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Cloning of *relA* from *Actinoplanes teichomyceticus* ATCC31121 and Its Application for Increases in Antibiotic Production

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The *relA* gene encoding the highly phosphorylated guanine nucleotide, ppGpp synthetase, which performs a central role in triggering antibiotic biosynthesis and morphological differentiation in *Streptomyces* species, was cloned from the non-*Streptomyces* actinomycetes, *Actinoplanes teichomyceticus* ATCC31121, which produces teicoplanin, and *relA* antibiotic production-increasing activity was confirmed. A 3.5-kb DNA fragment including an intact 2,466 bp open reading frame (ORF) evidencing high homology with the ppGpp synthetase (RelA) of *Streptomyces* species was acquired via polymerase chain reaction (PCR) using a newly designed primer based on the amino acid sequence of the previously studied RelA and Southern hybridization using the PCR product as a probe. *S. lividans* TK24 and *A. teichomyceticus* introduced with a high expression vector of the *relA* evidenced a 13- and 3-fold higher levels of actinorhodin and teicoplanin production, thus confirming for the first time that the high expression of *relA* derived from *A. teichomyceticus* is responsible for the induction of antibiotic production in *Streptomyces* species as well as in the non-*Streptomyces* actinomycetes, *A. teichomyceticus*.

Key words: *Actinoplanes teichomyceticus*, actinorhodin, cloning, *relA*, teicoplanin

Actinomycetes are Gram-positive filamentous soil prokaryotes, which are famous for their versatility in producing many different kinds of bioactive compounds, including more than 70% of the commercially important antibiotics [Kieser *et al*., 2000]. The *Streptomyces* genus comprises more than 95% of all actinomycetes isolated from nature, and thus has been the most well-studied [Lechevalier and Lechevalier, 1967]. The other 5% or less is non-*Streptomyces* actinomycetes, which are sometimes referred to as 'rare' actinomycetes. Recently, non-*Streptomyces* actinomycetes have received increased attention as very rich sources of medically important secondary metabolites and new antibiotic.

In bacteria, the highly phosphorylated guanine nucleotides, ppGpp, controls the stringent responses to carbon and energy starvation [Cashel *et al*., 1996]. The ppGpp synthetase (RelA) catalyzes ppGpp synthesis under amino acid limitation conditions, in association with ribosomes [Haseltine and Block, 1973]. The ppGpp synthetase/hydrolase (SpoT) is a bifunctional enzyme, which synthesizes ppGpp under carbon limitation conditions in a ribosome-independent manner, and also degrades ppGpp via manganese-dependent ppGpp pyrophosphohydrolase activity. ppGpp functions as a positive regulator of certain other transcriptions, and also as a negative regulator of rRNA transcription [Barker *et al*., 2001].

It has also been determined that ppGpp carries out a central function in the triggering of antibiotic biosynthesis and morphological differentiation in *Streptomyces* species [Strauch *et al*., 1991]. The *relA* null mutant of *Streptomyces coelicolor* M570 was completely defective in ppGpp synthesis and in the production of Act and Red under nitrogen limitation conditions [Chakraburtty and Bibb, 1997]. The disruption of *relA* in *Streptomyces antibioticus* yields a strain which grows more slowly and does not generate actinomycin [Hoyt and Jones, 1999]. On the other hand, the *relA*-null mutant of *Streptomyces clavuligerus* overproduces clavulanic acid and cephamycin C, even though its aerial mycelium formation and spore production are repressed as reported in another study [Gomez-Escribano *et al*., 2008]. However, although the RelA in *Streptomyces* species has been extensively studied, there have been no studies conducted thus far concerning the non-*Streptomyces* actinomycetes.

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In the present study, *relA* encoding ppGpp synthetase was, therefore, cloned from the representative non-*Streptomyces* actinomycetes, *Actinoplanes teichomyceticus* ATCC31121 producing teicoplanin, which has been clinically employed in the treatment of gram-positive pathogenic bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) [Bauernfeind and Petermüller, 1982; Borghi *et al*., 1989]. In order to evaluate the role of *relA* in the triggering of increased antibiotic biosynthesis, an *relA*-high expression vector was constructed and introduced into *Streptomyces lividans* TK24 and *A. teichomyceticus* via transconjugation, and the *in vivo* function of *relA* derived from *A. teichomyceticus* was evaluated via phenotypic comparison between the wild-type strain and the exconjugant harboring an *relA*-high expression vector.

Materials and Methods

Bacterial strains, plasmids, culture conditions, and conjugation. The *A. teichomyceticus* ATCC 31121 employed in this study was grown at 28°C in an ISP2 medium (g/L, 4 yeast extract, 10 malt extract, 4 dextrose, 20 agar, pH 7.0-7.4) for spore preparation. For genetic manipulation in *Escherichia coli*, the XL10-Gold strain (Stratagene, La Jolla, CA) was used. pUC19 was employed in the construction of a genomic library and for DNA sequencing. A site-specific integration vector, pSET152ET (6 kb) [Kieser *et al*., 2000], harboring the *oriT* of RK2, ØC31 *int* and *attP*, an apramycin resistance gene for selection in actinomycetes and *E. coli*, and a strong constitutive *ermE** promoter [Bibb *et al*., 1985], were employed in the construction of a high expression vector. This plasmid does not evidence the replicative functions of actinomycetes plasmids and can be maintained only at the chromosomally integrated state of recipient strains. To produce teicoplanin, 3×10^6 spores of *A. teichomyceticus* were inoculated into 20 mL of seed broth (g/L, 10 glucose, 4 yeast extract, 4 peptone, 2 KH_2PO_4, 4 K_2HPO_4, 0.5 $MgSO_4$) in 300-mL Erlenmeyer flasks and incubated for 72 h at 28°C with shaking (180 rpm). The main cultivation was conducted via the inoculation of 3 mL of the seed culture into 100 mL of main broth (g/L, 20 glucose, 5 yeast extract, 1.5 asparagine, 0.5 $MgSO_4$, 2 NaCl, 4 $CaCl_2\cdot2H_2O$) in 500 mL Baffle flasks, followed by 8 days of incubation at 28°C. For actinorhodin production, 20 mL of R4 medium (g/L, 1 yeast extract, 10 dextrose, 0.1 casamino acid, 3 proline, 1 $MgCl_4\cdot8H_2O$, 4 $CaCO_3\cdot2H_2O$, 0.2 K_2SO_4, 2 mL 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 conducted by inoculating 3 mL of the seed culture into 100 mL of R4 medium in 500 mL Baffle flasks, followed by 5 days of incubation at 28°C and 180 rpm. For transformation, conjugal DNA transfer into *A. teichomyceticus* was performed as described previously by Ha *et al*. [2008]. The methylation-deficient *E. coli* strain ET12567 (*dam-13::Tn9, dcm-6, hsdM, hsdS*) containing pUZ8002 was employed as the donor in intergeneric conjugations [Smokvina *et al*., 1990; Bierman *et al*., 1992; Motamedi *et al*., 1995]. pUZ8002 is an RK2 derivative with a defective *oriT* (*aph*); it is not self-transmissible but carries out a mobilization function for *oriT*-containing plasmids in trans.

Manipulation of DNA and molecular cloning of *relA*. DNA manipulations in *E. coli* and *A. teichomyceticus* were carried out as described by Sambrook and Russell [2001] and Kieser *et al*. [2000], respectively. Total DNA of *A. teichomyceticus* was obtained via the method of Rao *et al*. [1987]. In order to clone a *relA* gene from *A. teichomyceticus*, the degenerate primers F2 (5'-CGGAATTCGNYTSCAYAAYATGMGNACSATG-3') and R1 (5'-CGGAATTCCSARNCKRTGSGCSARSGG-3') were used (the underlined nucleotides were added to introduce an *Eco*RI site for cloning). Chromosomal DNAs isolated from the original strains and exconjugants harboring vectors were digested with *Bam*HI for Southern hybridization. Southern blot hybridization was conducted using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Co., Penzberg, Germany) according to the manufacturer's instructions, and a 0.5-kb probe for the *relA* was amplified by polymerase chain reaction (PCR). DNA sequencing was conducted via the dideoxy-chain termination method for both strands, using a sequencing kit (Amersham, Tokyo, Japan) and Cy5-labeled primers on a fluorescence DNA sequencer (ALFred; Pharmacia Biotech, Tokyo, Japan). Sequence analyses and homology comparisons were conducted on a personal computer using the GENETYX software package (Software Development Co., Ltd., Tokyo, Japan). In order to construct a high expression vector, a 2.6-kp *relA* fragment including the entire open reading frame (ORF) and its putative ribosomal binding site were inserted into the *Bam*HI and *Xba*I double-digested located immediately downstream of a strong constitutive *ermE** promoter in pSET152ET to generate pSC1, which can be integrated into the actinomycetes chromosome via site-specific recombination at the bacteriophage ØC31 attachment site (*attB*), and subsequently introduced via conjugation into *S. lividans* TK24 and *A. teichomyceticus* (Fig. 1).

Analysis of antibiotic production. The concentration of actinorhodin was determined via the methods described

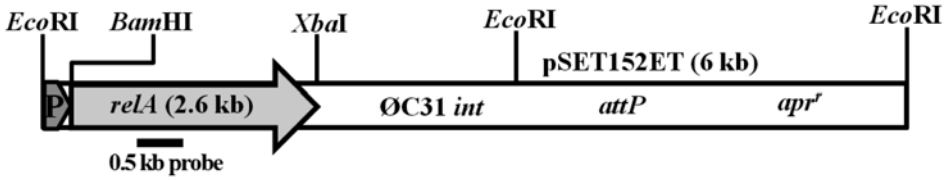

Fig. 1. Restriction map of *relA* high expression vector, pSC1. P, *ermE** constitutive promoter; *apr*^{r}, aparamycin resistance gene; ØC31 *int*, integrase gene; *attP*, attachment site of genomic *attB* site.

by Kieser *et al*. [2000]. For the assay of teicoplanin production, the culture broths were sampled by withdrawing 1 mL of culture broth once a day for 8 days, then centrifuged for 10 min at 4°C at 15000 rpm. For rapid analysis, antibiotic activity was assayed via a microbial paper-disc-agar diffusion method [Parenti *et al*., 1978], using *Bacillus subtilis* ATCC 6633 as a test organism. Qualitative evaluations were run using an HPLC (Hewlett Packard Series II 1090 instrument, Avondale, PA) equipped with an YMC-Pack ODS-A column (4.6×250 mm). 100-30% phase A (0.02 M NaH_2PO_4/acetonitrile, 95:5, v/v) and phase B (0.02 M NaH_2PO_4/acetonitrile, 25:75, v/v) were used for gradient elution for 40 min at a flow rate of 1 mL/min. The UV detector was set at 254 nm [Borghi *et al*., 1989; Heydorn *et al*., 1999]. Targocid (Hoechst Marion Roussel, Gerenzano, Italy), a lyophilized teicoplanin used for injection, was employed as a reference standard.

Nucleotide sequence accession number. The nucleotide sequence data of *relA* cloned in this paper has been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB306971.

Results and Discussion

Cloning and sequencing of *relA* from *A. teichomyceticus*. In order to clone *relA* from *A. teichomyceticus*, PCR was conducted using newly designed primers based on the highly conserved regions revealed by the alignment of the amino acid sequences of biochemically identified RelAs. An 870-bp PCR product was obtained and cloned into pUC19. The results of PCR product sequencing and database analysis evidenced a high level of sequence similarity to the internal segment of genes encoding RelA (data not shown). A 3.5-kb *Sal*I-*Kpn*I fragment was cloned into pUC19 via Southern and colony hybridizations using the PCR product as a probe against the genomic DNA of *A. teichomyceticus*. One complete ORF was confirmed via the nucleotide sequencing of the cloned fragment, in accordance with the characteristic codon usage of *Streptomyces* genes with extremely high G+C content (95.2%) as determined by the FRAME analysis developed by Bibb *et al*. [1984]. Although *A. teichomyceticus* is a non-*Streptomyces* actinomycetes, the ORF product exhibits the highest level of similarity to the ppGpp synthetases of the genus *Streptomyces*: *Streptomyces roseosporus* NRRL 15998 (97% identity, 99% similarity; accession no. ZP06582779), *Streptomyces flavogriseus* ATCC 33331 (96% identity, 99% similarity; accession no. ZP05807245), and *Streptomyces clavuligerus* ATCC 27064 (96% identity, 98% similarity; accession no. ZP05006525). The ORF cloned herein was therefore registered along with the *relA* gene derived from *A. teichomyceticus* in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB306971.

Construction of vector and exconjugants for *relA* high expression. In order to evaluate the effects of *relA* derived from *A. teichomyceticus* on the increase in antibiotic production in actinomycetes, the vector for *relA*-high expression, pSC1, was constructed with the integration vector, pSET152, including a strong constitutive *ermE** promoter (pSET152ET) as described in the Materials and Methods section, then introduced via conjugation into *S. lividans* TK24 and the original strain, *A. teichomyceticus* (Fig. 1). These plasmids do not exhibit the replicative functions of actinomycetes plasmids and can be maintained only in the chromosomally integrated state of recipient strains. *S. lividans* TK24 was used as a host to confirm the activity of the inducer gene for secondary metabolite production. This strain, a species related closely to *S. coelicolor*, harbors all genetic components required for actinorhodin biosynthesis, but does not generate actinorhodin under normal culture conditions, because its regulator gene cannot induce antibiotic production.

In an effort to confirm that the pSC1 vector was introduced correctly into the genomic DNA of host strains and remained stable throughout many generations, Southern hybridization analysis was conducted after the *Bam*HI digestion of chromosomal DNA. Hybridizing bands of approximately 8.6 and 7.2 kb were found for only the pSC1 vector and the exconjugant of *S. lividans* containing pSC1, respectively, whereas only one band was seen with the pSET152ET plasmid and *S. lividans* TK24 (Fig. 2A). Although S. *lividans* includes its original

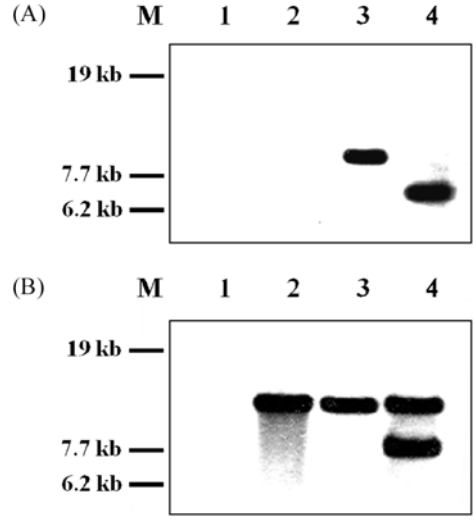

Fig. 2. Southern hybridization analysis of chromosomal DNA and vector digested with *Bam*HI. (A) Lane 1, pSET152ET vector; lane 2, *S. lividans* TK24 wild-type strain; lane 3, pSC1 vector; lane 4, exconjugant integrated with pSC1. (B) Lane 1, pSET152ET vector; lane 2, *A. teichomyceticus* wild-type strain; lane 3, exconjugant integrated with pSET152ET; lane 4, exconjugant integrated with pSC1. A 0.5-kb fragment of *relA* derived from *A. teichomyceticus* was used as the probe.

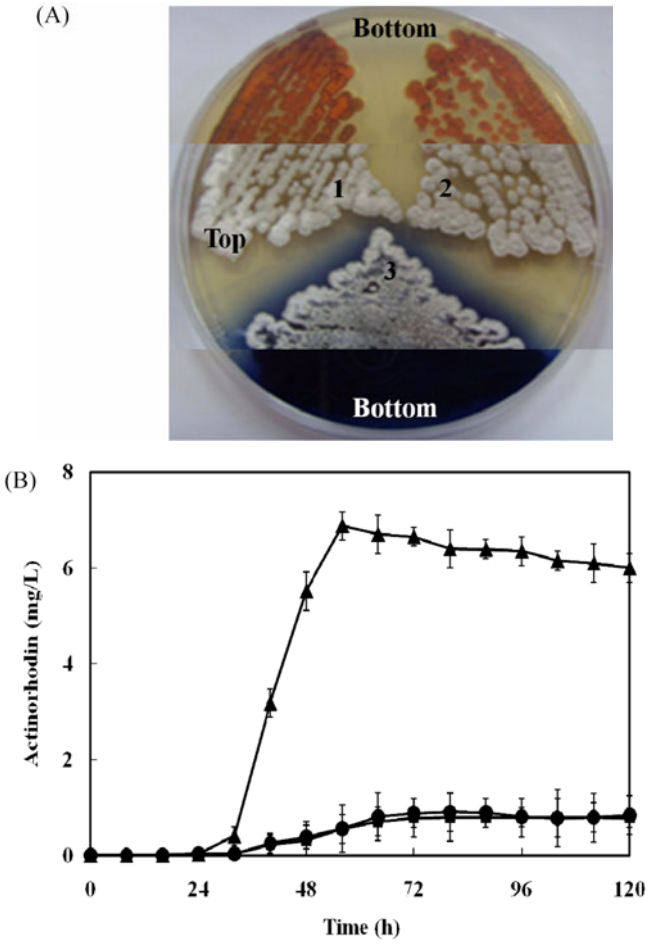

Fig. 3. Actinorhodin production of *S. lividans* TK24 on solid plate using R5 agar medium for 12 days at 28°C (A) and in liquid broth using R4 medium for 3 days at 28°C (B). (A) 1, *S. lividans* TK24 wild-type strain; 2, exconjugant integrated with pSET152ET; 3, exconjugant integrated with pSC1. (B) Solid circles, *S. lividans* TK24 wild-type strain; solid squares, exconjugant integrated with pSET152ET; solid triangles, exconjugant integrated with pSC1. Bars represent SD of three experiments (n=3).

relA gene, when the 0.5-kb fragment of *relA* derived from *A. teichomyceticus* was used as the probe, no non-specific band in Southern hybridization analysis of *S. lividans* chromosomal DNA was observed, because the RelA homology between the two strains was relatively low (68% identity, 80% similarity). Additionally, a hybridizing band of approximately 12 kb was detected for the original strain, *A. teichomyceticus*, and the exconjugant harboring the pSET152ET vector only, whereas in the exconjugant including pSC1, two hybridizing bands of 12 and 8 kb were confirmed, in accordance with our expectations (Fig. 2B); this shows that the pSC1 vector was correctly introduced at different sites from the locations of the original *relA* gene in the genomic DNA of *A. teichomyceticus* and have remained stable throughout many generations.

Effects of pSC1 introduction on antibiotic production in *S. lividans* TK24 and *A. teichomyceticus*. In order to confirm the effects of the *relA* derived from *A. teichomyceticus* on antibiotic production in *Streptomyces* strain, a pSC1 was introduced into *S. lividans* TK24, which does not produce actinorhodin due to the deficiency of antibiotic biosynthesis inducers, although it does harbor all of the genetic components necessary for actinorhodin biosynthesis. Introduction of pSC1 into *S. lividans* TK24 resulted in the induction of actinorhodin production (Fig. 3). On the R5 agar plates, the exconjugant containing pSC1 generated a strong blue pigment (actinorhodin), whereas other strains such as the wild-type strain and the exconjugant with pSET152ET vector only, did not (Fig. 3A). Identical results were also noted in liquid cultures using R4 medium (Fig. 3B). The exconjugant containing pSC1 showed a 13-fold higher actinorhodin production than other strains, without any difference in growth rates among them (data not shown). This result is similar to those observed in other *Streptomyces* strains, including *S. antibioticus*, *S. clavuligerus*, and *S. coelicolor* A3(2) [Chakraburtty and Bibb, 1997; Hoyt and Jones, 1999; Jin *et al*., 2004]. A disruption in *relA* from the genomes of *Streptomyces* strains induced reductions in both antibiotic production and growth rate; however, in the present study the high expression of *relA* in *S. lividans* only affected

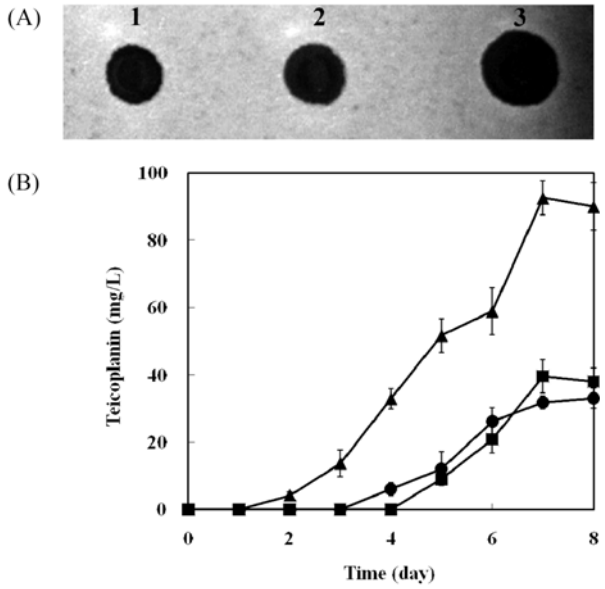

Fig. 4. Teicoplanin production of *A. teichomyceticus*. (A) Bioassay for teicoplanin production of *A. teichomyceticus* in liquid broth for 8 days was carried out via a microbial paper-disc-agar diffusion method, using *B. subtilis* ATCC 6633 as a test organism. 1, *A. teichomyceticus* wild-type strain; 2, exconjugant integrated with pSET152ET; 3, exconjugant integrated with pSC1. (B) Qualitative evaluation for teicoplanin production of *A. teichomyceticus* in liquid broth for 8 days was based on HPLC. Solid circles, *A. teichomyceticus* wild-type strain; solid squares, exconjugant integrated with pSET152ET; solid triangles, exconjugant integrated with pSC1. Bars represent SD of three experiments (n=3).

actinorhodin production. The increased ppGpp concentration in *S. coelicolor* A3(2) induced the activation of the transcription of *actII*-ORF4, the pathway-specific activator gene for actinorhodin production, but had no effect on growth rate [Hesketh *et al*., 2001]. This implies that ppGpp activates the transcription of *actII*-ORF4. The change in undecylprodigiosin production was also not observed with the introduction of pSC1 (data not shown). This result is similar to those noted in other studies [Martínez-Costa *et al*., 1996; Hesketh *et al*., 2001]. The induction of ppGpp synthesis did not affect the transcription of *redD*, the activator gene for undecylprodigiosin production, because its transcription is influenced by RedZ, a second pathway-specific regulatory protein for undecylprodigiosin production whose synthesis is also growth phase-dependent [White and Bibb, 1997].

Additionally, the introduction of pSC1 into the genome of the *A. teichomyceticus* wild-type strain induced an increase in teicoplanin production (Fig. 4). The exconjugant harboring pSC1 evidenced higher levels and earlier teicoplanin production than was observed in the wild type-strain and the exconjugant with pSET152 vector only, although their growth rates were almost identical (data not shown). The exconjugant with pSC1 began to produce teicoplanin 2 days earlier than the exconjugant harboring the vector only without the *relA* gene, and the amount of accumulated teicoplanin was elevated by 3-fold relative to that of the wild-type strain, confirming for the first time that high *relA* expression is also responsible for the induction of antibiotics production in *Streptomyces* species as well as the non-*Streptomyces* actinomycetes, *A. teichomyceticus*.

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