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SHORT COMMUNICATION

Functional expression of SCO7832 stimulates tautomycetin production via pathway-specific regulatory gene overexpression in *Streptomyces* sp. CK4412

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Abstract Comparative transcriptome analysis has revealed several acidic pH shock-induced genes presumably involved with stimulation of antibiotic production by *S. coelicolor* (Kim et al. Appl Microbiol Biotechnol 2007). *Streptomyces* sp. CK4412 produces a novel T cell-specific immunosuppressive compound, tautomycetin (TMC). When cultured at acidic pH medium, it also exhibited higher TMC productivity. To verify a gene responsible for acidic pH shock-induced TMC stimulation, a putative acid-shock-induced gene, SCO7832 encoding an Na^+/H^+ antiporter protein, was cloned under the influence of a strong constitutive *ermE** promoter in an integrative expression pSET152 vector. This was followed by its conjugation into the TMC-producing *Streptomyces* sp. CK4412. Comparing TMC production and antifungal activity of wild-type and the SCO7832-containing exconjugant revealed that SCO7832 stimulated TMC production more than 3.5-fold in *Streptomyces* sp. CK4412. The over-expression of SCO7832 did not affect the ratio between intra- and extracellular TMC productions. However, it significantly stimulated the expression of a TMC-specific positive regulatory gene. This implies that the stimulatory effect of SCO7832 functions in TMC-producing *Streptomyces* sp. CK4412 via up-regulation of a TMC pathway-specific positive regulatory gene, *tmcN* overexpression.

Keywords Antibiotic stimulation · SCO7832 · Tautomycetin · *Streptomyces* · pH-shock

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Introduction

Streptomycetes, the most ubiquitous morphologically differentiating gram-positive filamentous soil bacteria, are well known for producing a variety of commercially valuable enzymes and secondary metabolites, including antibiotics, anti-tumor agents, immunosuppressants and enzyme inhibitors [2, 9, 10, 25, 28]. *Streptomyces*-derived secondary metabolite production generally begins at the onset of the stationary growth phase and correlates temporally with the formation of aerial mycelia for cultures grown on the surfaces of solid media [1, 3, 24]. Previous studies have shown that the regulation of secondary metabolite production for *Streptomyces* sp. operates at several layers of control. This involves a complex regulatory network in response to external stimuli, such as nutrient depletion, salt shock, oxidative stress and acidic pH shock [8, 14, 17, 18, 26]. Some of these affect only one specific antibiotic production, whereas others affect several antibiotic productions along with morphological differentiation, suggesting that the two processes share some elements of genetic control, while others are unique. Among this regulatory network, the genes working at the proximal level usually reside within the respective biosynthetic gene cluster and are pathway-specific regulatory genes that only affect a single secondary metabolite biosynthetic pathway. The well-characterized pathway-specific

regulatory proteins including ActII-ORF4 for actinorhodin biosynthesis from *S. coelicolor* belong to the so-called *S*treptomyces Antibiotic Regulatory Proteins (SARPs) family [16]. Another important transcriptional family of regulators that belong to the so-called LAL (Large ATP-binding regulators of the LuxR) family have also been identified and characterized in several macrolide antibiotic pathways including TmcN for tautomycetin [20].

Tautomycetin (TMC) is a secondary metabolite produced by *Streptomyces* sp. CK4412 that has novel T cell-specific immunosuppressive activity as well as colorectal anticancer activity [22, 27]. TMC has a unique ester bond linkage between a terminal cyclic anhydride moiety and a linear polyketide chain that bears an unusual terminal alkene, whose chemical structure is identical to a previously reported antifungal compound produced by *S. griseochromogenes* (Fig. 1), [4]. Due to the superior pharmacokinetic potential of TMC, whose mechanism of action is different from CsA or FK506 [27], it is considered a new drug candidate with potent T cell-specific immunosuppressive activity. We previously isolated and characterized the entire TMC biosynthetic gene cluster from *Streptomyces* sp. CK4412. This revealed 2 ORFs that encoded a typical modular polyketide synthase (PKS) gene in addition to 12 ORFs located at both flanking regions, the deduced functions of which were consistent with TMC biosynthesis [5]. Recently, an entire biosynthetic pathway cluster for a structurally similar compound, tautomycin, was also identified for *S. spiroverticillatus* [23].

It has been reported that production of the antibiotic actinorhodin was significantly enhanced when an acidic pH shock was applied to surface cultures of *S. coelicolor* [20, 21]. Among many over-expressed genes identified from *S. coelicolor* DNA microarrays [19, 20], several Na^+/H^+ antiporter genes including SCO7832 were also identified as pH-shock-induced potential target genes. Moreover, the SCO7832 cloned in a high-copy plasmid pWHM3 in *S. lividans* stimulated the actinorhodin production in both plate and liquid cultures (Kim et al. unpublished data). Since a TMC-producing *Streptomyces* sp. CK4412 cultured at acidic pH also exhibited much stronger antifungal activity, as well as higher TMC production, than those cultured at neutral pH [12], a similar acidic pH shock induction could be involved in enhanced TMC production and/or secretion in *Streptomyces* sp. CK4412. Here we describe the cloning and functional overexpression of SCO7832 in TMC-producing *Streptomyces* sp. CK4412. We demonstrate that the stimulatory effect of SCO7832 functions via the up-regulation of a TMC pathway-specific positive regulatory gene. Overexpression of this regulatory gene suggests that enhanced TMC production is probably not due to direct enhancement of the antibiotic secretory mechanism in *Streptomyces* sp. CK4412.

Materials and methods

Bacterial strains and culture conditions

Streptomyces sp. CK4412, kindly provided by ForHuman-Tech Ltd., Korea, was used as a TMC-producing strain [27]. *S. coelicolor* total chromosomal DNA was used for SCO7832 gene cloning. Both strains were cultured at 28°C in either R2YE or MS (2% soy bean and 2% mannitol)

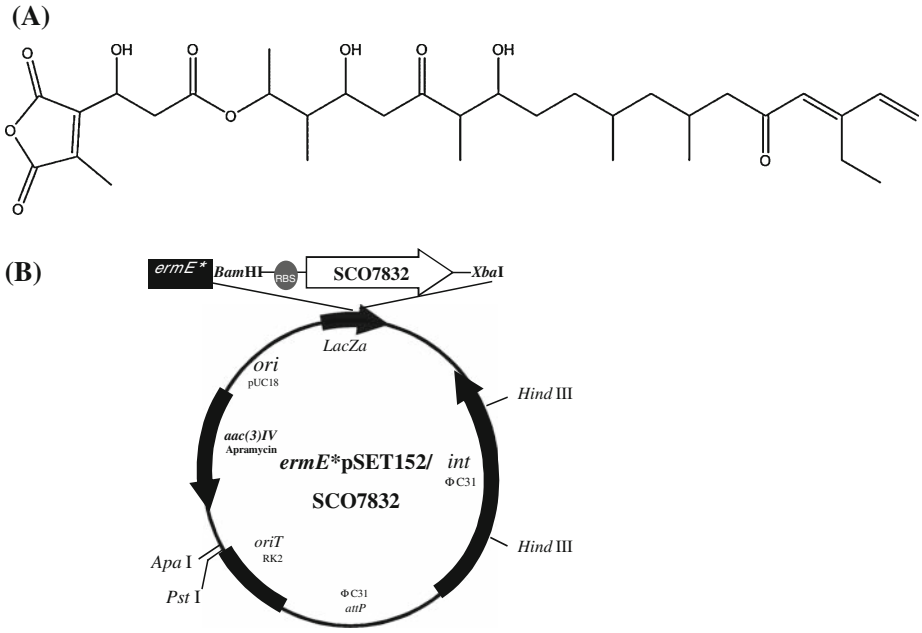

Fig. 1 **a** The structure of tautomycetin (TMC). **b** Map of pSET152 derivative integrative plasmid containing the *erm*E* promoter and SCO7832

liquid medium [16]. For TMC production, *Streptomyces* sp. CK4412 cells were pre-cultured for 3 days in R2YE and then cultured for 4 more days in MS medium. *Escherichia coli* DH5α strain was used for DNA cloning and plasmid propagation. *E. coli* ET12567/pUZ8002 (*dam2, dcm2, hrdM*) was used as the transient host for *E. coli–Streptomyces* conjugation [6]. All *E. coli* strains were cultured at 37°C in Luria broth or on Luria agar, supplemented with the appropriate antibiotics when needed [16].

Construction of an integrative plasmid for SCO7832 overexpression

For heterologous functional overexpression of SCO7832 in *Streptomyces* sp. CK4412, a 1.5-kb DNA fragment including the entire SCO7832 gene was amplified by PCR using genomic DNA from *S. coelicolor* as a template and the primer pair $P_{SCO7832}$-forward (5′-GGATCCACGAACGGT TATGTCAGCT-3′) and $P_{SCO7832}$-reverse (5′-TCTAGAG TCACAGGCGAAGG 3′). The underlined sequences indicate *Bam*HI and *Xba*I restriction sites, respectively. PCR was performed in a final volume of 20 µl containing 0.4 µM of each primer, 0.25 mM of each of the 4 dNTPs (Roche), 1 µl of extracted DNA, 1 U of Ex Taq polymerase (TaKaRa, Japan) in its recommended reaction buffer, and 10% DMSO. Amplifications were performed in a Thermal Cycler (BioRad, USA) according to the following profile: 30 cycles of 60 s at 95°C, 60 s at 55°C, and 70 s at 75°C. The amplified PCR product was analyzed by electrophoresis in 1% (w/v) agarose gel and purified via a DNA extraction kit (COSMO, Korea). This was ligated into pMD18-T (TaKaRa), followed by complete nucleotide sequencing confirmation by Genotech Korea. The PCR-amplified SCO7832 gene was cloned into the *Bam*HI and *Xba*I double-digested pSET152 derivative integrative plasmid [7, 15] containing a strong constitutive promoter *ermE** (Fig. 1).

HPLC quantification and antifungal bioassay for TMC

For HPLC analysis of intra- and extra-cellular TMC production, both the culture broth supernatant and the cell pellet were separately extracted with equal volumes of either chloroform for the broth or acetone for the pellet [13]. The extracts were dried using a rotoevaporator and then resuspended in methanol. Extracts were fractionated by HPLC using isocratic conditions with methanol: water: buffer (1% diethylamine-formic acid pH 7.3) = 75: 15: 10 on a Genesis C18 4-µm column with UV detection at 273 nm. TMC production was also evaluated by a biological assay against *Aspergillus niger* as an indicator using a paper disc containing the same culture broth extract used in the HPLC assay [13]. The paper disc was placed on top of *A. niger* that had been incubated on ME medium (0.05% malt extract, 0.05% glucose, 0.001% peptone in 1L ddH_2O) for 6 h at 30°C, followed by measurement of the inhibition zone after overnight incubation at 30°C.

Isolation of total RNA and gene expression analysis by RT-PCR

Streptomyces sp. CK4412 containing an empty vector and the SCO7832 containing *Streptomyces* sp. CK4412 exconjugant were grown for 72 h in R2YE medium. The cultures were washed twice with 1 volume of sterile water. The mycelia were harvested by centrifugation and immediately frozen by immersion in liquid nitrogen. The frozen mycelia were broken by shearing in a mortar, and the frozen lysate was added to RLT buffer (Qiagen) in the presence of 1.0% β-mercaptoethanol. RNeasy mini spin columns were used for RNA isolation according to the manufacturer's instructions. RNA preparations were treated with DNase I (Qiagen) to eliminate possible chromosomal DNA contamination.

DNase I-treated RNA (7 µg) was used as a template for reverse transcription (RT) at 50°C with an AVM Reverse Transcriptase XL (TaKaRa, Japan) and random hexamers. The conditions for cDNA synthesis were as follows: 30°C for 10 min, 50°C for 1 h, 99°C for 2 min, 5°C for 5 min. The resulting cDNA was used for PCR amplification under the following conditions: denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 35 s for 25 cycles. Each primer pair for TMC biosynthetic genes was carefully designed to generate a PCR product of approximately 150 to 200 bp using a genscript site (http://www.genscript.com/ssl-bin/app/primer). The complete RT-PCR primer sequences pairs are listed in Table 1.

Results and discussion

SCO7832, which is present as a duplicate at the opposite end of the *S. coelicolor* linear chromosome (SCO0015), encodes a 410 aa Na^+/H^+ antiporter protein (http://strepdb.streptomyces.org.uk). Generally, Na^+/H^+ antiporter proteins are thought to function as pH stabilizers in bacterial cells by controlling the H^+ concentration upon a pH-shift in the culture [11, 29]. Although the detailed mechanisms for Na^+/H^+ antiporter proteins in *Streptomyces* species are not known, they are also proposed to play important roles for maintaining cell viability, inducing secondary metabolite biosynthesis and promoting metabolite secretion upon an acid pH shock [21].

To demonstrate that SCO7832 was, indeed, responsible for acid pH shock-induced TMC stimulation, we engineered the wild-type strain of *Streptomyces* sp. CK4412 by

Table 1 The complete RT-PCR primer sequences pairs

Primers	Sequence (5′-3′)	Description
tmcB-F	TCCGGTGGTGTCGAACTTGA	Forward primer for *tmcB*
tmcB-R	GCATCGGTGCACTGTTGTCC	Reverse primer for *tmcB*
tmcC-F	GTGCTGGTGTGGCTGCACTT	Forward primer for *tmcC*
tmcC-R	ATCTGGTCGAGCAGGGCAAG	Reverse primer for *tmcC*
tmcJ-F	CGAGACCCATCTCGTGCTGA	Forward primer for *tmcJ*
tmcJ-R	CGAGCGTCTTCATGGTGCAG	Reverse primer for *tmcJ*
tmcN-F	GGACGAGACCCGGAGGAGTT	Forward primer for *tmcN*
tmcN-R	TGACGCAATGTCCTGACGTG	Reverse primer for *tmcN*
rRNA-F	GACTCCTACGGGAGGCAGCA	Forward primer for rRNA
rRNA-R	CGCCCAATAATTCCGGACAA	Reverse primer for rRNA

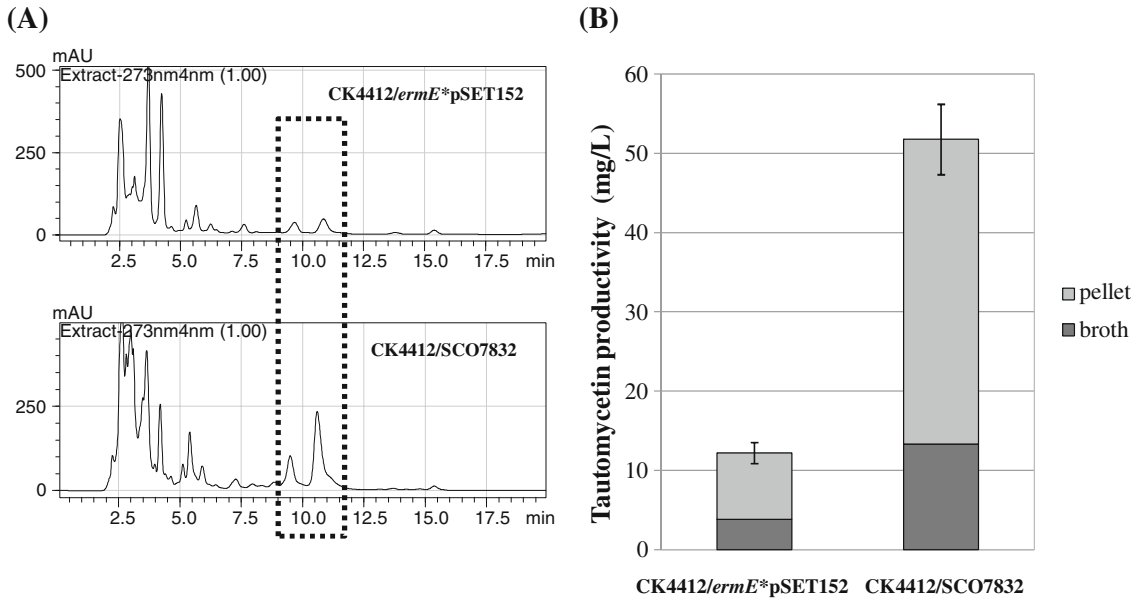

Fig. 2 HPLC chromatograms **a** from chloroform-extracted broths of the strains containing an empty vector (CK4412/*ermE**pSET152) and the SCO7832-containing strain (CK4412/SCO7832). **b** Intra- and extra-cellular TMC productions measured by quantitative HPLC analyses (average of triplicates) from the strains containing an empty vector (CK4412/ermE*pSET152) and the SCO7832-containing strain (CK4412/SCO7832)

expressing SCO7832 under the influence of a strong constitutive *ermE** promoter in the integrative expression pSET152 vector. This was followed by conjugation into TMC-producing *Streptomyces* sp. CK4412. Comparing TMC production and antifungal activity of wild-type and the SCO7832-containing exconjugant showed that SCO7832 stimulated TMC production more than 3.5-fold in *Streptomyces* sp. CK4412 (Fig. 2a, b). Moreover, the over-expression of SCO7832 did not affect the ratio between intra- and extra-cellular TMC productions (Fig. 2c). Although pH shock was proved to be involved in stimulation of both secretion and biosynthesis of actinorhodin in *S. coelicolor* [20], its TMC stimulatory mechanism in *Streptomyces* sp. CK4412 seems to be limited to only the stimulation of TMC biosynthesis, implying that the SCO7832-induced TMC stimulatory effect might not be directly related to the metabolite secretory system in *Streptomyces* sp. CK4412.

To elaborate on the SCO7832-induced TMC stimulatory mechanism, total RNA samples were prepared from wild-type *Streptomyces* sp. CK4412 containing an empty vector and the SCO7832-overexpressing *Streptomyces* sp. CK4412 exconjugant for RT-PCR gene expression analysis. Primers for RT-PCR were specific for sequences within *tmc* genes (Table 1) and were designed to produce cDNAs of approximately 200 bp. A primer pair designed to amplify a cDNA for the 16 s rRNA gene was used as an internal control. Transcripts were analyzed for the four genes located in four different putative operons within the *tmc* cluster [5]. By RT-PCR analysis, the transcripts of all four genes, including *tmcB, tmcC, tmcJ,* and *tmcN*, were detected from the empty vector *Streptomyces* sp. CK4412

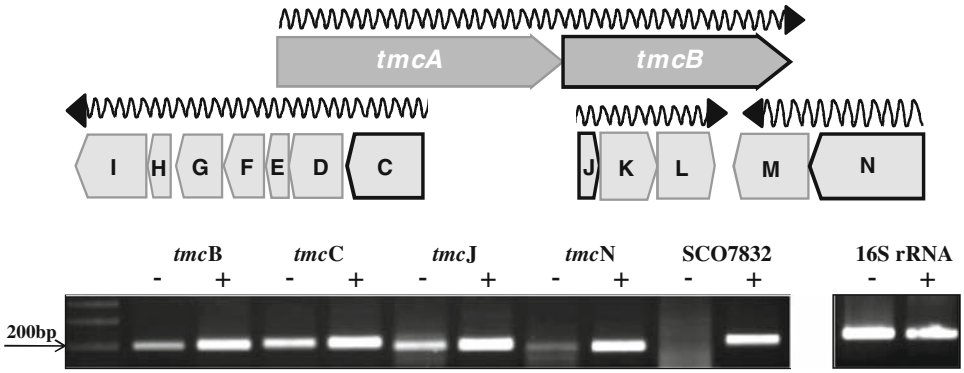

Fig. 3 RT-PCR gene expression analyses for *Streptomyces* sp. CK4412/*ermE**pSET152 (−) and *Streptomyces* sp. CK4412/SCO7832 (+) strains as described in Materials and Methods. Transcription of the 16 s *rRNA* gene was also assessed as an internal control. A diagram with the organization of the genes within the TMC cluster and their putative transcripts is also shown

wild type, while the transcription pattern for the SCO7832-overexpressing *Streptomyces* sp. CK4412 exconjugant was enhanced for all four genes, especially *tmcN* (Fig. 3). Because the *tmcN* was recently identified as a key pathway-specific positive regulatory gene that controls the remainder of the TMC biosynthetic genes [13], the stimulatory effect of SCO7832 is believed to function via up-regulation of *tmcN* transcription in *Streptomyces* sp. CK4412. According to the recent results of transcriptional and proteomic analyses, an acidic pH shock was considered to be one of the strongest stresses to influence a wide range of sigma factors and shock-related proteins, including general stress response proteins. The up-regulation of the sigma factors and shock proteins already found to be related to actinorhodin biosynthesis was considered to have contributed to enhanced actinorhodin productivity by mediating the pH shock signal to regulators or biosynthesis genes for actinorhodin production [19], implying that the detailed TMC stimulatory network initiated by SCO7832 overexpression in *Streptomyces* sp. CK4412 could be also very complex and complicated; this mechanism remains to be further determined.

In conclusion, the functional expression described here is an efficient approach to identify previously unknown acidic pH shock-induced genes. One of the key factors for further development of TMC as a medicinal agent is the limited amount of this natural product available through fermentation methods. Although the wild-type *Streptomyces* sp. CK4412 strain containing an extra copy of *tmcN* led to an approximately 5.5-fold increase in TMC biosynthesis [13], additional manipulations of a positive regulator, such as SCO7832, may result in further improvements in TMC production. Moreover, additional manipulations of a newly identified positive regulator, such as SCO7832, may result in further improvements in the production of pharmaceuticals produced by industrial *Streptomyces* strains, including those for which complete genome sequence information and knowledge of regulatory mechanisms at the molecular level are not currently available.

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