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Stimulation of Actinorhodin Production by *Streptomyces lividans* with a Chromosomally-Integrated Antibiotic Regulatory Gene *afsR2*

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Abstract An actinorhodin nonproducing *Streptomyces lividans* was converted to an actinorhodin overproducer through a single chromosomal integration of an antibiotic regulatory gene, *afsR2*. This strain exhibited early actinorhodin production and an average of 37.5% higher productivity than the *S. lividans* containing multiple copies of *afsR2* plasmid in a glucose-containing liquid culture.

Key words: *afsR2*, chromosomal integration, actinorhodin, *Streptomyces lividans*

Streptomycetes are Gram-positive filamentous soil bacteria which undergo unique morphological differentiation coinciding with the production of many valuable secondary metabolites including antibiotics, anticancer drugs, and immunosupressors [1, 2]. It has been reported that more than 70% of microbial antibiotics originate from streptomycetes and physiologically related actinomycetes, implying that streptomycetes are one of the most important groups of industrial microorganisms [3]. Among various streptomycetes species, *S. coelicolor* has been the most thoroughly characterized strain both genetically and biochemically, partially due to the fact that it produces four structurally different antibiotics including a deep-blue pigment called actinorhodin, a dimer of 16-carbon aromatic polyketide antibiotics [5, 10]. Recently, the actinorhodin biosynthetic and regulatory mechanisms were examined at the transcriptional levels using a DNA microarray system [8].

S. lividans, a close relative to *S. coelicolor*, with little production of actinorhodin in a typical glucose-containing culture condition, has also been used extensively for streptomycetes cloning and as an expression host [4]. In particular, *S. lividans* has been successfully used as a host system to isolate several global antibiotic regulatory genes including *afsR2*, of which the multiple copies stimulated actinorhodin biosynthesis in *S. lividans* [18]. The *afsR2*, also known as *afsS* in *S. coelicolor*, is located immediately 3' to *afsR* and encodes a 63-amino-acid protein of unknown function [13, 16]. Although the detailed function and regulatory mechanism of *afsR2* are still unknown, the *afsR2* overexpression using a specific culture condition significantly stimulated actinorhodin production in *S. lividans* [12]. This antibiotics-overproducing strain improvement approach using *afsR2* has been used successfully in avermectin-producing *Streptomyces avermetilis* strains [14]. Unfortunately, the actinorhodin overproduction *via afsR2* expression in *S. lividans* is only possible in a specific culture condition such as a minimal plate medium containing glycerol as the sole carbon source [12]. From a general strain improvement perspective, an antibiotics-overproducing streptomycetes strain would be more versatile if it is free of plasmid as well as glucose repression during the culture condition.

In this brief communication, an actinorhodin nonproducing *Streptomyces lividans* was converted to an actinorhodin overproducer through a single chromosomal integration of an antibiotic regulatory gene, *afsR2*. This strain also exhibited early actinorhodin production and an average of 37.5% higher productivity than the *S. lividans* containing multiple copies of *afsR2* plasmid in a glucose-containing liquid culture. These results suggest that a chromosomal integration of a single copy of an antibiotic regulatory gene is a promising method for the development of a stable antibiotic-overproducing streptomycetes strain.

To integrate the antibiotic regulatory gene, *afsR2*, into the *S. lividans* TK21 chromosome, the complete open reading frame and promoter region of *afsR2* was first amplified using *S. lividans* total chromosomal DNA as a

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template for polymerase chain reaction (PCR) with a pair of primers (*Bam*HI-containing forward primer *afsR2*-1:5'-GGATCCGTCGACCGGTGGCCGGG-3'; *Pst*I-containing reserve primer *afsR2*-2:5'-CTGCAGGGTCACCGTCCC-CGCGGACG-3'). The PCR was performed for 30 cycles with a Rapid Thermocycler (Idaho Technology, U.S.A.) using a routinely-used high G+C DNA amplification program; denature at 96°C for 30 sec, annealing at 40°C for 30 sec, and extension at 72°C for 35 sec [17]. The amplified 0.58-kb *afsR2* gene fragment was cloned into pGEM-T plasmid (Promega, U.S.A.), followed by the complete sequencing confirmation. The 0.58-kb *Bam*HI and *Pst*I fragment was then subcloned into a streptomycetes-*E. coli* shuttle vector pWHM3 [6] in an opposite direction of the thiostrepton promoter (named pESK206). The pESK206 was transformed into *S. lividans* TK21 through a PEG-assisted protoplast transformation, followed by the thiostrepton antibiotic selection [17].

Among more than 100 individual thiostrepton-resistant *S. lividans* transformants, one unique transformant with unusually blue phenotype was selected as a putative pESK206-integrated *S. lividans* strain (named *S. lividans* ESK206). The *S. lividans* ESK206 strain exhibited very healthy growth and sporulation phenotype on R2YE plate medium (Fig. 1). In addition, the *S. lividans* ESK206 consistently secreted a significant amount of deep-blue pigment actinorhodin on the plate even after several rounds of regeneration, similar to the actinorhodin-overproducing

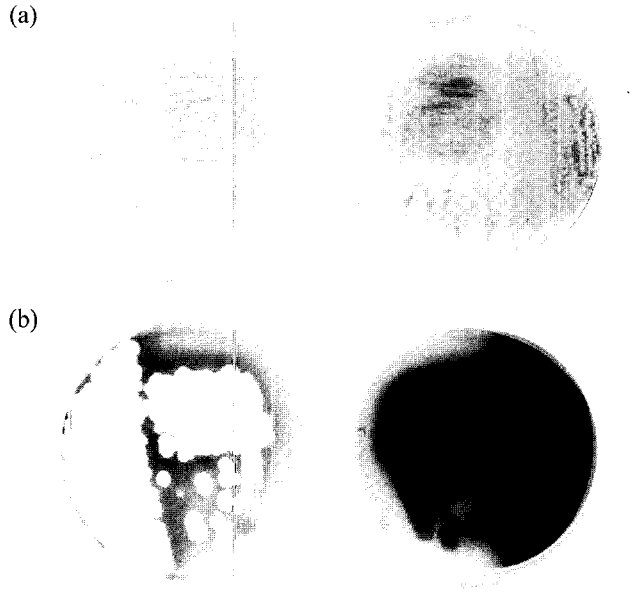

Fig. 1. Actinorhodin production by the wild-type *S. lividans* TK21 (a) and the *S. lividans* ESK206 (b).
The same plate was photographed twice; colony-side-up (left) and colony-side-down (right). Each strain was streaked on R2YE media plate, followed by a week of incubation at 30°C. Each plate was placed upside down for ammonia fuming [12] as well as photography.

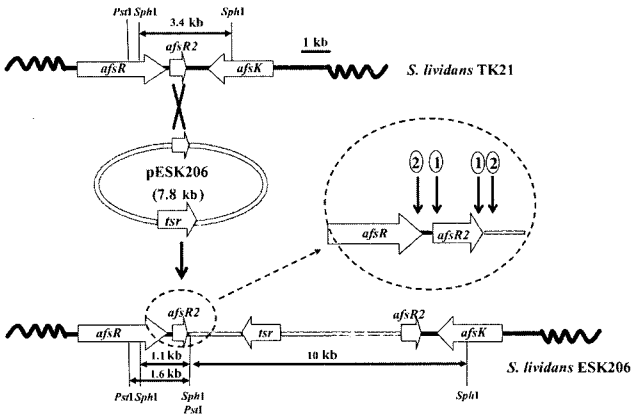

Fig. 2. Restriction maps and schematic presentation of pESK206 integration into the *S. lividans* TK21 chromosome *via afsR2* homologous recombination.
The small arrows with number 1 and 2 shown within the dotted-circle indicate the locations of PCR primers.

S. coelicolor strain (Fig. 1). To genetically confirm the *afsR2* integration in *S. lividans* ESK206, both PCR analysis and Southern hybridization were performed. PCR analysis used total *S. lividans* ESK206 genomic DNA as a template and two pairs of primers: primer pair No. 1 for the amplification of internal region of *afsR2* (control) and primer pair No. 2 for the amplification between the plasmid region and the chromosomal region (Fig. 2). As shown in Fig. 3a, identical 0.58-kb DNA fragments were PCR-amplified from the chromosomes isolated from both *S. lividans* TK21 and *S. lividans* ESK206 using primer pair No.1. However, a 0.6-kb DNA fragment was PCR-amplified only from the chromosome isolated from *S. lividans* ESK206 using primer pair No. 2 (Fig. 3a), indicating the stable chromosomal integration of pESK206 into the *S. lividans* TK21 chromosome *via* homologous recombination (Fig. 3a). The southern hybridization result also supported the site-specific homologous integration of *afsR2* (Fig. 3b). The *Sph*I-digested pattern of *S. lividans* TK21 (3.4 kb signal in Lane 2 of Fig. 3b) was clearly distinguished from that of *S. lividans* ESK206 (1.1 kb signal in Lane 3 of Fig. 3b). An additional 10 kb signal in the *Sph*I-digested *S. lividans* ESK206 was not clearly visible probably due to insufficient membrane transfer of the large-size DNA fragment. The *S. lividans* ESK206 strain was stably maintained with and without thiostrepton selection in the culture and was confirmed to contain no extra copies of pEKS206 plasmid (data not shown).

To quantitatively compare the actinorhodin productivity between the *S. lividans* TK21 containing multiple copies of *afsR2* gene (pESK206) and the *afsR2*-integrated *S. lividans* ESK206, each strain was cultured in 25 ml of R2YE liquid culture at 30°C for 12 days with constant shaking. One ml sample from each culture was taken every two days for the measurement of dry cell weight and

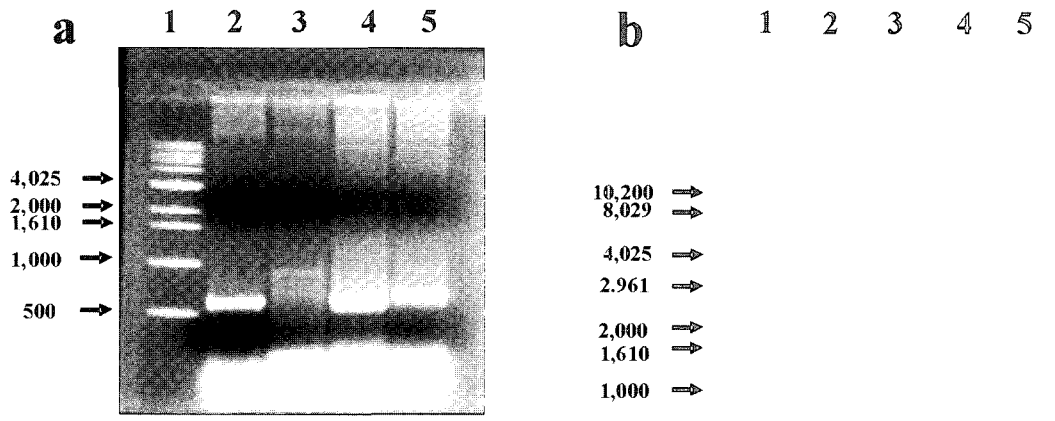

Fig. 3. a) PCR-amplified fragments in EtBr-stained 1.5% agarose gel: Lane 1, molecular weight size marker (λ/*Hin*dIII); Lane 2, *S. lividans* TK21 with primer No. 1; Lane 3, *S. lividans* TK21 with primer No. 2; Lane 4, *S. lividans* ESK206 with primer No. 1; Lane 5, *S. lividans* ESK206 with primer No. 2. b) Southern hybridization with *afsR2* as a probe: Lane 1, *Pst*I-digested *S. lividans* TK21; Lane 2, *Sph*I-digested *S. lividans* TK21; Lane 3, *Sph*I-digested *S. lividans* ESK206; Lane 4, *Pst*I-digested *S. lividans* ESK206; Lane 5, *afsR2*-probe pESK206.

actinorhodin production, of which the detailed assay methods were described elsewhere [12]. As shown in Fig. 4a, *S. lividans* ESK206 started to produce actinorhodin 2 days earlier than the pESK206-containing *S. lividans* TK21, and also maintained an average of 37.5% higher actinorhodin productivity since Day 6 of inoculation. A similar pattern of higher actinorhodin productivity by *S. lividans* ESK206 was repeatedly and consistently observed. As expected, however, the *S. lividans* TK21 containing only a cloning plasmid pWHM3 failed to produce actinorhodin during the same period of culture (Fig. 4a). In addition, the total RNA samples from *S. lividans*/pWHM3, *S. lividans*/pMOV532, and *S. lividans* ESK206 were also isolated at Day 8 of liquid culture (RNeasy Mini Kit, QIAGEN, U.S.A.), followed by the reverse-transcriptase PCR (One Step RNA PCR Kit, TAKARA Bio, Korea) using *afsR2* internal primers (primer-1: ATGAGCGACAAGATGAAGGA; primer-2: CTACTTGCCGTCGCCGTCCA). As shown in Fig. 4b, the *afsR2* transcript was only detected from actinorhodin-producing strains, *S. lividans*/pMOV532 and *S. lividans*

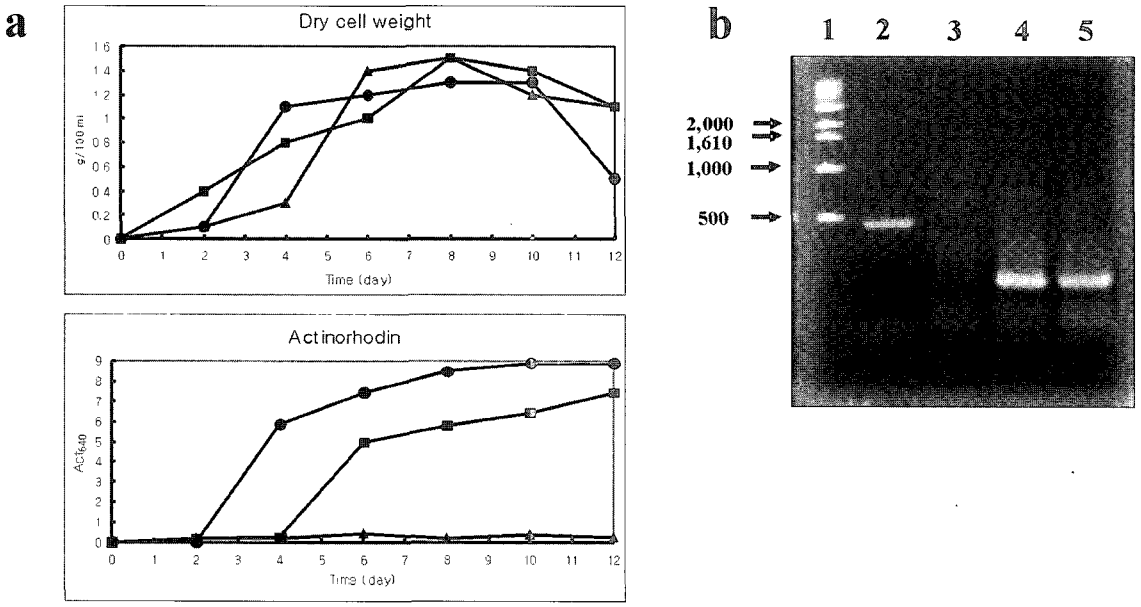

Fig. 4. a) Measurement of dry cell weight (top) and actinorhodin production (bottom) in R2YE liquid culture by pWHM3-containing *S. lividans* TK21 (closed triangle), pESK206-containing *S. lividans* TK21 (closed square), and *S. lividans* ESK206 (closed circle). b) RT-PCR-amplified fragments in EtBr-stained 1.5% agarose gel: Lane 1, molecular weight size marker (λ/*Hin*dIII); Lane 2, RT-PCR control (provided by One Step RNA PCR Kit, TAKARA Bio. Korea); Lane 3, RT-PCR from *S. lividans* TK21/pWHM3; Lane 4, *S. lividans* TK21/pMOV532; Lane 5, *S. lividans* ESK206.

ESK206, but not from *S. lividans*/pWHM3. These results suggest that the chromosomal integration of a single copy of *afsR2* stimulates its transcription level by an unknown mechanism, eventually leading to actinorhodin overproduction. Although the strain improvement strategy described in this article could be used for other secondary metabolite overproduction in streptomycetes, an actinorhodin overproduction by a single copy integration of *afsR2* could be a *S. lividans*-specific phenomenon.

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