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ORIGINAL RESEARCH PAPER

In vivo functions of the γ-butyrolactone autoregulator receptor in *Streptomyces ambofaciens* producing spiramycin

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Abstract A gene encoding a γ-butyrolactone autoregulator receptor was cloned in to *E. coli* from *Streptomyces ambofaciens* producing spiramycin, a macrolide antibiotic used in both veterinary medicine and human medicine. A 714-bp intact receptor gene (*saaR*) was obtained by PCR and genomic Southern hybridization with the 100-bp PCR product as a probe. To clarify the in vivo function of *saaR*, a *saaR*-disrupted strain was constructed by means of homologous recombination, and phenotypes were compared with those of the wild-type strain. The number of *saaR*-disruptant spores was 4-fold less than that of the wild-type strain. In addition, *saaR* deletion from the *S. ambofaciens* chromosome resulted in complete loss of spiramycin production suggesting that *saaR* is a rare positive regulator, controlling both spiramycin biosynthesis and sporulation.

Keywords Autoregulator receptor · γ-Butyrolactone · Spiramycin · Sporulation · *Streptomyces ambofaciens*

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Introduction

The γ-butyrolactone autoregulators found in the genus *Streptomyces* control the production of secondary metabolites and/or morphological differentiation. 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 *Streptomyces* hormones. From in vitro studies these autoregulator receptors are dimeric DNA-binding proteins that, in the absence of autoregulators, recognize and bind to the specific DNA sequences located in the promoter region of target genes (Kinoshita et al. 1999; Kitani et al. 1999; Takano et al. 2001). Autoregulator-binding to the corresponding receptors causes the receptor to dissociate from the DNA, which in turn allows transcription of the target genes.

From the in vivo study of actinomycetes, the disruption of autoregulator receptor genes from *Kitasatospora setae* and *Streptomyces lavendulae* FRI-5 resulted in the overproduction of secondary metabolites (bafilomycin in *K. setae*, and nucleoside antibiotics in *S. lavendulae* FRI-5), while no apparent effect was observed on growth or morphological differentiation, indicating that the corresponding autoregulator receptors only act as negative regulators on the biosynthesis of secondary metabolites. Such non-involvement of autoregulator receptors in morphological differentiation has also been reported for *S. virginiae* (Nakano et al.

1998) and *S. coelicolor* A3(2) (Takano et al. 2001); but not for *S. griseus* and *S. natalensis*, in which disruption of the autoregulator receptor gene resulted in 10- and 4.6-fold higher productions of antibiotic, as well as earlier sporulation and 10-fold more abundant spore production, respectively (Onaka et al. 1995; Lee et al. 2005).

In the present study, a gene encoding an autoregulator receptor from *S. ambofaciens* producing spiramycin, a commercially important macrolide antibiotic consisting of a 16-membered lactone ring and three amino-sugar residues, was cloned by PCR using primers designed for the two highly conserved regions of *Streptomyces* autoregulator receptors, along with genomic Southern hybridization. The in vivo function of the autoregulator receptor in *S. ambofaciens* was also identified by phenotypic comparison between the receptor gene-disruptants and the wild-type.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Streptomyces ambofaciens ATCC15154 was grown at 28°C in a HT medium (g/l, 1 g yeast extract, 1 g beef extract, 2 g NZ-amine A, 10 g dextrin, 20 g agar) for preparation of spores. *E. coli* strain XL10-Gold (Stratagene, UK) was used as the general cloning host. pUC19 was used to construct a genomic library and for DNA sequencing. For the seed culture the preparation, spores of *S. ambofaciens* (5×10^6) were inoculated into 20 ml of vegetative medium (Ford et al. 1990), and then incubated at 28°C for 24 h on a rotary shaker (180 rpm). The main cultivation was performed by inoculating 2.1 ml of seed culture into 70 ml vegetative medium at 28°C for 132 h.

DNA manipulation and sequencing

The degenerate primers F (5′-CGCGGATCCSGCNG CGGCCNNGGTSTTCGA-3′) and R (5′-CGCGGAT CCGTGGAANTASANSGCNCCCTT-3′) were used to clone an autoregulator receptor gene from *S. ambofaciens* (Fig. 1). Southern blot hybridization was performed using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Co., Penzberg, Germany) according to the protocol supplied by the manufacturer. DNA sequencing was performed on a fluorescence DNA sequencer (ALFred; Amersham Pharmacia Biotech, USA) on double-stranded templates derived from different clones in pUC19, using the dideoxy-chain termination method with a thermosequenase cycle sequencing kit (Amersham Pharmacia Biotech, USA) and Cy5-labelled primers. A partial genomic library was constructed with size-fractionated *Eco*RI fragments (4.5 kb) and pUC19, using *E. coli* XL10-Gold as a host, and screened by colony hybridization with a 100-bp PCR fragment.

Construction of a *saaR* disrupted strain

To construct Δ*saaR*, the cloned 4.5-kb fragment was digested with *Eco*81I to remove a 714-bp *saaR* fragment (Fig. 2), and then ligated. The entire 3.8-kb insert containing Δ*saaR* was recovered and inserted into the *Eco*RI sites of pKC1132, a homologous recombination vector containing *oriT* of RK2 and an apramycin resistance gene for selection in actinomycetes and *E. coli* (Bierman et al. 1992), to give pMK201 (Fig. 3a). *E. coli* ET12567/pUZ8002 transformed with pMK201 was conjugated with *S. ambofaciens*. Exconjugants, in which the plasmid pMK201 had integrated at the *saaR* locus by a single crossover via homologous recombination, were selected with apramycin. After three rounds of incubation at 28°C on HT medium in the absence of apramycin, putative *saaR*-disrupted strains formed from the second crossover were detected by their apramycin sensitivity. Because all the *saaR*-disrupted strains showed identical behavior, such as morphology and growth, one of the strains was chosen for detailed analyses (strain MK1) (Fig. 3a).

Morphological assessment and analysis of spiramycin

To analyze morphological differentiation, 10^6 spores each of the wild-type strain and the *saaR*-disrupted strain KM101 were grown on HT, MS (Hobbs et al. 1989) and ISP2 medium, respectively, and were cultivated at 28°C for 7 days. After making the spore suspension, spore counting was done by plating the

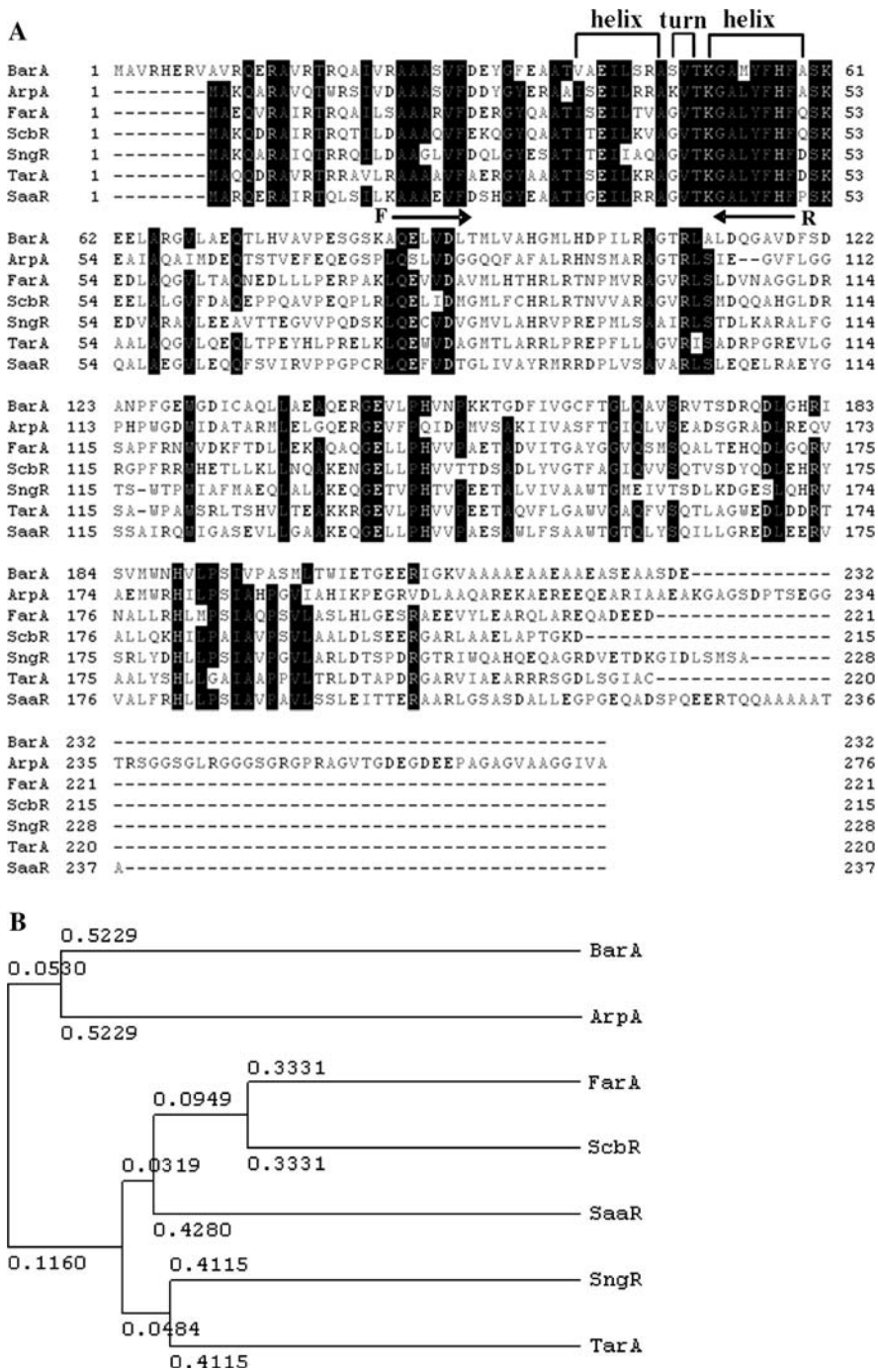

Fig. 1 Alignment of overall amino acid sequences (**a**), and phylogenetic tree (**b**) of BarA, ArpA, FarA, ScbR, SngR, TarA, and SaaR. The alignment and the phylogenetic tree were created by Genetyx software (GENETYX CO., Tokyo, Japan). Identical amino acids are indicated by *black boxes*. *Inverted arrows* represent the regions of designed primers. BarA, VB-specific receptor (BarA) of *S. virginiae*; ArpA, A-factor receptor (ArpA) of *S. griseus*; FarA, IM-2 specific receptor (FarA) of *S. lavendulae* FRI-5; ScbR, SCB1-receptor (ScbR) of *S. coelicolor* A3(2); SngR, receptor of *S. natalensis*; tarA, receptor of *S. tendae* ; SaaR, receptor of *S. ambofaciens* (this study)

serially diluted spore suspension. Values represented the average from three independent experiments, and the error rate was less than 5%. Growth in the liquid culture was measured as the OD_{600}. To assess spiramycin production in the culture broths, the seed culture and the main culture were performed as described above. The broth of the main culture was centrifuged at 13,000*g* for 10 min at 4°C to remove mycelia, and the supernatant was used for bioassay on nutrient agar (g/l, 3 g beef extract, 5 g peptone, 15 g agar, pH 7.0), with *Micrococcus luteus* as a test organism. The clear-zone was measured after

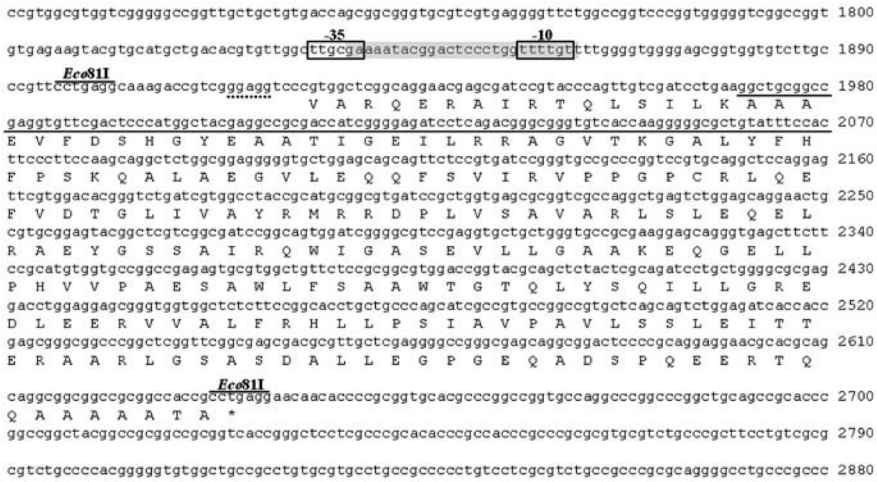

Fig. 2 Nucleotide and deduced amino acid (one-letter notation) sequences of *saaR* in the cloned 4.5-kb *Eco*RI fragment. An asterisk denotes a translational stop codon. Location of putative −10 and −35 sequences are boxed. A putative 26-bp receptor-binding sequence is indicated by the *light gray box*. A probable ribosome-binding sequence, ggagg, is present six nucleotides upstream of the putative ATG start codon (under *dotted line*). The regions amplified by the initial PCR with the designed primers are shown by the *underline*

incubation for 20 h at 30°C. Commercial spiramycin (Sigma) was used as a standard.

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB362564.

Results and discussion

Cloning and sequencing of *saaR*

To search for a receptor gene from *S. ambofaciens*, primers were designed for the two highly conserved amino acid s4 autoregulator receptors [BarA of *S. virginiae* (Okamoto et al. 1995), FarA of *S. lavendulae* (Waki et al. 1997), ScbR of *S. coelicolor* A3(2) (Takano et al. 2001), and ArpA of *S. griseus* (Onaka et al. 1995)] with codon usage data derived from 64 *Streptomyces* genes (Fig. 1) (Wright and Bibb 1992). A 100-bp PCR product clearly encoding the targeted region of a putative autoregulator receptor was obtained, and a 4.5-kb *Eco*RI fragment was cloned using the PCR fragment as a probe, yielding pMK101, as described in the 'Materials and methods' (Fig. 3a).

When the cloned 4.5-kb fragment was sequenced, three complete open reading frames (ORFs) (*orf1* to *orf3*) were identified. In addition, it appeared that the fragment cloned from *S. ambofaciens* ATCC15154 identified to that of *S. ambofaciens* ATCC23877, including a putative γ-butyrolactone-binding protein (accession number AAR30170), by means of sequence matching in GenBank detected by BLAST. The *orf1* consisting of 714 bp is predicted to encode a 237-amino acid protein, which shows high similarity to several autoregulator receptor proteins such as FarA of *S. lavendulae* FRI-5 (50% identity, 63% similarity), ScbR of *S. coelicolor* A3(2) (48% identity, 66% similarity), ArpA of *S. griseus* (43% identity, 62% similarity), and BarA of *S. virginiae* (40% identity, 61% similarity). The *orf2* and *orf3* product identified to a putative acetyltransferase (accession number CAI78116) and a SimX2-like protein (accession number AAR30169) of *S. ambofaciens* ATCC23877, respectively. In addition, the Orf1 has a well-conserved helix-turn-helix (HTH) DNA binding motif (Fig. 1), and its estimated pI value is 5.3, which agreed well with pI values of ~5 for all *Streptomyces* autoregulator receptors (Choi et al. 2004). Therefore, this *orf1* was named *saaR* (*Streptomyces*

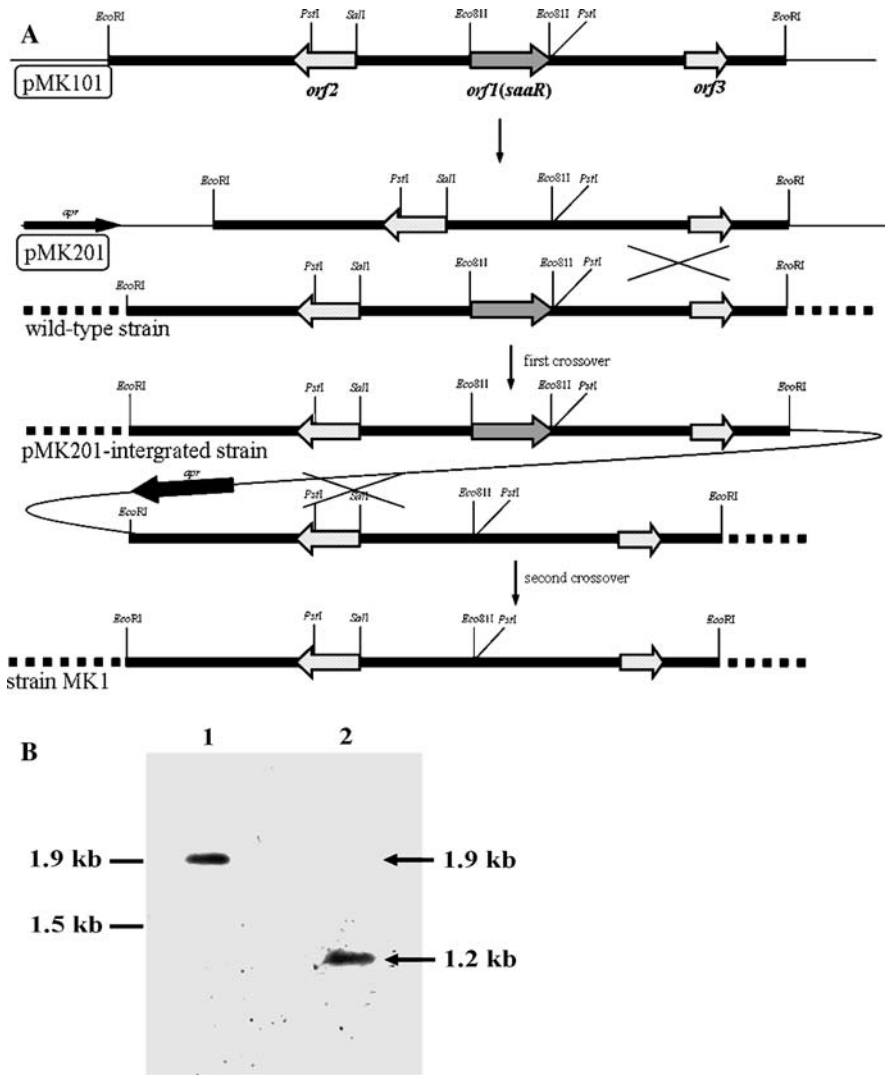

Fig. 3 Gene replacement of the *S. ambofaciens saaR* gene with deleted *saaR* by homologous recombination. (**a**) Schematic representation of the strategy used for the disruption of *saaR*. The *light gray arrows* indicate the location and orientation of *saaR*. (**b**) Southern hybridization analysis of *Pst*I-digested chromosomal DNA from a *S. ambofaciens* wild-type strain (lane 1) and a *saaR*-disruptant, strain MK1 (lane 2). The probe used was the 0.8-kb *Sal*I-*Eco81*I fragment

ambofaciens γ-butyrolactone-autoregulator receptor), and predicted to encode a real autoregulator receptor protein.

In *Streptomyces*, autoregulator receptor proteins usually bind to the promoter region of the receptor gene, and regulate their own transcription depending on the presence of autoregulators (Kinoshita et al. 1999; Kitani et al. 1999; Takano et al. 2001). To estimate whether similar regulation might operate in *saaR*, a putative receptor-binding sequence was identified in the 5′-upstream region of *saaR* using the consensus-binding sequence of Thompson et al. (TNANAWACNNACYNNNCGGTTTKTTT) (Folcher et al. 2001). A 26-bp sequence (GAAAATACG GACTCCCTGGTTTTGTT) was found at 61–86 bp upstream of the *saaR* initiation codon, which was localized between the putative −10 and −35, namely, typical transcriptional promoter sequences (Fig. 2), suggesting that the transcription of *saaR* is likely to be autoregulated via SaaR protein.

Disruption of the *saaR* gene and phenotypic analyses

To determine the in vivo function of SaaR in *S. ambofaciens*, the chromosomal *saaR* gene was disrupted as described in the 'Materials and methods', resulting in a *saaR*-disrupted strain (Δ*saaR*, strain MK1) (Fig. 3a). As shown in Fig. 3b, it was confirmed by

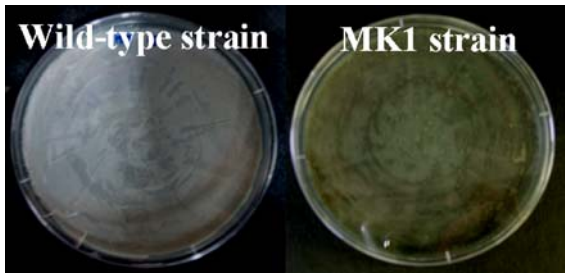

Fig. 4 Effect of *saaR*-disruption on morphological differentiation. Spores (1×10^6) of each strain were plated on HT agar, followed by incubation for 7 days at 28°C

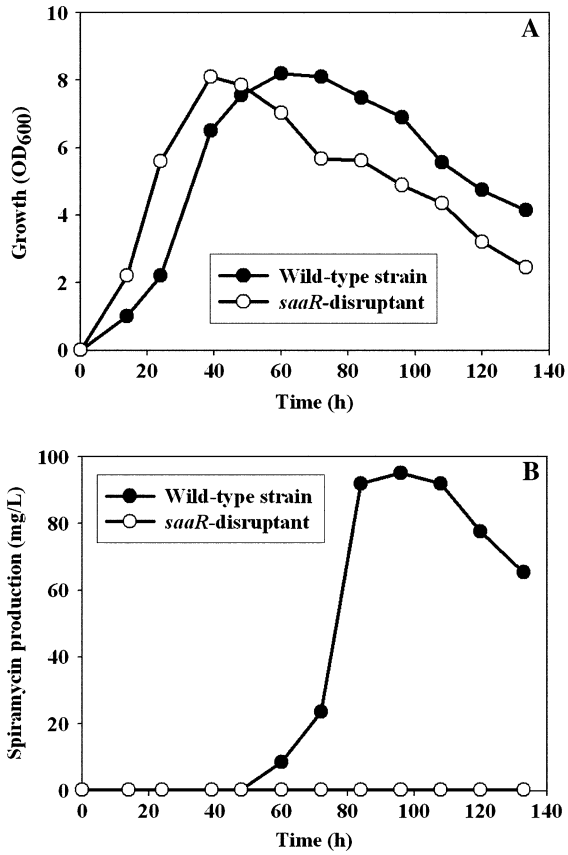

Fig. 5 Growth curves (**a**) and natamycin production (**b**) in liquid culture of a wild-type strain (*solid circles*) and a *saaR*-disruptant (strain MK1; *open circles*). Three independent *saaR*-disruptants showed identical patterns, and the representative data from strains MK1 are shown

Southern blot analysis that the *Pst*I fragment of strain MK1 was 0.7-kb shorter than that of the wild-type strain because a 737-bp fragment, including the whole *saaR* (714 bp), was removed by *Eco*81I and then self-ligated from *S. ambofaciens* chromosome.

To observe the morphological differentiation, the morphological characteristics of the wild-type strain and the strain MK1 were carefully compared on solid media in order to clarify whether *saaR* is involved in the morphological differentiation of *S. ambofaciens*. When the strains were grown on HT agar medium, the number of strain MK1 spores decreased 4-fold in comparison to the wild-type strain (HT agar 6.4×10^7 spores/cm^2 for the wild-type strain; 1.6×10^7 spores/cm^2 for MK1) (Fig. 4). A similar result was also obtained from the MS and ISP2 agar (MS agar 6.9×10^7 spores/cm^2 and ISP2 agar 2.9×10^7 spores/cm^2 for the wild-type strain; MS agar 1.8×10^7 spores/cm^2 and ISP2 agar 6.6×10^6 spores/cm^2 for MK1). In addition, although the growth of strain MK1 in liquid medium was more rapid than that of the wild-type strain, *saaR* deletion from the *S. ambofaciens* chromosome resulted in complete loss of spiramycin production (Fig. 5). Therefore, these results indicate that *saaR* plays an important role as a rare positive regulator, controlling both sporulation and antibiotic production in *S. ambofaciens*.

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References

Bierman M, Logan R, O′Brien K, Seno ET, Rao RN, Schoner BE (1992) Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene 116:43–49

Choi SU, Lee CK, Hwang YI, Kinoshita H, Nihira T (2004) Cloning and functional analysis by gene disruption of a gene encoding a γ-butyrolactone autoregulator receptor from *Kitasatospora setae*. J Bacteriol 186:3423–3430

Folcher M, Gaillard H, Nguyen LT, Nguyen KT, Lacroix P, Bamas-Jacques N, Rinkel M, Thompson CJ (2001) Pleiotropic functions of a *Streptomyces pristinaespiralis* autoregulator receptor in development, antibiotic biosynthesis, and expression of a superoxide dismutase. J Biol Chem 276:44297–44306

Ford LM, Eaton TE, Godfrey OW (1990) Selection of *Streptomyces ambofaciens* mutants that produce large quantities of spiramycin and determination of optimal conditions for spiramycin production. Appl Environ Microbiol 56:3511–3514

Hobbs G, Frazer CM, Gardner DCJ, Cullum JA, Oliver SG (1989) Dispersed growth of *Streptomyces* in liquid culture. Appl Microbiol Biotechnol 31:272–277

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) Practical *Streptomyces* genetics. The John Innes Foundation, Norwich

Kinoshita H, Tsuji T, Ipposhi H, Nihira T, Yamada Y (1999) Characterization of binding sequences for butyrolactone autoregulator receptors in streptomycetes. J Bacteriol 181:5075–5080

Kitani S, Kinoshita H, Nihira T, Yamada Y (1999) In vitro analysis of the butyrolactone autoregulator receptor protein (FarA) of *Streptomyces lavendulae* FRI-5 reveals that FarA acts as a DNA-binding transcriptional regulator that controls its own synthesis. J Bacteriol 181:5081–5084

Lee KM, Lee CK, Choi SU, Park HR, Kitani S, Nihira T, Hwang YI (2005) Cloning and in vivo functional analysis by disruption of a gene encoding the gamma-butyrolactone autoregulator receptor from *Streptomyces natalensis*. Arch Microbiol 184:249–257

Nakano H, Takehara E, Nihira T, Yamada Y (1998) Gene replacement analysis of the *Streptomyces virginiae* barA gene encoding the butyrolactone autoregulator receptor reveals that BarA acts as a repressor in virginiamycin biosynthesis. J Bacteriol 180:3317–3322

Okamoto S, Nakamura K, Nihira T, Yamada Y (1995) Virginiae butanolide binding protein from *Streptomyces virginiae*. J Biol Chem 270:12319–12326

Onaka H, Ando N, Nihira T, Yamada Y, Beppu T, Horinouchi S (1995) Cloning and characterization of the A-factor receptor gene from *Streptomyces griseus*. J Bacteriol 177:6083–6092

Takano E, Chakraburtty R, Nihira T, Yamada Y, Bibb M (2001) A complex role for the γ-butyrolactone SCB1 in regulating antibiotic production in *Streptomyces coelicolor* A3(2). Mol Microbiol 41:1015–1028

Waki M, Nihira T, Yamada Y (1997) Cloning and Characterization of the gene (*farA*) encoding the receptor for an extracellular regulatory factor (IM-2) from *Streptomyces* sp. Strain FRI-5. J Bacteriol 179:5131–5137

Wright F, Bibb MJ (1992) Codon usage in the G + C-rich *Streptomyces* genome. Gene 113:55–65