In vivo Functional Analysis of γ-Butyrolactone Autoregulator Receptor Gene (*scaR*) in *Streptomyces clavuligerus*

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A γ-butyrolactone autoregulator receptor has a common activity as DNA-binding transcriptional repressors controlling secondary metabolism and/or morphological differentiation in *Streptomyces*. A gene (*scaR*) encoding it was cloned from *Streptomyces clavuligerus*, a clavulanic acid producer, and was *in vitro* characterized in a previous report. In this study to clarify the *in vivo* function of ScaR, a γ-butyrolactone autoregulator receptor of *Streptomyces clavuligerus*, we constructed a *scaR*-deleted strain by means of homologous recombination. No difference in morphology was found between the wild-type strain and the *scaR*-disruptant, but the *scaR*-disruptant showed higher clavulanic acid production. This indicates that the ScaR in *S. clavuligerus* acts as a negative regulator of the biosynthesis of clavulanic acid, but plays no role in morphological differentiation.

Key words – *Streptomyces clavuligerus*, clavulanic acid, autoregulator receptor, negative regulator

Actinomycetes are gram-positive filamentous bacteria which are able to produce a variety of secondary metabolites and the morphological complexity. These phenotypes are controlled by low-molecular weight compounds called butyrolactone autoregulators[14]. The effectiveness of these autoregulators, which are active at nanomolar concentrations, as well as the presence of the specific receptor proteins as mediators of autoregulator signaling, implies that these γ-butyrolactone autoregulators should be regarded as hormones for *Streptomyces*. The discovered receptor proteins include BarA as a virginiae butanolide (VB)-specific receptor in *S. virginiae*[10], FarA as an IM-2-specific receptor in *S. lavendulae* FRI-5[9], ScbR as an SCB1-specific receptor in *S. coelicolor* A3(2)[15], and ArpA as an A-factor-specific receptor in *S. griseus*[11]. *In vitro* studies of receptors for these autoregulator have indicated that the receptors are dimeric DNA-binding proteins which, in the absence of autoregulators, recognize and bind to specific DNA sequences situated in the promoter regions of target genes[6,8]. As result of binding of an autoregulator to the corresponding receptor, the receptor causes the to be dissociated from the DNA, which allows subsequent transcription of the target genes to occur. Although a common autoregulator-dependent cascade has not yet been completely elucidated, all of the autoregulator receptors share a common activity as DNA-binding transcriptional repressors.

S. clavuligerus ATCC27064 is used industrially for the production of clavulanic acid, a commercially important β-lactam compound with potent β-lactamase inhibitory activity which is useful against β-lactamase-producing microorganisms when combined with conventional β-lactam antibiotics[12]. In a previous report[5], we cloned and, *in vitro* only, characterized the gene (*scaR*; *Streptomyces clavuligerus* autoregulator receptor) encoding the γ-butyrolactone autoregulator receptor of *S. clavuligerus*. The molecular mass of the purified rScaR protein was determined to 27 kDa as determined by SDS-PAGE, and 54 kDa by using molecular sieve HPLC under nondenatured conditions at a low protein concentration, which indicates that the majority of the native ScaR is present in the dimeric form. To investigate autoregulator binding activity of rScaR, an autoregulator binding assay was conducted. A binding assay with tritium-labeled autoregulators indicated that IM-2-type compounds with a long C2 side-chain were the most effective ligands for rScaR. This indicates that the β-lactam producer, *S. clavuligerus*, contains a gene for the γ-butyrolactone autoregulator receptor[5].

In this study, to clarify the function of ScaR *in vivo*, a *scaR* deletion mutant of *S. clavuligerus* was constructed using homologous recombination, and a phenotypic compar-

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ison between the wild-type strain and a scaR deletion mutant was performed.

Materials and Methods

Bacterial strains, plasmids, growth conditions, and conjugal transfer of DNA from *Escherichia coli* to *S. clavuligerus*

S. clavuligerus was grown at 28°C on ISP2 medium (DIFCO, Detroit, MI) for spore formation and in 100 ml of yeast extract-malt extract medium (YEME) containing 10 mM $MgCl_2$[4] for production of clavulanic acid. Plasmid pUC19 was used for genetic manipulation and *E. coli* DH5 α was used for transformation of derivative plasmids from pUC19. Conjugal transfer of DNA into *S. clavuligerus* was performed as described by Kieser et al.[4]. ET12567 (*dam-13::Tn9, dcm-6, hsdM, hsdS*), a methylation-deficient *E. coli* strain containing pUZ8002, was used as the donor for intergeneric conjugations[7]. pKC1132 (3.5 kb)[1], a homologous recombination vector containing *oriT* of RK2 and an apramycin resistance gene for selection in Actinomycetes and *E. coli*, and pSET152 (5.7 kb)[1], a site-specific integration vector containing ΦC31 *int* and *attP*, were used to construct the deletion mutants and the complemented strains, respectively. These plasmids do not contain the replicative functions as a plasmid in Actinomycetes but can only be maintained in recipient strains in the chromosomally-integrated state. All chemicals used in this study were used by reagents of high-performance liquid chromatography (HPLC) grade and were purchased from either Takara Shuzo Co. (Otsu, Japan) or Wako Pure Chemical Industrial, Ltd. (Osaka, Japan).

Determination of transcriptional start sites

To identify transcriptional start sites (TSS) of *scaR*, primer extension analysis was performed as described by Sambrook et al.[13]. The oligonucleotide 5'-GTCCGGATGGCTCGCTCCT-3' (complementary nucleotide sequence to nt +29 to +11 relative to the putative *scaR* start codon) was 5'-end labeled with [γ-^{32}P]ATP and polynucleotide kinase. The labeled oligonucleotide was annealed to total RNA of *S. clavuligerus* isolated from the 24 h cultured cells, and was reverse-transcribed by Rous-associated virus 2 reverse transcriptase (Takara, Otsu, Japan). Each ^{32}P-labeled oligonucleotide was used for making a sequence ladder with a BcaBEST Dideoxy Sequencing Kit (Takara, Otsu, Japan) using a 4.2-kb *Sal*I fragment containing the *scaR* as a template[5]. The ladder and the primer-extended product were separated on a 6% polyacrylamide-8 M urea gel.

Construction of a *scaR*-disrupted strain

To assess the role of ScaR in *S. clavuligerus in vivo*, the 702-bp chromosomal *scaR* gene, was replaced with a 1.1-kb thiostrepton resistance gene (*tsr*) on the chromosomal DNA of the wild-type strain by means of homologous recombination, generating a *scaR*-disrupted strain (strain CK101) (Fig. 2A). A 1.5-kb *Hin*dIII-*Xba*I fragment of the 5' upstream of *scaR* and a 1.5-kb *Eco*RI-*Eco*RV fragment of the 3' downstream of *scaR* were amplified by PCR. These fragments were then subcloned into the *Hin*dIII-*Xba*I sites and *Eco*RI-*Sma*I sites of pIJ8606 and named to pCK1. The 6.8-kb on pCK1 contains a 1.5-kb fragment which corresponds to the *scaR*-downstream region, a 1.5-kb fragment which corresponds to the *scaR*-upstream region, and a 1.1-kb *tsr* which replaced a *scaR* gene. An *Eco*RI-*Hin*dIII fragment was recovered from pCK1 and inserted into the *Eco*RI-*Hin*dIII sites of pKC1132, generating pCK2. *E. coli* ET12567 (pUZ8002) harboring pCK2 was conjugated with *S. clavuligerus*, and exconjugants in which the pCK2 plasmid had been presumptively integrated at the *scaR* locus by a single crossover via homologous recombination were selected with apramycin. After three rounds of incubation at 28°C on ISP2 agar in the absence of apramycin, the putative *scaR*-disrupted strains (strain CK101 as the representative strain) which had been formed from the second crossover were detected according to their apramycin sensitivity and thiostrepton resistance, and were analyzed by Southern hybridization.

Morphological assessment and analysis of clavulanic acid production

To analyze morphological differentiation, spores of the wild-type strain and the *scaR*-disrupted strain (strain CK101) were streaked on ISP2 medium, ISP4 medium, MS agar[4], R5 agar, and modified SMMS agar (supplemental minimum medium, solid), and were cultivated for 7 days at 28°C. Produced clavulanic acid was separated by HPLC (model K-1001; KNAUER, Berlin, German) on a C_{18} reversed-phase column (HIGGINS ANALYTICAL, Inc., Mountain View, CA) and the concentration was measured at 311 nm by a UV-detector (model K-1001; KNAUER, Berlin, German) after reacting with imidazole solution for 15 min

at room temperature. The used buffer was composed of 0.1 M KH_2PO_4/1% acetonitrile (pH 3.2), and the flow rate was 1 ml/min[3].

Results and Discussion

Transcriptional start site (TSS) of *scaR* and a plausible ScaR-binding sequence

Primer extension analysis was carried out with total RNA isolated from culture of 24 h mycelia to identify the TSS of the *scaR* transcript (Fig. 1A). The *scaR* transcript had two adjacent TSS at TT situated 54 and 55 bp upstream from the ATG initiation codon. Typical transcriptional promoter sequences, namely, -10 (TATAGT) and -35 (TGGCAG), were found at appropriate positions (Fig. 1B). In *Streptomyces*, autoregulator receptor proteins normally bind to the promoter region of the receptor gene and regulate their own transcription depending on the presence of autoregulators[8]. To estimate whether similar regulation might function with *scaR*, a plausible receptor-binding sequence was evaluated in the 5'-upstream region of *scaR* using the consensus-binding sequence of Thompson et al. (TNANAWACNNACYNNNCGGTTTKTTT)[2]. A probable 26-bp sequence (TGTCATTCAGACCCTTCGGTTTCTTT) was found at 35-60 bp upstream of the *scaR* initiation codon, which was localized between the typical transcriptional promoter region and the translational start codon (Fig. 1B), suggesting that *scaR* transcription is likely to be autoregulated via ScaR protein[8].

Phenotypic analyses of a *scaR*-disrupted strain

To generate a *scaR*-disrupted strain (strain CK101), the 702-bp chromosomal *scaR* gene was replaced with a 1.1-kb *tsr* on the chromosomal DNA of the wild-type strain by means of homologous recombination as described in Materials and Methods (Fig. 2A). Southern blot analysis of CK101 using a PCR-amplified 464-bp fragment of the 5' upstream of *scaR* as a probe showed that a 3.3-kb *Kpn*I-*Eco*RI fragment in the wild-type strain shifted to a 3.7-kb fragment in strain CK101 (Fig. 2B), which confirmed that the CK101 chromosome contained only the deleted *scaR* gene.

The growth characteristics of strain CK101 in liquid culture of YEME containing 10 mM $MgCl_2$ and on agar media of ISP2 medium, ISP4 medium, MS agar, R5 agar, and modified SMMS agar were similar to those of the wild-type strain (Fig. 3A), indicating that *scaR* does not participate in essential primary metabolism nor in the control of

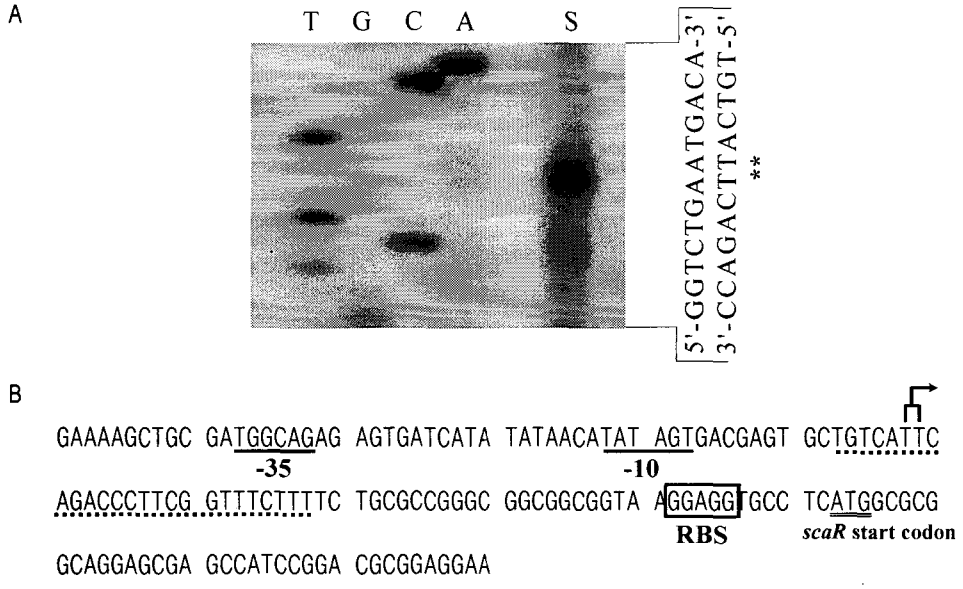

Fig. 1. Identification of the *scaR* transcriptional start sites (A) by primer extension analysis and the promoter region (B). (A) Lanes A, C, G, and T represent a sequencing ladder generated by the same primer. The primer extension reaction was carried out with total RNA prepared from 24 h culture of *S. clavuligerus* (lane S). The asterisks indicate the transcriptional start sites. (B) Locations of the TSS of *scaR* are indicated by arrows; putative -10 and -35 sequences for *scaR* are underlined. A plausible 26-bp receptor-binding sequence is indicated by a dotted underline. The putative ribosome-binding site (RBS) of *scaR* is boxed. The putative ATG start codon for *scaR* is double-underlined.

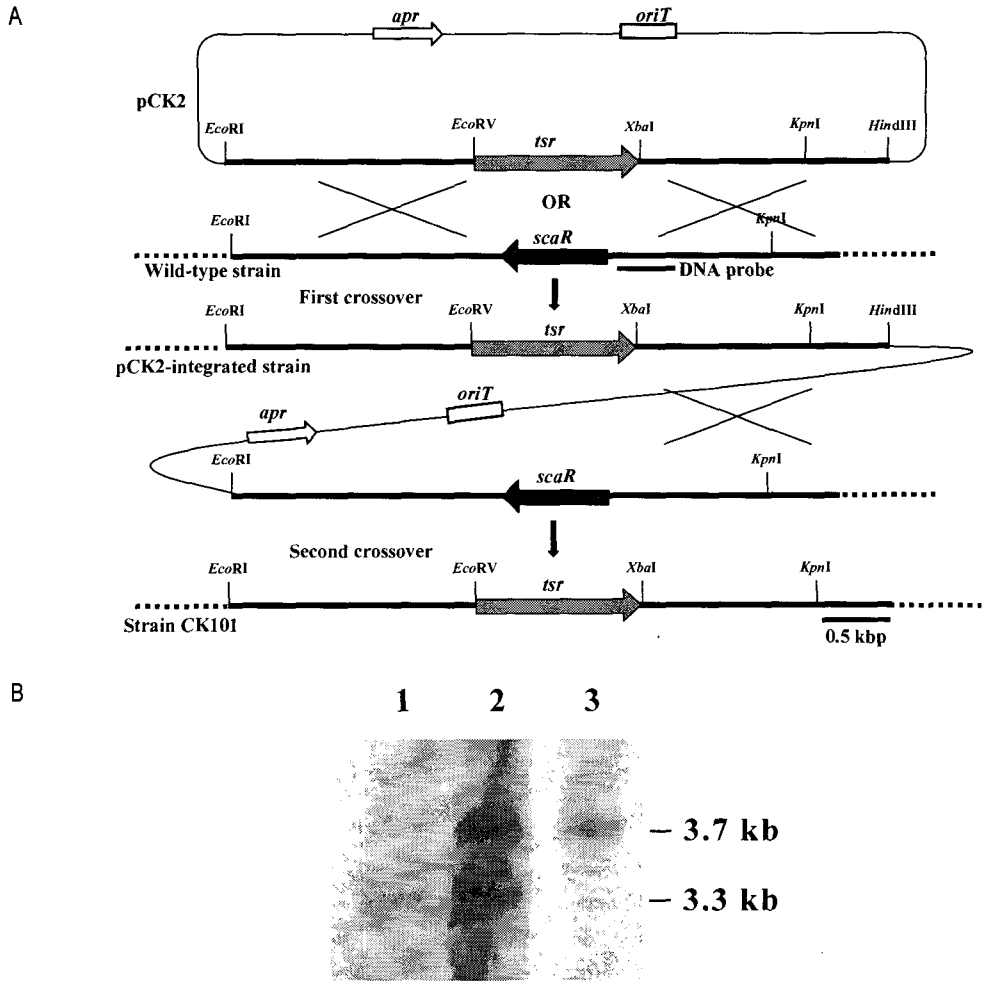

Fig. 2. Gene replacement of the *scaR* gene of *S. clavuligerus* with mutated *scaR* by homologous recombination. (A) Schematic representation of the strategy used for the disruption of *scaR*. The solid arrows indicate the location and orientation of *scaR*, the dark gray arrows represent the thiostrepton-resistance gene, the open arrows represent apramycin-resistance gene, and the open box represents the *oriT* sequence. (B) Southern hybridization analysis of chromosomal DNA digested with *Eco*RI-*Kpn*I from the *S. clavuligerus* wild-type strain (lane 1), the pCK2-integrated strain (lane 2), and a *scaR*-disruptant, strain CK101 (lane 3). The 464-bp fragment of the 5′ upstream of *scaR* was used as a probe.

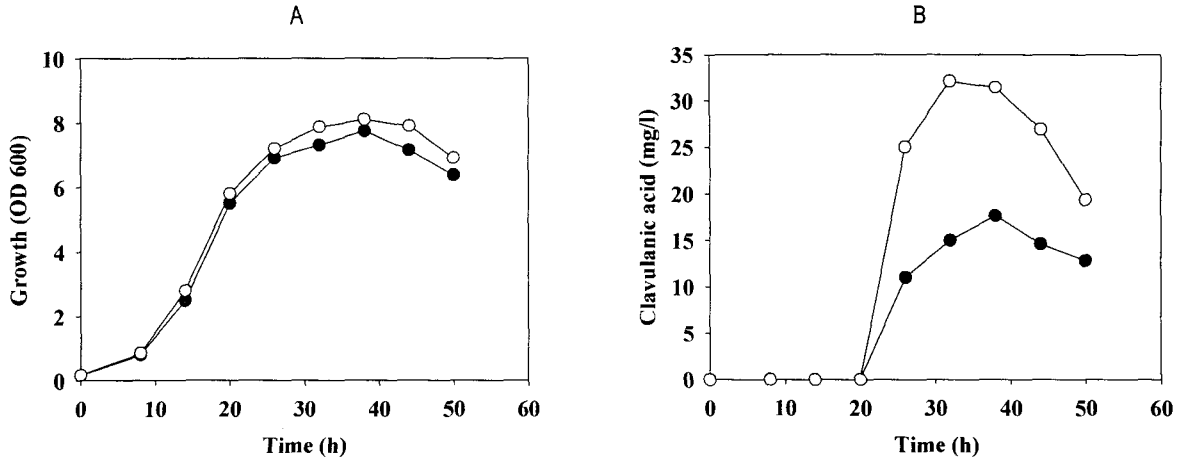

Fig. 3. Growth curves (A) and clavulanic acid production (B) in liquid culture of a wild-type strain (solid circles) and a strain CK101 (open circles).

morphological differentiation in *S. clavuligerus*. However, when clavulanic acid production by the *scaR*-disruptant (strain CK101) was measured by HPLC, the amount of clavulanic acid produced was higher than that by the wild-type strain (Fig. 3B). These results indicate that the loss of ScaR function is responsible for the change in clavulanic acid production, and an autoregulator receptor ScaR likely acts as a negative regulator of the biosynthesis of clavulanic acid, but plays no role in morphological differentiation. Such non-involvement of autoregulator receptors in morphological differentiation has also been reported for *S. lavendulae* FRI-5[9] and *S. coelicolor* A3(2)[15], but not for *S. griseus*[11], in which disruption of the A-factor receptor gene resulted in 10-fold higher production of streptomycin but also earlier sporulation.

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초록 : *Streptomyces clavuligerus*의 γ-butyrolactone autoregulator receptor 유전자에 대한 *in vivo* 기능 분석

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방선균에서 DNA 전사 억제인자로써 작용하여 이차대사산물의 생산뿐만 아니라 형태분화를 조절하는 γ-butyrolactone autoregulator receptor를 암호화하는 유전자(*scaR*)를 clavulanic acid 생산 균주, *Streptomyces clavuligerus*로부터 클로닝하고 *in vitro*에서 ScaR의 특징을 연구하여 보고한 바 있다. 따라서 본 연구에서는 ScaR의 *in vivo* 기능을 분석하기위해 상동재조합방법을 이용하여 *scaR*이 제거된 변이주를 제작하고 야생균주와 표현형을 비교해 보았다. 그 결과, *Streptomyces clavuligerus*의 형태분화에서는 큰 차이를 나타내지 않았지만 clavulanic acid의 생산에 있어서는 *scaR* 파괴 변이주가 야생균주에 비해 생산이 증가된 경향을 보였다. 그러므로 ScaR이 *S. clavuligerus*의 형태분화에는 영향을 미치지 않지만 clavulanic acid 생합성에는 negative regulator로 작용한다는 것을 본 연구를 통해 명확하게 확인할 수 있었다.