belong to this family [18], which includes DnrI of the daunorubicin pathway [17], ActII-Orf4 of the actinorhodin pathway [2], Aur1PR3 of the auricin pathway [22], and NanR1 and NanR2 of the nanchangmycin pathway [34].

A few years after the identification of SARPs, a new kind of pathway-specific regulator called PAS-LuxR was identified. This regulator combined an N-terminal PAS domain with a C-terminal helix–turn–helix (HTH) motif of the LuxR type. PAS domains exist widely in the kingdom as signal sensors to respond toward changes in energy, light, oxygen, redox potential, and small ligands [31]. PimM (named ScnRII in *S. chattanoogensis* [9]) of the pimaricin pathway [1] is the archetype of this novel class of regulators, and this PAS-LuxR class of proteins includes several other polyketide synthase (PKS) pathway regulators, such as NysRIV of the nystatin pathway [30] and AURJ3 M of the aureofuscin pathway [32]. Some of these regulators have been manipulated to improve the production of antibiotics. For example, the insertion of one additional copy of

Table 1 Strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Source or references
Strains		
E. coli		
DH5α	F$^-$λ$^-$*recA1* Δ(*lacZYA-argF*) U169 *hsdR17* ($r_k^-m_k^+$) *thi-1 gyrA96 supE44 endA1 relA1* φ80d*lacZ* Δ *M15 phoA deoR*	Invitrogen
ET12567	Methylation defective, strain used in *E.coli-Streptomyces* intergeneric conjugation	[16]
S. ahygroscopicus		
H42	Tetramycin and nystatin producer	CGMCC 4.7082
Δ*ttmRIV*	H42 with *ttmRIV* disrupted	This work
Saccharomyces cerevisiae	Anti-microbiology assay	ATCC 9763
Plasmids		
pIJ2925	Gene cloning vector, Amp^R	[11]
pKC1139	Gene disruption vector, Am^R	[3]
pSPU241	pIJ2925 containing *ermEp** promoter and terminator	This work
pSET152	*φC31 int* and *attP*, Am^R	[3]
pET-32a	*E. coli* expression vector, Amp^R	Novagen
pKCRIV	*ttmRIV* disruption plasmid, pKC1139 containing *ttmRIV* upstream and downstream fragments	This work
pSPURIV	pSPU241 containing a single copy of *ttmRIV*	This work
pSETCRIV	*ttmRIV* gene complementation plasmid, pSET152 containing a single copy of *ttmRIV* with native promoter	This work
pSETERIV	*ttmRIV* gene over-expression plasmid, pSET152 containing *ermEp** and single copy of *ttmRIV*	This work
pET-RIV	pET-32a containing the coding region of *ttmRIV*	This work

Amp^R, ampicillin resistance; Am^R, apramycin resistance

scnRII into the *S. chattanoogensis* L10 strain boosted pimaricin production by 3.3-fold in YSG medium and 4.6-fold in YEME medium without sucrose [9], and overexpression of *aurj3 M* in *S. aureofuscus* SYAU0709 increased aureofuscin production by 6.0-fold [32].

The fermentation products of *S. ahygroscopicus* contain two kinds of polyene macrolide compounds: tetramycin and nystatin [21]. Tetramycin is a 26-member tetraene antibiotic with two components: tetramycin A and tetramycin B [8, 20]. This antibiotic is applied to treat some diseases of crop plants in China [36]. The tetramycin gene cluster had been reported from the genome of *S. hygrospinosus* var. *beijingensis* ACCC40068 [6] and *S. ahygroscopicus* CGMCC 4.7082 [21]. Nystatin is a 38-member tetraene antibiotic containing four components [10] used to treat fungal infections. Nystatin A1 is the main component of nystatin produced by *S. ahygroscopicus*. Overall, tetramycin and nystatin share similar pathways and precursors [4, 21].

The protein product encoded by *ttmRIV* belongs to the PAS-LuxR type, as revealed by in silico analysis. The present study describes the characterization of *ttmRIV*, a tetramycin pathway-specific positive regulatory gene in the tetramycin gene cluster. The regulatory mechanism of TtmRIV was also demonstrated through reverse transcription-polymerase chain reaction (RT-PCR) analysis of tetramycin gene expression and electrophoretic mobility shift assay (EMSA). The production of nystatin A1 and tetramycin A were enhanced by functional manipulations of the *ttmRIV* gene.

Materials and methods

Bacterial strains and growth conditions

The strains and plasmids used in this work are listed in Table 1. *Escherichia coli* DH5α was used as the plasmid host for plasmid subcloning. *Escherichia coli* ET12567 (pUZ8002) was used as a donor for *E. coli–Streptomyces* intergeneric conjugations. *Escherichia coli* strains were grown on Luria–Bertani (LB) agar or liquid medium containing the appropriate antibiotic for selection at 37 °C. Gause's synthetic agar was used for sporulation of *S. ahygroscopicus* and its derivative mutant. All *Streptomyces* strains were cultured through two-stage fermentation with shaking at 220 rpm and 28 °C. The components of seed and fermentation medium were as described by Ren et al. [21]. After incubation at 28 °C for 24 h, seed cultures were used

to inoculate fermentation medium to 10 % (v/v), and incubated for 96 h.

Genetic procedures

General procedures for DNA manipulation were conducted as described by Sambrook and Russell [24]. Intergeneric conjugation between *E. coli* and *S. ahygroscopicus* was performed as described by Kieser et al. [12].

Plasmid construction for *ttmRIV* disruption

The primers used to construct the plasmids for *ttmRIV* disruption are listed in Table 2. H42 total DNA was used as the template for PCR. Primers RIV-P1 and RIV-P2 were used to amplify a 1.50 kb RIVS1 fragment containing the regions upstream of *ttmS2* and downstream of *ttmRIV*. The RIVS1 fragment was digested with *Hin*dIII and *Xba*I. A 1.43 kb PCR product called RIVS3 containing the regions upstream of *ttmRIV* and downstream of *ttmRIII*, amplified with primers RIV-P3 and RIV-P4, was digested with *Xba*I and *Bam*HI. The resulting *Hin*dIII-*Xba*I and *Xba*I-*Bam*HI fragments were ligated into *Hin*dIII-*Bam*HI cut pKC1139 to generate the *ttmRIV* in-frame deletion of plasmid pKCRIV.

Plasmid construction for gene complementation in mutants and increasing copy number of *ttmRIV* gene in wild-type.

The primers used in this section are listed in Table 2. The template for PCR was H42 total DNA. Primers NPCRIVS and NPCRIVAS were used to amplify a 1.34 kb NPRIV fragment covering the putative promoter and the whole *ttmRIV* gene. The NPRIV was digested with *Eco*RV and *Xba*I. The resulting *Eco*RV-*Xba*I fragment was ligated into pSET152, also digested with *Eco*RV-*Xba*I, to generate the *ttmRIV* complementary plasmid pSETCRIV. A 0.64 kb PCR product, the entire *ttmRIV* gene, amplified with primers PRIV-CS and PRIV-CAS, was digested with *Xba*I and *Hin*dIII and cloned into the same sites of pSPU241 to yield pSPURIV. The 1.32 kb insert included the *ermEp** promoter, *ttmRIV* gene, and the terminator, and was recovered as a *Bgl*II fragment from pSPURIV and inserted into the *Bam*HI site of pSET152, resulting a site-specific integrating type of overexpressed plasmid pSETERIV.

Total RNA isolation and gene expression analysis via RT-PCR

Particulate matter in the fermentation medium made it difficult to harvest the mycelia for total RNA isolation. Therefore, H42 and its mutants were cultured on fermentation agar medium plates covered with cellophane sheets. To determine the time when tetramycin was actively produced, a time–production curve of the H42 strain on fermentation agar medium plates was obtained by antimicrobial bioassay with *Saccharomyces cerevisiae* as the indicator organism and nystatin as the reference standard. H42 and its mutants were cultured at 28 °C, and samples were collected from the cultures when tetramycin was actively produced. Mycelia were scraped from the cellophane and immediately frozen by immersion in liquid nitrogen. The total RNA was isolated using RNAiso Plus (TaKaRa), followed by DNaseI (RNase-free, TaKaRa) treatment to eliminate genomic DNA contamination.

Primers P-*lysA*-S and P-*lysA*-AS (Supplementary material, Table S2) were designed to amplify a 577 bp fragment inside the *lysA* gene (a primary metabolism gene encoding diaminopimelate decarboxylase) as control. All of the

Table 2 Primers used for gene deletion, complementation, over-expression and *E. coli* expression

Name	Sequence	Description
RIV-P1	CCCAAGCTTGGGGTCGCCGAGGGTGGT	Construction of the plasmid pKCRIV
RIV-P2	GCTCTAGATGCTGCGGAAACTGAAGG	
RIV-P3	GCTCTAGACAGCACGGAAATGCATAT	
RIV-P4	CGGGATCCGTGGACGGCATCAACACC	
YP1	CGCAGCGTCTCCGAGGACTT	Identification of Δ*ttmRIV*
YP2	CGCACGGCGAACCAACAGA	
YP3	CGTCGGACCACAGGATTAGGGA	
YP4	CCGCAGGGACAGCACCATCT	
NPCRIVS	CTAGTCTAGACGGGGGTGGTCAGGGAAT	Construction of the plasmid pSETCRIV
NPCRIVAS	GCGATATCCGCAAGTTGGGCGTAGCG	
PRIV-CS	GCTCTAGAGTGCTGGATCCCGCTCTGAC	Construction of the plasmid pSETERIV
PRIV-CAS	CCCAAGCTTTTACTTGATGAAGTCGTCCA	
RIV-S	CATGCCATGGTGGATCCCGCTCTGAC	Construction of the plasmid pET-32aRIV
RIV-AS	CCCAAGCTTTTACTTGATGAAGTCGTCCAC	

The underlines represent the digest sites for constructing the plasmids

DNaseI-treated RNA samples were determined to be DNA-free by PCR with the primers above. The cDNA synthesis of the tetramycin mRNAs was accomplished using the PrimeScript RT reagent kit (TaKaRa). Gene-specific primers were designed for each gene in the tetramycin biosynthetic gene cluster to investigate their transcription in the mutants and were tested using PCR with chromosomal DNA as the template (Supplementary material, Table S1). The primers for the intergenic regions predicted to be within operons were designed as described by Schmeling et al. [27] (Supplementary material, Table S1). The reaction mixtures and cycling parameters were as follows: first-strand cDNA synthesis with 4 μL of 5× PrimeScript buffer and 1 μL of PrimeScript RT Enzyme Mix I; 500 ng of total RNA and RNase-free H_2O in a total volume of up to 20 μL; reverse transcription reaction at 37 °C for 15 min followed by heating at 85 °C for 5 s to inactivate the reverse transcriptase; and PCR amplification with 0.5 μM of each specific primer and 2 μL of the above first-strand cDNA synthesis mixture in a total volume of 20 μL, 25 cycles of 94 °C for 30 s, 50 to 65 °C (depending on the set of primers used) for 30 s, and 72 °C for 1 min. The PCR products were detected by 0.7 % (w/v) agarose gel electrophoresis with ethidium bromide. These products were observed and photographed using a UV transilluminator.

HPLC and MS analyses of polyene macrolide products

Culture broths were centrifuged to obtain the mycelia and then washed once with distilled water. Similar volumes of methanol were added to the water-washed mycelia. Samples were shaken for 15 min at 37 °C to extract the products. Supernatants containing the products were used for high-performance liquid chromatography (HPLC) analysis, which was performed using a Shimadzu LC-10ATVP HPLC with a Shimadzu SPD-10AVP UV detector set at 304 nm, combined with a Diamonsil RP-C18 column (10 μm; 200 × 4.6 mm). The elution consisted of methanol–water-formic acid (60:40:1). The retention times for tetramycin B, nystatin A1, and tetramycin A were 14.9, 26.1, and 34 min, respectively. High-resolution mass spectra (HR-ESI–MS) analyses of polyene macrolides were performed using a Bruker micrOTOF-Q 125 mass spectrometer.

Heterologous expression and purification of protein

TtmRIV was produced at high level in *E. coli* BL21 (DE3) cells. The *ttmRIV* gene was amplified with primers RIV-32a-S and RIV-32a-AS, H42 total DNA as the template, and the fragment was digested with *Nco*I and *Hin*dIII and cloned into the same sites of pET-32a to generate the expression vector pET-RIV. To express the protein, *E. coli* BL21 (DE3, pET-RIV) and the corresponding control strain BL 21 (DE3, pET-32a) were cultured at 37 °C in 400 mL of LB medium containing 100 μg/mL of ampicillin. When an OD_{600} of 0.5 was reached, expression induction was performed by adding IPTG to a final concentration of 0.05 mM for an additional 9 h culture at 20 °C. Cells were harvested, resuspended in PBS buffer (containing 50 mM NaH_2PO_4; 300 mM NaCl; and 10 mM imidazole, pH 8.0), and lysed using a SCIENTZ-IID ultrasonic cell disruptor. After the sample was filtered through a 0.45 μm membrane, the soluble fraction was applied to a Ni Sepharose™ 6 Fast Flow (GE Healthcare) column. Protein was eluted with 500 mM imidazole in PBS buffer, desalted by dialysis, and stored in distilled water at −80 °C before use.

Electrophoretic mobility shift assays (EMSA)

DNA binding tests were performed using EMSA. The DNA fragments used for EMSA were amplified through PCR using primers labeled with biotin (Supplementary material, Table S2). Up to 6 μg His_6-TrxA or His_6-TrxA-TtmRIV and 2 ng probes were mixed in a reaction system containing binding buffer at 25 °C for 20 min. Reactants were then analyzed on 6 % TBE polyacrylamide gels with 0.5× TBE as a running buffer at 30 °C for 1 h. The procedure was performed in accordance with the guidelines in the LightShift Chemiluminescent EMSA kit manual (Thermo Scientific).

Results and discussion

In silico analysis of putative regulator TtmRIV

The structure of tetramycin is very similar to that of pimaricin except for the C4–C5 and C24 substituent groups (Fig. 1a). This suggests that the biosynthetic pathways as well as the types of regulatory proteins, may be similar for both compounds. In the tetramycin gene cluster, the expected regulatory genes corresponding to *pimM* and *pimR* of the pimaricin pathway have not been identified, but four predicted regulatory genes, namely, *ttmRI*, *ttmRII*, *ttmRIII*, and *ttmRIV*, were present. Computer-assisted analysis of the *ttmRIV* gene product revealed the following positive regulators of known polyene macrolide antibiotics showing strong homology to TtmRIV (Fig. 1c, d): NysRIV (67.2 % identity) of the nystatin-producing strain *S. noursei* ATCC11455 [30], AmphRIV (64.02 % identity) of the amphotericin-producing strain *S. nodosus* ATCC14899 [7], and FscRI (65.08 % identity) of the FR-008/candicidin-producing strain *Streptomyces* sp. FR-008 [35]. All these regulators combined an N terminal PAS domain with a C terminal HTH motif of the LuxR type. Interestingly, TtmRIV was 18 amino acids longer than its counterparts at the

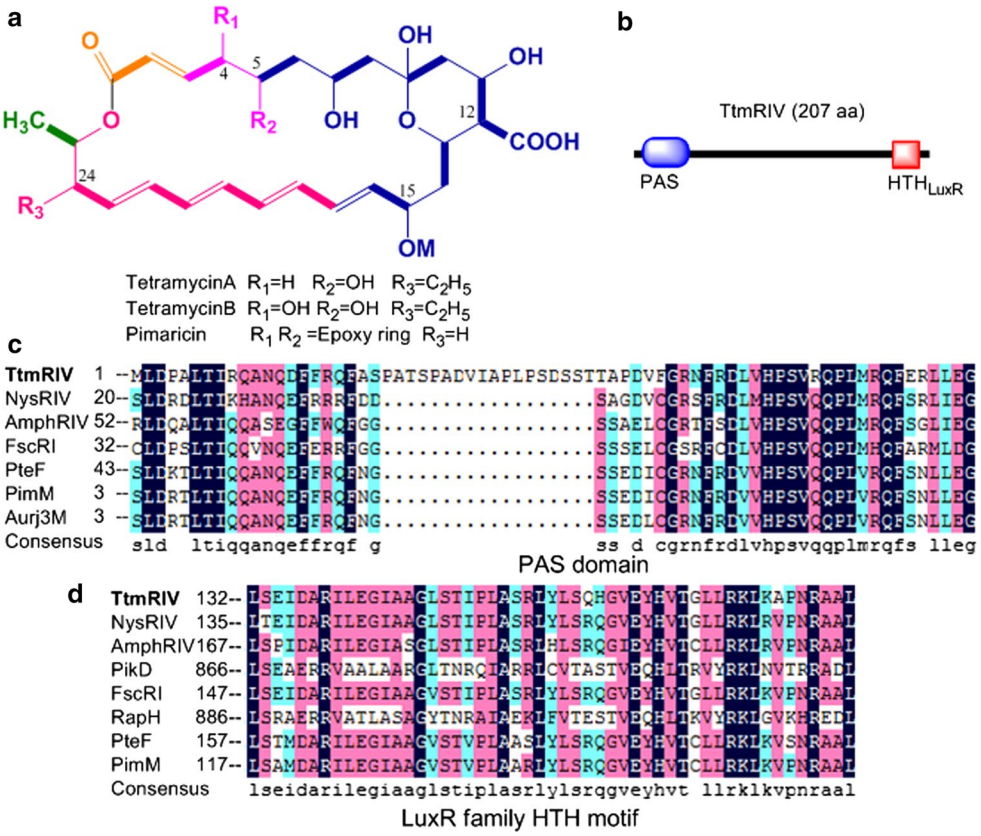


Fig. 1 Domain structure and amino acid sequence alignments of the parts of TtmRIV protein. **a** Tetramycin and pimaricin structure. **b** Schematic of TtmRIV protein. PAS, signal sensor, HTH$_{LuxR}$, DNA binding domain. **c**, **d** Sequence comparison of the N-terminal PAS domains and C-terminal HTH$_{LuxR}$, TtmRIV and other regulators of polyene biosynthetic pathway, Ttm (tetramycin, JX827252), Nys (nystatin, AF263912), Amph (amphotericin, AY639386), Fsc (candicidin, AY310323), Pte (pentaene filipin, BA000030), Pim (pimaricin, AM493721), Aurj (aureofuscin, EU697915), Pik (pikromycin, AF079139), and Rap (rapamycin, X86780). The *numbers* indicated the location of the amino acid residues from the N terminus of the protein

N terminus (Fig. 1b). The genes *ttmRI*, *ttmRII*, and *ttmRIII* encoded three positive LAL-family regulators, which did not improve tetramycin production (unpublished data) as the potential maximum threshold concentration of positive regulators similar to AveR for avermectin in *S. avermitilis* [13].

Inactivation of *ttmRIV* enhanced nystatinA1 biosynthesis

The function of the protein TtmRIV in the tetramycin pathway was confirmed by disrupting its encoding gene through in-frame deletion. A 501 bp internal fragment of *ttmRIV* was deleted in the gene inactivation strain, and recombinant strains were confirmed through PCR analysis by using the primers YP1/YP2 and YP3/YP4 (Table 2; Fig. 2a, b). The length of the deletion fragment was designed to keep the mutant gene in-frame to circumvent the potential possibility of polar effects. Complementation and RT-PCR analyses did not find any polar effects in the gene-deletion mutant Δ*ttmRIV*.

The *ttmRIV* inactivation abolished tetramycin biosynthesis in H42, whereas the Δ*ttmRIV* strain produced 2.1-fold more nystatin A1 than the parental strain (Fig. 3a; Supplementary material, Figs. S1 and S2). To confirm whether the disappearance of tetramycin was directly caused by

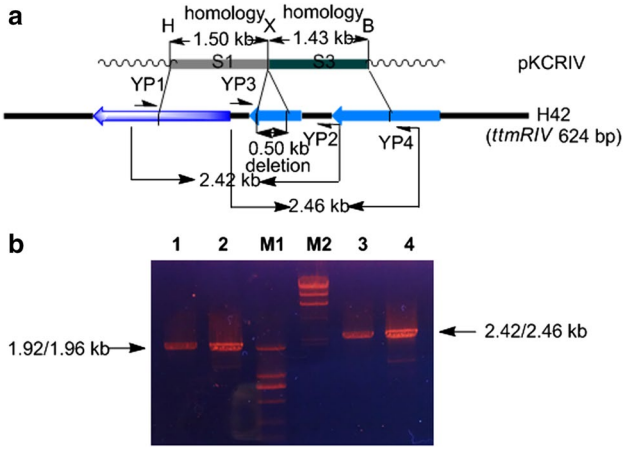
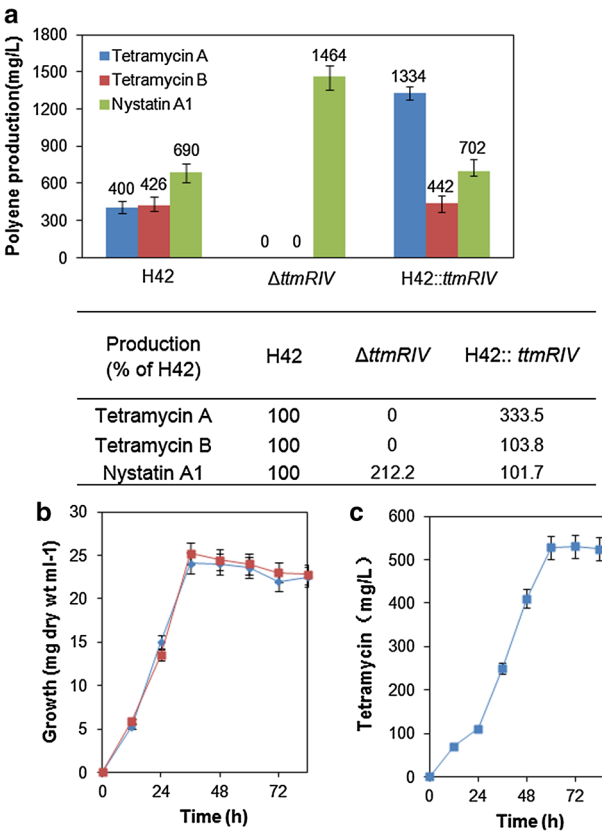


Fig. 2 Targeted gene disruption in H42. **a** Schematic of PCR-targeted gene disruption of *ttmRIV*. The confirmation primers were designated as YP1/YP2 and YP3/YP4. The PCR products of YP1/YP2 and YP3/YP4 with H42 total DNA were 2.42 and 2.46 kb, respectively, and with Δ*ttmRIV* total DNA were 1.92 and 1.96 kb, respectively. The deletion part was 0.5 kb. H, *Hin*dIII; X, *Xba*I; and B, *Bam*HI. **b** Confirmation of constructed mutant strain by PCR. The PCR products were amplified using the primers YP1/YP2 and YP3/YP4; *lanes 1–2*, Δ*ttmRIV*/YP1YP2-YP3YP4; *lanes 3–4*, H42/YP1YP2-YP3YP4; *lane M1*, DL2000 DNA ladder (TaKaRa; 2000, 1000, 750, 500, 250, and 100 bp); *lane M2*, λ-*Hin*dIII digest (TaKaRa, 23130, 9416, 6557, 4361, 2322, 2027, 564, and 125 bp)

the *ttmRIV* gene deletion, a complementation experiment was performed by expressing *ttmRIV* from its native promoter in the integrative plasmid pSET152 introduced into the Δ*ttmRIV* H42 strain, with unmodified pSET152 in Δ*ttmRIV* H42 as control. Tetramycin production was restored to H42 wild type levels in the complemented strain (Supplementary material, Fig. S1), whereas the deletion strain containing an empty vector (unpublished data) was similar to that of Δ*ttmRIV* H42. Additionally, *ttmRIV* deletion did not affect the growth (Fig. 3b) and phenotype of H42.



Production (% of H42)	H42	Δ*ttmRIV*	H42:: *ttmRIV*
Tetramycin A	100	0	333.5
Tetramycin B	100	0	103.8
Nystatin A1	100	212.2	101.7

Fig. 3 Secondary metabolite products and growth of *Streptomyces*. **a** Comparison of tetramycin and nystatin A1 production and average rates in the extracts from the H42 strain and its derivative mutants. Δ*ttmRIV*, the deleted *ttmRIV* strain; H42::*ttmRIV*, the overexpressed *ttmRIV* strain. This production of polyene was determined through HPLC assay using nystatin as the standard or substituted standard (for tetramycin). Nystatin A1 production was 1464 mg/L in Δ*ttmRIV* (2.1-fold of H42), whereas tetramycin A production was 1334 mg/L in H42::*ttmRIV* (3.3- fold of H42). **b** The growth curves of H42 and *ttmRIV* mutant strain in seed medium. Growth was monitored by measuring the mycelial dry weight. Growth curves of the H42 strain (*diamonds*), the Δ*ttmRIV* strain (*squares*). **c** Production analysis of tetramycin using H42 in the solid fermentation medium. This *curve* was determined using anti-microbiology assay against *Saccharomyces cerevisiae* with nystatin as the substituted standard. *Error bars* indicate standard deviations. Average data from three parallel experiments are presented

Nystatin A1 enhancement in the Δ*ttmRIV* strain may have resulted from the redirection of precursor fluxes, such as was seen in the *ttmS1* disruption [21]. This phenomenon also was observed in *S. avermitilis* mutants. The disruption of the positive regulatory genes *olmRI* and *olmRII* abolished the production of oligomycin but increased that of avermectin [33]. By contrast, *aveR* deletion decreased the production of avermectin but increased that of oligomycin [37].

Transcriptional control of tetramycin biosynthetic pathway genes

To evaluate the possible role of *ttmRIV* in the gene expression of tetramycin biosynthetic gene cluster, RT-PCR was utilized, and gene-specific primers for RT-PCR were designed to produce 500 to 600 bp products. An additional pair of primers for the *lysA* gene was used as the internal control [1]. In the case of *ttmRIV*, transcripts were analyzed using primers P-RIV-S and P-RIV-AS located before and after the deletion. The transcripts from the 20 genes of the tetramycin biosynthetic gene cluster, including *ttmRIV,*

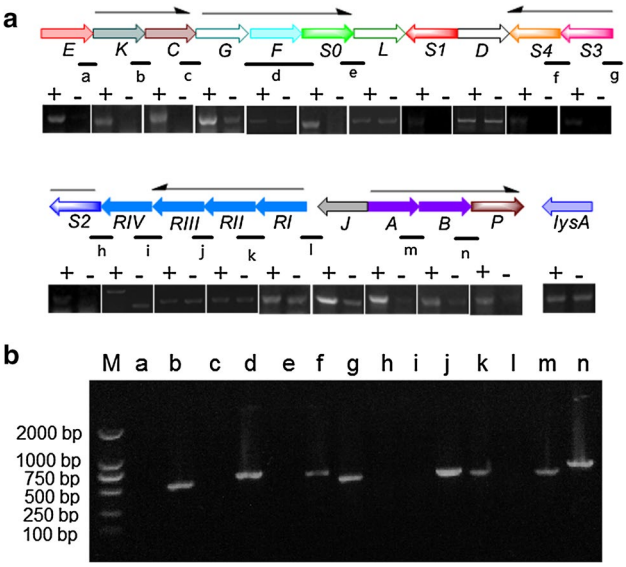


Fig. 4 RT-PCR results. **a** Gene expression analysis of the *ttm* gene cluster using RT-PCR. Analysis was performed on H42 (+) and Δ*ttmRIV* (−) strains as indicated in " Materials and methods" section. *lysA* gene transcription (encoding diaminopimelate decarboxylase) was assessed as an internal control. The *arrows* indicate the organization of the genes within the *ttm* cluster. The *single arrows* above the genes represent the putative transcripts, namely, *ttmK-C*, *ttmG-F-S0*, *ttmS2-S3-S4*, *ttmRI-RII-RIII*, and *ttmA-B-P*. The *short lines* below the gene cluster represent the designed segments to analyze the operons. **b** Operon analysis through RT-PCR. The present products of the intergenic regions showed that the two genes underwent co-transcription, otherwise their transcripts were independent. M, DL2000 DNA Marker (TaKaRa). The fragment *d* contained a part of *ttmG*, the whole *ttmF*, and a part of *ttmS0* as *ttmF* was only 192 bp

were analyzed after 25 PCR cycles. These analyses were performed at least thrice for each primer pair (Fig. 4).

The culture age of the mycelia at harvest was decided depending on time–production curves, and 45 h was chosen as the time when tetramycin was actively produced (Fig. 3c). All the 20 genes were transcribed at 45 h in H42; however, when the transcription pattern in Δ*ttmRIV* was analyzed, no or low-level transcripts were found for the genes *ttmE*, *ttmK*, *ttmC*, *ttmG*, *ttmS0*, *ttmS1*, *ttmS2*, *ttmS3*, *ttmS4*, *ttmJ*, *ttmA*, *ttmB*, and *ttmP*. The RT-PCR reactions carried out in this study were not quantitative, but since the reactions were stopped after 25 cycles, and before end points had been reached, this suggested that the expression of these genes was directly or indirectly controlled by TtmRIV. Considering the functions of the genes above, all of the type I PKS genes in the tetramycin pathway were influenced by the *ttmRIV* mutant. Moreover, *ttmS0* was the gene required for chain initiation, whereas *ttmS1*, *ttmS2*, *ttmS3*, and *ttmS4* were the genes responsible for the 12 rounds of chain elongation. This observation suggested that TtmRIV controls tetramycin biosynthesis by regulating the transcripts of all the genes involved in the backbone biosynthesis. In addition, *ttmC*, *ttmJ*, and *ttmK* were the genes required for mycosamine biosynthesis and the attachment of the mycosamine moiety [21], and *ttmG* was responsible for carboxylation of methyl group at C12 of tetramycin. The attachment of mycosamine to C15 must occur after the carboxylation of methyl group at C12, as the unpublished data for the modification steps order of the tetramycin pathway, as well as those reported regarding the nystatin and amphotericin pathways [5, 10]. Therefore, the regulator TtmRIV controlled most post-modification steps of the backbone by regulating the three key genes. The other genes, such as *ttmA* and *ttmB*, which encode the putative heterodimer ABC transporters, were involved in tetramycin secretion, whereas *ttmP* provided the four-carbon extender unit during tetramycin biosynthesis and was affected by the lack of TtmRIV. In the case of putative LAL family regulatory genes *ttmRI*, *ttmRII*, *ttmRIII*, and *ttmRIV*, their transcriptions were not influenced by *ttmRIV* disruption, indicating that the functions of the other family regulatory genes were independent of *ttmRIV*, and similarly, TtmRIV was not autoregulated. Finally, *ttmL*, a gene that showed no effect on tetramycin production in the tetramycin biosynthetic gene cluster [6], also showed transcription that was not affected by the *ttmRIV* gene deletion. In conclusion, these results demonstrated that *ttmRIV* encoded a PAS-LuxR-like pathway-specific transcription activator of the tetramycin pathway in *S. ahygroscopicus*.

Furthermore, several operons in the tetramycin biosynthetic gene cluster were predicted on the basis of the RT-PCR results (Fig. 4). The present products of the fragments b, d, f, g, j, k, m, and n showed that the two adjacent genes of them underwent co-transcription. On the contrary, the genes located two flanks of the fragments a, c, e, h, i, and l transcribed independently. Five potential operons were identified in this gene cluster, namely, *ttmK-C*, *ttmG-F-S0*, *ttmS2-S3-S4*, *ttmRI-RII-RIII*, and *ttmA-B-P*. Antibiotic biosynthesis genes were also generally controlled as operons by the pathway-specific regulators in other *Streptomyces* strains. For instance, SsaA regulated the sansanmycin pathway by controlling several operons in *Streptomyces* sp. SS [15], Aur1P triggered the expression of an operon containing 22 structural genes (*aur1A-aur1U*) in the auricin cluster in *S. aureofaciens* [19], and StrR controlled streptomycin production in the same manner in *S. griseus* [23].

DNA and protein binding assays

On the basis of the RT-PCR results and putative transcripts mentioned above, four DNA fragments covering the putative promoters of five genes were amplified and labeled with biotin as probes. For the EMSA experiments, a non-specific probe and His_6-TrxA (Fig. 5a) were set as a negative control reaction. For the His_6-TrxA-TtmRIV-containing reactions (Fig. 5a), retarded bands appeared in all of the binding reactions with the four probes, which included

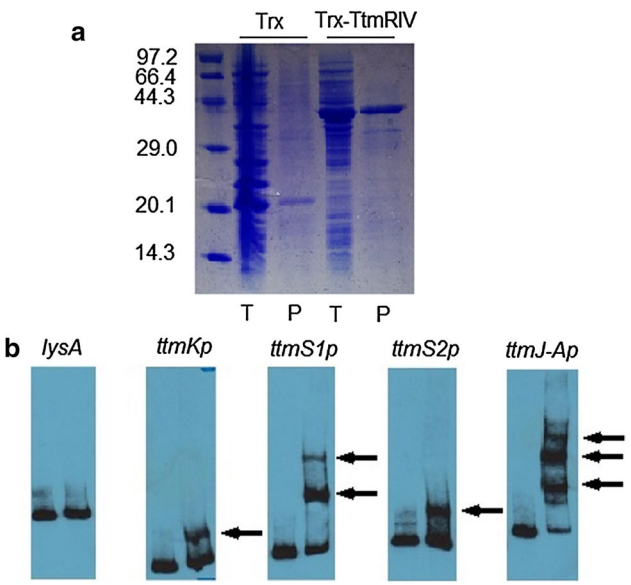


Fig. 5 Binding analysis between TtmRIV and the five promoters using EMSA. **a** Purification of Trx and fusion protein Trx-TtmRIV in *E. coli* BL21. *Lanes T*, total *E. coli* cell extract; *lanes P*, purified proteins after affinity Ni column; and left lane, molecular size markers (in kDa). **b** DNA binding assay results (EMSA) of Trx-TtmRIV binding to different putative promoter regions. *Left lane*, control with 6 μg of protein Trx; *right lane*, 6 μg of Trx-TtmRIV protein. The *lysA* region was used as a negative control. The *arrows* indicate the DNA–protein complexes. Promoter names are indicated above the pictures. All binding experiments were performed using 2 ng of biotin-labeled DNA probe

ttmKp (promoter of gene *ttmK*, one retardation band), *ttmS1p* (promoter of gene *ttmS1*, two retardation bands), *ttmS2p* (promoter of gene *ttmS2*, one retardation band), and *ttmJ-A* (promoters of genes *ttmJ* and *ttmA*, three retardation bands) (Fig. 5b). Meanwhile, no retardation bands were found in the nonspecific probe reactions. One shifted band appeared upon the incubation of His_6-TrxA-TtmRIV with the *ttmKp* or *ttmS2p* promoter, suggesting that this regulator directly binds to a single site within these regions. In the case of the *ttmS1p* promoter, two retardation bands were observed, whereas three retardation bands were obtained with the *ttmJ-Ap* bidirectional promoter region. More than one shifted band suggested the possibility that more than one binding site could exist in these promoter regions or the proteins were able to form the dimer or multimer which do not affect their bioactivity.

The PAS–LuxR transcriptional regulators in several polyene macrolide biosynthesis pathways show functional conservation by binding to the double strands of the target promoters. The binding sites span 16 nucleotides and adjusted consensus CTVGGGAWWTCCCBAG (where V is A, C, or G; W is A or T; and B is C, G, or T) [25]. *In silico* analysis of the sequences of the four probes showed significant similarity with the corresponding promoters in the pimaricin cluster [26]. Furthermore, five potential binding sites were determined on the four single strands (*ttmJ-Ap* contained two binding sites). All of the sites were located within the range of the probes in nucleotide positions −180 to −90 with respect to the genes translational ATG/GTG start sites (Fig. 6a).

a

Promoter	Strand	Alignment	Identity	*Ri* score
ttmKp	+	-106<TACGGATTTTCCGGTG>-91	75.0%	13.95
ttmKp	-	-108<CCGGAAAATCCGTAGA>-93	81.3%	12.81
ttmS1p	+	-112<TACGGAAACCCGCAGT>-97	93.8%	13.22
ttmS1p	-	-114<TGCGGGTTTCCGTAGT>-99	93.8%	12.76
ttmS2p	+	-159<TACGGAATTCCCTGAC>-144	87.5%	17.39
ttmS2p	-	-161<CAGGGAATTCCGTAGA>-146	87.5%	12.43
ttmAp	+	-115<TACGGATTTTCCGGTG>-100	87.5%	14.95
ttmAp	-	-117<CCGGAAAATCCGTAGT>-102	81.3%	3.09
ttmJp	+	-157<CCGGAATTGCTTGACG>-142	87.5%	19.24
ttmJp	-	-179<TACGGAATTCCGGAGT>-164	87.5%	13.95

b

Fig. 6 Putative binding sites of TtmRIV. **a** The putative binding sites and their information content (*Ri*). The nucleic acids in the *yellow shade* means they are different with the location on the PimM binding sites. The identity values showed the consistency of TtmRIV and PimM binding sites on the corresponding genes. **b** Sequence logo of TtmRIV binding sites. The logo was created with the 10 putative binding sites listed above. The height of each *letter* is proportional to the frequency of the base, and the height of the *letter stack* shows the conservation in bits at that position [28]. The total information ($R_{sequence}$) for the binding site is 13.22 bits (0.83 bits per base)

One shifted band was obtained in each of the *ttmKp* and *ttmS2p* promoter regions, which is in agreement with the presence of one binding site in each region. In the case of *ttmJ-Ap* and *ttmS1p*, the number of shifted bands exceeded the number of proposed binding sites, which might be explained by weak DNA–protein interactions that are stabilized once in the polyacrylamide gel [14], although the possibility of being derived from potential pseudo-binding sites cannot be excluded. There should not be the proteins dimer or multimer who play roles in the appearance of multi retardation bands of *ttmJ* and *ttmA*, and *ttmS1* as only one retardation band was present in *ttmK* and *ttmS1*binding reactions.

The individual information content (*Ri*) [28] of the binding sites demonstrated the requirements shown by TtmRIV for DNA recognition, in that the TtmRIV binding sites showed high *Ri* values ranging from 3.09 bits in the reverse strand of the *ttmAp* promoter to 19.24 bits in the *ttmJp* promoter. The total information ($R_{sequence}$) for the series binding site showed 13.22 ± 2 (0.83 bits per base). This site spanned 14 nucleotides, displayed dyad symmetry, and adjusted to the consensus YVSGGAWWTCCSBR (where Y is C or T, V is A, C, or G; S is C or G; W is A or T; B is C, G, or T; and R is A or G). A sequence logo [29] is illustrated in Fig. 6b, and the identity of this logo with that previously reported suggests that TtmRIV demonstrates the same working principle and belongs to the PAS-LuxR type.

Overexpression of *ttmRIV* boosts tetramycin A production

At present, tetramycin is mainly used as an agricultural antibiotic, but the similar structures of tetramycin and pimaricin also suggest it has potential for usage as a food additive. Therefore, the overexpression of *ttmRIV* with *emrEp** in H42 was proposed as a means to improve tetramycin production. Compared with the production in strain H42, tetramycin A production increased to 3.3-fold in the engineered strain, while that of tetramycin B (1.03-fold of H42) showed almost no difference (Fig. 3a; Supplementary material, Figs. S1 and S2). As indicated by the RT-PCR results, the transcription of *ttmD,* the gene responsible for transforming tetramycin A to tetramycin B, did not change after *ttmRIV* deletion. This result may explain why the increased copy number of *ttmRIV* in the engineered strain of H42 enhanced tetramycin A production but not tetramycin B production.

Conclusions

PAS-LuxR-type regulators bind to the upstream region of their target genes to activate transcription. This study reported a positive regulator, TtmRIV, belonging to this

type and helped to explain the regulatory mechanisms involved through RT-PCR studies and EMSA. Increasing the copy number of *ttmRIV* in H42 improved tetramycin A production, whereas *ttmRIV* deletion improved nystatin A1 production. These results provided further evidence that manipulating the key regulatory genes may be a particularly valuable strategy in genetic engineering of high production strains.

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