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Cloning of *metK* from *Actinoplanes teichomyceticus* ATCC31121 and Effect of Its High Expression on Antibiotic Production

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A *metK* gene encoding *S*-adenosyl-L-methionine synthetase was cloned from the non-*Streptomyces* actinomycetes, *Actinoplanes teichomyceticus* ATCC31121. In order to evaluate the effect of the *metK* expression on antibiotic production in actinomycetes, an expression vector harboring the *metK* gene was constructed and introduced into *Streptomyces lividans* TK24 and *A. teichomyceticus*, and the antibiotic production of the exconjugants was assessed. As a result, it was determined that the expression of *metK* induced 17-fold and 2.2-fold increases in actinorhodin production from *S. lividans* TK24 and teicoplanin production from *A. teichomyceticus*, respectively, compared with the control strains.

Keywords: *Actinoplanes teichomyceticus*, teicoplanin, *metK*, cloning, actinorhodin

S-Adenosylmethionine (SAM) is synthesized *via* a metabolic reaction involving adenosine triphosphate and L-methionine which is catalyzed by *S*-adenosyl-L-methionine synthetase (SAM-s) and encoded by the *metK* gene [11]. It is one of the most versatile compounds in living organisms, in addition to its widely recognized role as a methyl donor in DNA, RNA, histones, and other proteins [6]. It has also been shown to enhance the production of secondary metabolites in *Streptomyces* strains [14]. The overexpression of the *metK* gene in *S. coelicolor* A3(2) has been shown to enhance the production of actinorhodin in agar plates. This was caused by the induction of *act*II-ORF4, a transcription activator of actinorhodin biosynthetic gene clusters [10, 14]. Additonally, overexpression of the *metK* gene stimulated the formation of novobiocin, concomitant with an increase in the intracellular SAM concentration; the addition of external SAM did not affect novobiocin accumulation [20]. The introduction of high-copy-number plasmids harboring *metK* genes resulted in 1.4~2.1-fold greater levels of doxorubicin production in *S. peucetius* var. *caesius* [13]. However, despite the SAM-s in *Streptomyces* species have been studied extensively, no studies have yet been conducted on non-*Streptomyces* actinomycetes, which are occasionally referred to as "rare" actinomycetes, although they have received some recent attention as very rich sources of medically important secondary metabolites and novel antibiotics.

In this study, *metK* encoding SAM-s was cloned from a representative non-*Streptomyces* actinomycetes, *Actinoplanes teichomyceticus* ATCC31121; this strain produces teicoplanin, which is clinically employed for the treatment of Gram-positive pathogenic bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) [1, 5]. In order to assess the effects of *metK* related to increased antibiotic biosynthesis, a *metK* high expression vector was constructed and introduced into *S. lividans* TK24 and *A. teichomyceticus* by conjugal transfer, and then the *in vivo* function of *metK* derived from *A. teichomyceticus* was evaluated *via* phenotypic comparison between the wild-type strain and the exconjugant harboring the *metK* high expression vector.

The *A. teichomyceticus* ATCC31121 employed in this study was cultivated at 28°C in an ISP2 medium (0.4% yeast extract, 1% malt extract, 0.4% dextrose, and 2% agar, adjusted to pH 7.0−7.4) for the preparation of spores. For genetic manipulation in *E. coli*, strain XL10-Gold (Stratagene, UK) was used. pUC19 was used to construct a genomic library and for DNA sequencing. DNA manipulations in *E. coli* and in *A. teichomyceticus* were conducted in accordance with the previous descriptions of Kieser *et al.* [9] and Sambrook and Russell [17], respectively. Total DNA of *A. teichomyceticus* was acquired *via* the method of Rao *et al.* [16]. For transformation, conjugal DNA transfer into *A. teichomyceticus* was carried out as previously described by Ha *et al.* [7]. The methylation-deficient *E. coli* strain ET12567 (*dam-13::Tn9*, *dcm-6*, *hsdM*, *hsdS*)

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harboring pUZ8002 was employed as the donor in intergeneric conjugations [4, 12, 18].

In order to clone *metK* from *A. teichomyceticus*, PCR was carried out using the newly designed degenerate primers F2 (5'-CGGAATTCARGGNCAYCCSGAYAARATC-3') and R2 (5'-CGGAATTCTCNACYTTSSWSGGRTCYTT-3') (the underlined nucleotides were added to introduce an *Eco*RI site for cloning) based on the highly conserved regions revealed by alignment of the amino acid sequences of biochemically identified SAM-s. An 839 bp PCR product was obtained and cloned into pUC19. Sequencing of the PCR product and database analysis revealed high sequence similarity to the internal segment of the gene encoding SAM-s (data not shown). A 4.6 kb *Pst*I-*Kpn*I fragment was cloned into pUC19 *via* Southern and Colony hybridization using the PCR product as a probe against the genomic DNA of *A. teichomyceticus*. Two complete and one truncated ORFs (*orf1~orf3*) were confirmed *via* the nucleotide sequencing of the cloned fragment, consistent with the characteristic codon usage of *Streptomyces* genes with extremely high G + C content (91.2%, 93.9%, and 85.8% for *orf1* to *orf3*, respectively) as determined by the FRAME analysis developed by Bibb *et al*. [2]. The ORF1 shows the highest degree of similarity to a phosphopantothenoylcysteine decarboxylase of *Micromonospora aurantiaca* ATCC27029 (76% identity, 83% similarity), the ORF2 of an *S*-adenosylmethionine synthetase of *Micromonospora aurantiaca* ATCC27029 (91% identity, 95% similarity), and the truncated ORF3 of the primosomal protein N' of *Streptosporangium roseum* DSM 43021 (68% identity, 79% similarity), respectively. The *orf2* cloned in this study was therefore registered as the *metK* gene derived from *A. teichomyceticus* in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB470732.

In order to evaluate the effects of *metK* derived from *A. teichomyceticus* on antibiotic production in actinomycetes, the vector to overexpress the *metK* gene, pDS1, was constructed *via* the insertion of a 1.5 kb *metK* fragment encompassing the entire *orf2* gene and its putative ribosomal binding site into the *Bam*HI and *Xba*I double-digested fragment in pSET152ET (6 kb) [3, 9], located immediately downstream of a strong constitutive *ermE** promoter, and subsequently introduced *via* conjugation into the host actinomycetes (Fig. 1). These plasmids do not exhibit the replicative functions of actinomycetes plasmids and can be maintained only in the chromosomally integrated state of the recipient strains. *Via* PCR and Southern hybridization, it was confirmed that the pDS1 vector was correctly introduced at different sites from the locations of the original *metK* gene in the genomic DNA of host strains, and remained stable throughout many generations (data not shown).

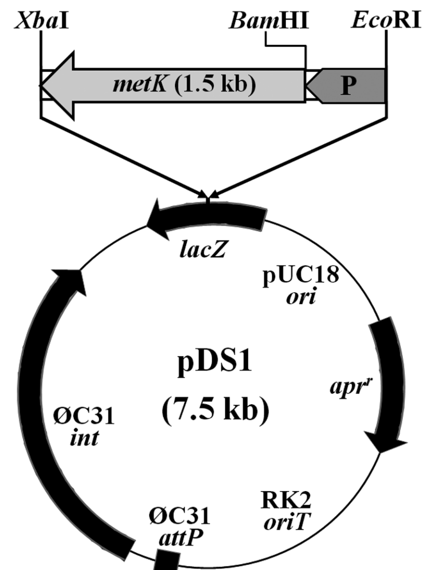

Fig. 1. Vector construction, pDS1, for high *metK* expression.
P, *ermE** constitutive promoter; *apr^r*, aparamycin resistance gene; ØC31 *int*, integrase gene; *attP*, attachment site of genomic *attB* site.

In order to confirm the effects of the *metK* derived from *A. teichomyceticus* on actinorhodin production, a pDS1 was introduced into *S. lividans* TK24, which was employed as a host for the confirmation of inducer gene activity for secondary metabolite production. *S. lividans*, a species closely related to *S. coelicolor*, features all of the genetic components necessary for actinorhodin biosynthesis, but does not produce actinorhodin under normal culture conditions because its regulator gene is insufficient to induce antibiotic production. For actinorhodin production, 20 ml of R4 medium (0.1% yeast extract, 1% dextrose, 0.01% casamino acid, 0.3% proline, 0.1% $MgCl_4·8H_2O$, 0.4% $CaCO_3·2H_2O$, 0.02% K_2SO_4, and 0.2% trace elements) in 300 ml Erlenmeyer flasks was inoculated with 5×10^5 spores of *S. lividans* TK24 and incubated for 48 h at 28°C with shaking (180 rpm). The main cultivation was performed *via* the inoculation of 3 ml of the seed culture into 100 ml of R4 medium in 500 ml baffled flasks, followed by 5 days of incubation at 28°C and 180 rpm. The actinorhodin concentration was determined *via* the method described previously by Kieser *et al*. [9]. As shown in Fig. 2, the introduction of pDS1 into *S. lividans* TK24 resulted in the induction of actinorhodin production. On the R5 agar plates, the exconjugant harboring pDS1 generated a strong blue pigment (actinorhodin), but other strains, such as the wild-type strain and the exconjugant with pSET152ET vector only, did not (Fig. 2A). Identical results were also noted in liquid cultures using R4 medium (Fig. 2B). The exconjugant harboring pDS1 displayed higher levels and earlier actinorhodin production activity than were observed

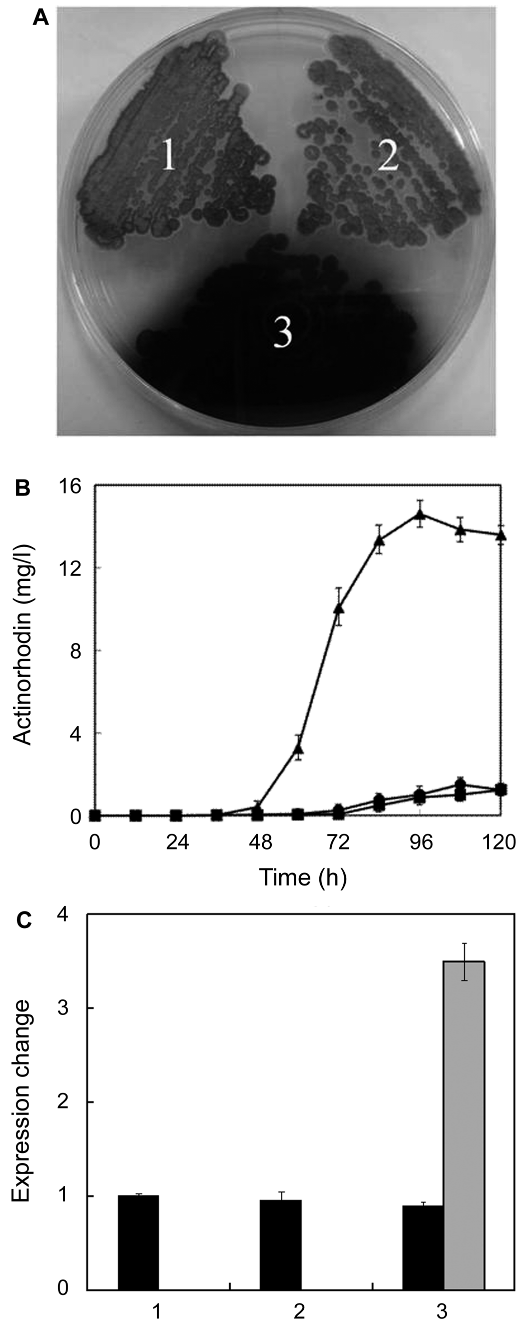

Fig. 2. Actinorhodin production and transcript amount of *metK* from *S. lividans* TK24.

(**A**) Actinorhodin production of *S. lividans* TK24 on solid plate using R5 agar medium for 12 days at 28°C (1, *S. lividans* TK24 wild-type strain; 2, exconjugant integrated with pSET152ET; 3, exconjugant integrated with pDS1). (**B**) Actinorhodin production of *S. lividans* TK24 in liquid broth using R4 medium for 5 days at 28°C (Solid circles, *S. lividans* TK24 wild-type strain; solid squares, exconjugant integrated with pSET152ET; solid triangles, exconjugant integrated with pDS1). (**C**) Transcriptional comparison of *metK* from *S. lividans* TK24 cultivated for 72 h by real-time qRT-PCR (1, *S. lividans* TK24 wild-type strain; 2, exconjugant integrated with pSET152ET; 3, exconjugant integrated with pDS1). Black and gray bar graphs indicate the transcript amounts of *metK* of *S. lividans* TK24 and *metK* derived from *A. teichomyceticus*, respectively. Bars represent the SD of three experiments (n=3).

in the wild-type strain and in the exconjugant with pSET152 vector only, although their growth rates were almost identical (data not shown). The exconjugant harboring pDS1 began to generate actinorhodin 36 h earlier than the exconjugant harboring the vector only without the *metK* gene, and the amount of accumulated actinorhodin was increased 17-fold. This result is similar to those observed in other *Streptomyces* strains, including *S. actuosus*, *S. coelicolor* A3(2), and *S. coelicolor* M512 [14, 19, 20].

To determine the amount of *metK* transcript in control strains and exconjugant containing pDS1, total RNA was prepared from the control strains and exconjugants grown for 72 h in R4 medium. According to the manufacturer's instructions, the SYBR greenER qPCR Supermix universal kit (Invitrogen, Carlsbad, CA, USA) was employed for the amplification of the synthesized cDNA. Primers for real-time qPCR were designed to produce cDNAs of approximately 200 bp for each *metK* and *lysA* (as an internal control of real-time PCR). We employed the Sequence Detection System software (SDS version 2.1, PE Applied Biosystems, Foster, CA, USA) for the analysis and quantification of gene expression data generated by quantitative real-time (qRT) PCR. The amplification efficiency for each target (*metK* of *S. lividans* TK24 and *metK* derived from *A. teichomyceticus*) and reference gene (*lysA*) was derived from a standard curve plotted as the cycle threshold (C_T) versus copies. The results are shown as ratios of each target mRNA to *lysA* mRNA. Transcript amounts of *metK* derived from *A. teichomyceticus* was approximately 3.5-fold higher than that of the *metK* of *S. lividans* TK24 in the exconjugant harboring pDS1, and was not detected in the wild-type strain and in the exconjugant harboring only the pSET152ET vector, as expected; this indicates that the increase in the transcript amounts of *metK* derived from *A. teichomyceticus* is responsible for the acceleration of actinorhodin biosynthesis (Fig. 2C).

Additionally, a pDS1 was introduced into the genome of the *A. teichomyceticus* wild-type strain in order to evaluate the effects of *metK* on the increase in teicoplanin production. To produce teicoplanin, 3×10^6 spores of *A. teichomyceticus* were inoculated into 20 ml of seed broth (1% glucose, 0.4% yeast extract, 0.4% peptone, 0.2% KH_2PO_4, 0.4% K_2HPO_4, and 0.05% $MgSO_4$) in 300 ml Erlenmeyer flasks and incubated for 72 h at 28°C with shaking (180 rpm). The main cultivation was carried out by inoculating 3 ml of the seed culture into 100 ml of main broth (2% glucose, 0.5% yeast extract, 0.15% asparagine, 0.05% $MgSO_4$, 0.2% NaCl, and 0.4% $CaCl_2{\cdot}2H_2O$) in 500 ml baffled flasks, followed by 8 days of incubation at 28°C. For the teicoplanin production assay, the culture broths were sampled for 8 days and 1 ml of culture broth was withdrawn every day, and then centrifuged for 10 min at 4°C at 15,000 rpm. For rapid analysis, antibiotic activity was evaluated *via* a microbial paper-disc-agar diffusion method

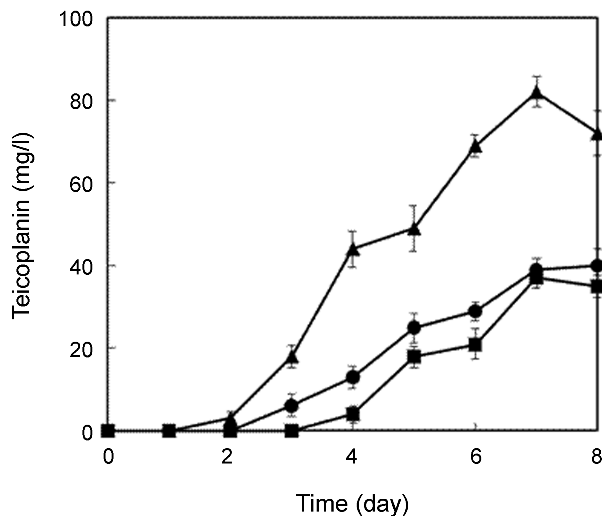

Fig. 3. Teicoplanin production by *A. teichomyceticus* in liquid broth for 8 days at 28°C.

Solid circles, *A. teichomyceticus* wild-type strain; solid squares, exconjugant integrated with pSET152ET; solid triangles, exconjugant integrated with pDS1. Bars represent the SD of three experiments (n=3).

[15], using *Bacillus subtilis* ATCC6633 as a test organism. Qualitative evaluations were conducted based on HPLC using a Hewlett Packard Series II 1090 instrument with an YMC-Pack ODS-A column (4.6 × 250 mm). A gradient of 100 ~ 30% phase A [0.02 mol/l NaH_2PO_4/CH_3CN, 95:5 (v/v)] in phase B [0.02 mol/l NaH_2PO_4/CH_3CN, 25:75 (v/v)] required 40 min at a flow rate of 1 ml/min. The UV detector was set at 254 nm [5, 8]. Targocid (Hoechst Marion Roussel, Italy), a lyophilized teicoplanin used for injection, was used as a reference standard. As shown in Fig. 3, the introduction of pDS1 into *A. teichomyceticus* induced an increase in teicoplanin production. The exconjugant harboring pDS1 evidenced 1 day earlier and 2.2-fold higher teicoplanin production than was observed in the exconjugant harboring the vector only without the *metK* gene, with no differences in growth rates among them (data not shown).

The results of this study demonstrate that a *metK* gene encoding SAM-s was cloned for the first time from a non-*Streptomyces* actinomycete, *Actinoplanes teichomyceticus*; high expression of this gene may increase actinorhodin production in *S. lividans* TK24 and teicoplanin production in *A. teichomyceticus*.

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