



SrrB, a Pseudo-Receptor Protein, Acts as a Negative Regulator for Lankacidin and Lankamycin Production in *Streptomyces rochei*

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Specialty section:

This article was submitted to
Antimicrobials, Resistance and
Chemotherapy,
a section of the journal
Frontiers in Microbiology

Received: 17 December 2019

Accepted: 30 April 2020

Published: 09 June 2020

Citation:

Misaki Y, Yamamoto S, Suzuki T,
Iwakuni M, Sasaki H, Takahashi Y,
Inada K, Kinashi H and Arakawa K
(2020) SrrB, a Pseudo-Receptor
Protein, Acts as a Negative Regulator
for Lankacidin and Lankamycin
Production in *Streptomyces rochei*.
Front. Microbiol. 11:1089.
doi: 10.3389/fmicb.2020.01089

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Streptomyces rochei 7434AN4, a producer of lankacidin (LC) and lankamycin (LM), carries many regulatory genes including a biosynthesis gene for signaling molecules SRBs (*srrX*), an SRB receptor gene (*srrA*), and a SARP (*Streptomyces* antibiotic regulatory protein) family activator gene (*srrY*). Our previous study revealed that the main regulatory cascade goes from *srrX* through *srrA* to *srrY*, leading to LC production, whereas *srrY* further regulates a second SARP gene *srrZ* to synthesize LM. In this study we extensively investigated the function of *srrB*, a pseudo-receptor gene, by analyzing antibiotic production and transcription. Metabolite analysis showed that the *srrB* mutation increased both LC and LM production over four-folds. Transcription, gel shift, and DNase I footprinting experiments revealed that *srrB* and *srrY* are expressed under the SRB/SrrA regulatory system, and at the later stage, SrrB represses *srrY* expression by binding to the promoter region of *srrY*. These findings confirmed that SrrB acts as a negative regulator of the activator gene *srrY* to control LC and LM production at the later stage of fermentation in *S. rochei*.

Keywords: *Streptomyces*, regulatory cascade, pseudo-receptor, antibiotic, biosynthesis

INTRODUCTION

Secondary metabolites production is strictly controlled by small diffusible signaling molecules that constitute signaling-molecule/receptor regulatory systems in *Streptomyces* species (Bibb, 2005; Takano, 2006; Horinouchi and Beppu, 2007; Martín and Liras, 2019). The most-studied signaling-molecule/receptor system is A-factor/ArpA in *Streptomyces griseus* for streptomycin and grinoxone production (Ohnishi et al., 1999, 2005). In the absence of A-factor, ArpA protein specifically binds to the promoter region of the target activator gene *adpA* and represses its transcription. When A-factor reaches a critical concentration, A-factor/ArpA complex dissociates from the promoter region of *adpA*, leading to the onset of *adpA* transcription. Then, the gene product of *adpA* binds to its targets (AdpA-regulons) to activate streptomycin and grinoxone production and morphological differentiation (Ohnishi et al., 1999, 2005). Gene sets involved in the signaling-molecule-dependent regulatory pathways for

secondary metabolite production are listed in **Table 1** (Arakawa, 2018; Xu and Yang, 2019); e.g., streptomycin and grizalone production in *S. griseus* (Hara and Beppu, 1982; Onaka et al., 1995; Ohnishi et al., 1999, 2005), lankamycin (LM) and lankacidin (LC) (**Figure 1**) in *Streptomyces rochei* (Arakawa et al., 2007, 2012; Yamamoto et al., 2008; Suzuki et al., 2010), tylosin in *Streptomyces fradiae* (Bate et al., 1999, 2002; Stratigopoulos and Cundliffe, 2002), coelimycin P-1 in *Streptomyces coelicolor* (Takano et al., 2001, 2005; Hsiao et al., 2009; Gottelt et al., 2010; Li et al., 2015), actinorhodin and undecylprodigiosin in *Streptomyces coelicolor* (Xu et al., 2010; Wang et al., 2011), virginiamycin in *Streptomyces virginiae* (Kondo et al., 1989; Kinoshita et al., 1997; Kawauchi et al., 2000), jadomycin in *Streptomyces venezuelae* (Yang et al., 1995; Wang and Vining, 2003; Wang et al., 2009; Xu et al., 2010; Zou et al., 2014), kinamycin in *Streptomyces ambofaciens* (Aigle et al., 2005; Bunet et al., 2008, 2011), and avermectin in *Streptomyces avermitilis* (Kitani et al., 2011; Wang J. B., et al., 2014; Zhu et al., 2016).

Streptomyces rochei 7434AN4 produces two structurally unrelated polyketide antibiotics, LM and LC (**Figure 1A**) and carries three linear plasmids pSLA2-L, -M, and -S (Kinashi et al., 1994). Together with the biosynthetic genes for LM and LC, many regulatory genes including a biosynthetic gene for signaling molecules SRBs (**Figure 1B**) (*srrX*), six *tetR*-type repressor genes (*srrA*, *srrB*, *srrC*, *srrD*, *srrE*, and *srrF*), and three SARP (*Streptomyces* antibiotic regulatory protein) family activator genes (*srrY*, *srrZ*, and *srrW*) are located on giant linear plasmid pSLA2-L (210,614 bp) (Mochizuki et al., 2003). Our group revealed that SRBs/SrrA complex dissociates from the promoter region of *srrY*, leading to the activation of LC production (Yamamoto et al., 2008), whereas the gene product of *srrY* further activates a second activator gene *srrZ* to produce LM (Suzuki et al., 2010). In addition, mutation of an additional receptor gene *srrB* greatly increased the production of both LC and LM (Arakawa et al., 2007). This finding suggested that *srrB* negatively regulates LC and LM production, however, its functional role has not been clarified.

In this study, we extensively investigated the function of *srrB* by analyzing antibiotic production and transcription, the results of which indicated that SrrB acts as a negative regulator by binding to the promoter region of the activator gene *srrY* to control LC and LM production at the later stage of fermentation in *S. rochei*.

MATERIALS AND METHODS

Bacterial Strains and DNA Manipulation

S. rochei strain 51252 carrying only the linear plasmid pSLA2-L was used as the parent strain (Kinashi et al., 1994). All strains, plasmids, and oligonucleotides used in this study are listed in **Table 2**. *Streptomyces* strains were grown in YM medium (0.4% yeast extract, 1.0% malt extract, 0.4% D-glucose, pH 7.3) for antibiotic production and RNA isolation. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium supplemented with ampicillin (100 µg/ml), apramycin (50 µg/ml), and/or chloramphenicol (25 µg/ml) when necessary. For protoplasts preparation, *Streptomyces* strains were grown in YEME medium

(Kieser et al., 2000). Protoplasts were regenerated on R1M plates (Zhang et al., 1997). DNA manipulations for *E. coli* (Sambrook et al., 1989) and *Streptomyces* (Kieser et al., 2000) were performed according to the standard protocols. PCR amplification was done on a 2720 Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) with KOD-Plus- DNA polymerase (Toyobo, Osaka, Japan).

Construction of Mutant and Plasmid Construction of *srrA* and *srrB* Double Mutant

The target plasmid pKAR3014 that carries in-frame deletion of *srrA* in *E. coli-Streptomyces* shuttle vector pRES18 (Ishikawa et al., 1996) was constructed as described previously (Arakawa et al., 2007). Targeted mutagenesis was performed as follows. Plasmid pKAR3014 was transformed into protoplasts of *S. rochei* strain KA07 (*srrB* mutant), and thiostrepton-resistant strains were obtained. Among these transformants, single-crossover strains were selected by Southern hybridization. Some single-crossover colonies were continuously grown in YEME liquid medium to facilitate a second crossover. Finally, thiostrepton sensitive strains were selected as double crossover strains, to obtain a strain TS03 (*srrAB* mutant). Gene disruption was checked by Southern hybridization analysis using DIG DNA Labeling and Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany).

Construction of *srrY* and *srrB* Double Mutant

The target plasmid pKAR3055 that carries in-frame deletion of *srrY* in pRES18 was constructed as described previously (Yamamoto et al., 2008). This plasmid was transformed into protoplasts of *S. rochei* strain KA07, and an *srrB-srrY* double mutant KA64 was constructed in a similar manipulation as above mentioned.

Construction of *in vivo srrB* Expression Plasmid

The *srrB* gene was amplified using cosmid A8 (Mochizuki et al., 2003) and primers, *srrB*-8600f1 and *srrB*-8600r1. The resulting PCR product was digested with *NdeI* and *XbaI* and cloned into pIJ8600, an *E. coli-Streptomyces* shuttle vector carrying a *tipA* promoter (Sun et al., 1999), to obtain pKAR3065.

This plasmid was introduced into strain 51252, and transformants were cultured for 24 h at 28°C in YM liquid medium with 10 µg/ml apramycin. Thiostrepton (10 µg/ml as final concentration) was added at 24 h to induce *srrB* expression. After cultivation for additional 24 h, the broth filtrate was extracted twice with equal volume of ethyl acetate. The combined organic phase was dried with Na₂SO₄, and concentrated *in vacuo* to obtain crude extracts.

Construction of *srrB* Overexpression Plasmid in *E. coli*

The *srrB*-coding sequence was PCR amplified using the template cosmid A8 (Mochizuki et al., 2003) and primers, KAR7903OE and KAR7902OE. The amplified fragment was digested with *BglII* and *EcoRI* and cloned into pET32b(+), a (His)₆-tagged expression vector, to obtain pKAR3036.

TABLE 1 | Gene sets of signaling molecule(s)/receptor/pseudo-receptor for secondary metabolite production in *Streptomyces* species.

	Signaling molecule synthesis gene	Signaling molecule(s)	Receptor gene	Pseudo-receptor gene (pl of gene product)	Secondary metabolite(s)
<i>S. griseus</i>	<i>afsA</i>	A-factor	<i>arpA</i>		Streptomycin, grizazone
<i>S. rochei</i>	<i>srrX</i>	SRB1, SRB2	<i>srrA</i>	<i>srrB</i> (11.2)	Lankacidin, lankamycin
<i>S. fradiae</i>	ND	ND	<i>tylP</i>	<i>tylQ</i> (6.4)	Tylosin
<i>S. coelicolor</i>	<i>scbA</i>	SCB1-3	<i>scbR</i>	<i>scbR2</i> (5.8)	Coelimycin P-1, actinorhodin, undecylprodigiosin
<i>S. coelicolor</i>	<i>mmfL</i>	MMFs	<i>mmfR</i>		Methylenomycin
<i>S. virginiae</i>	<i>barX</i>	Virginia butanolides	<i>barA</i>	<i>barB</i> (10.2)	Virginiamycin
<i>S. venezuelae</i>	<i>jadW1</i>	SVB1	<i>jadR3</i>	<i>jadR2</i> (7.8)	Jadomycin
<i>S. lavendulae</i>	<i>farA</i>	IM-2	<i>farR</i>	<i>farR2</i> (9.7)	Showdomycin
<i>S. ambofaciens</i>	ND	ND	<i>alpZ</i>	<i>alpW</i> (11.6)	Kinamycins
<i>S. avermitilis</i>	<i>aco</i>	Avenolide	<i>avaR1</i>	<i>avaR2</i> (9.6)	Avermectins

ND, Not determined.

Isolation and Analysis of Metabolites

The 48-h cultures of *S. rochei* strains were harvested, and the supernatant was extracted twice with equal volume of ethyl acetate. The crude extracts were purified by Sephadex LH-20 chromatography (1 × 40 cm, GE Healthcare, Chicago, IL) with methanol. Then the fractions containing antibiotics were purified by silica gel chromatography with chloroform-methanol (80:1–10:1, v/v). NMR spectra were recorded on an ECA-500 spectrometer (JEOL, Tokyo, Japan) equipped with a field gradient accessory. Chloroform-*d* and methanol-*d*₄ were used as solvents. Chemical shifts were recorded in δ value based on the solvent signals ($\delta_C = 77.0$ in CDCl₃, $\delta_C = 49.0$ in CD₃OD, and $\delta_H = 3.30$ in residual CH₃OH) or an internal standard tetramethylsilane ($\delta_H = 0$). High resolution ESI-MS spectra were measured by a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). The ¹H- and ¹³C-NMR assignments for lankamycin (1), lankacidin C (2), lankacidin A (3), lankacidinol A (4), iso-lankacidinol (5), and lankacidinol (6) have already been reported (Suzuki et al., 2010; Arakawa et al., 2011; Yamamoto et al., 2018).

SRB Assay

Two strains KA61 ($\Delta srrY$) and KA64 ($\Delta srrY\Delta srrB$) were cultured at 28°C for 30 h, and the supernatant (60 ml) was acidified to pH 3 and extracted with equal volume of ethyl acetate twice. The combined organic phase was concentrated in vacuo. Appropriately diluted culture extract (100 μ l) was added to the fresh culture (5 ml) of strain KA20, an *srrX*-deficient strain, and cultured at 28°C for 36 h to restore LM and LC production.

Time-Course Analysis

S. rochei strains were grown in YM liquid medium and harvested at various time periods at 12–36 h. Cells were used for measurement of dry cell weight (dcw) and isolation of total RNA, while the culture supernatant was for measurement of antibiotic production.

Measurement of DCW

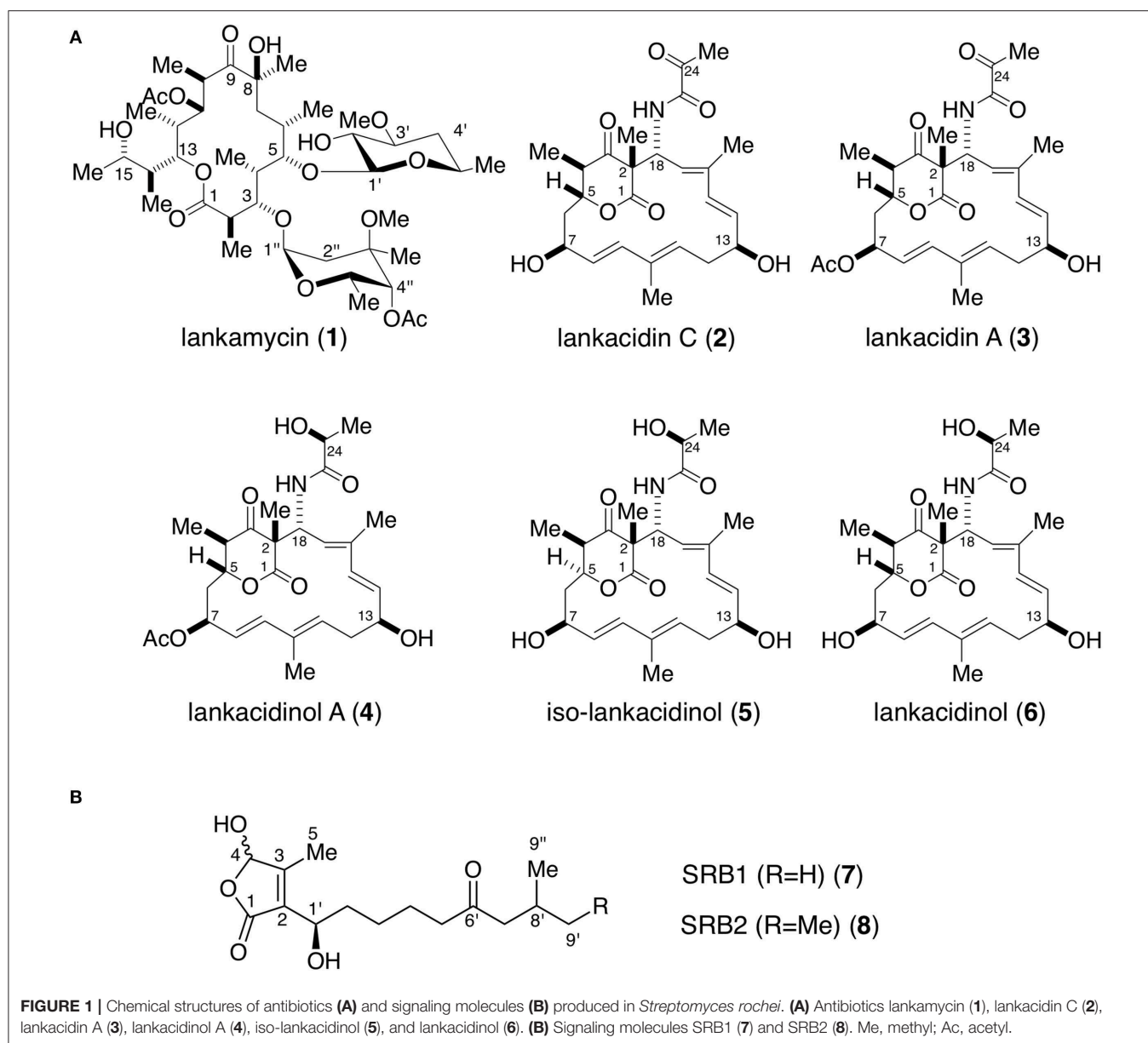
Cultures were collected at various time periods and centrifuged at 5,000 rpm for 10 min. The resulting pellet was washed twice with 10.3% sucrose, and then placed in a 60°C dry oven until the weight reaches to a constant value.

RNA Preparation and Reverse Transcription-PCR (RT-PCR)

S. rochei strains were cultured at 28°C in YM liquid medium for various time periods. Total RNAs were extracted from cells with a TRI reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Trace amounts of DNA were removed with RNase-free DNase I (Takara, Kyoto, Japan). The concentration of purified RNA was determined by UV absorbance at 260 nm using Ultrospec 3300 pro spectrometer (GE Healthcare). The cDNAs were synthesized using Transcriptor Reverse Transcriptase (Roche Diagnostics). Each reaction mixture contained 1 μ g of total RNA and 0.08 A₂₆₀ units random primer. Each mixture was sequentially treated at 85°C for 5 min, at 25°C for 10 min, and 55°C for 45 min for the cDNA synthesis. The 16S rRNA was used as an internal standard (Lane, 1991; Turner et al., 1999).

5' Rapid Amplification of cDNA Ends (5' RACE)

Transcriptional start site (TSS) of *srrB* was determined using 5' RACE System, Version 2.0 (Invitrogen, Carlsbad, CA, USA). Total RNA was prepared from a 24-h culture sample of parent strain. One microgram of total RNA was converted to the cDNA using specific primer *srrB*-GSP1, and the resultant was treated with ribonuclease and purified through spin column to afford cDNA. A homopolymeric tail was then added to the 3'-end of cDNA using terminal deoxynucleotidyl transferase and dCTP. PCR was performed with poly C tailed cDNA as a template using abridged anchor primer and



inner specific primer RT79-R2. TSS was determined from nucleotide sequence of amplified PCR product using ABI PRISM 310 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA).

Overexpression and Purification of SrrB Protein

E. coli BL21(DE3)pLysS was used as hosts for plasmid pKAR3036. Cells were grown in LB liquid medium supplemented with 100 µg/ml ampicillin and 25 µg/ml chloramphenicol at 37°C to $OD_{600} = 0.6$ and then were induced with 1 mM isopropyl β-thiogalactopyranoside (IPTG). Cultivation was continued for 12 h at 16°C, and then cells were harvested and disrupted by SONIFER 250 ultrasonic homogenizer (Branson Ultrasonics

Corporation, Danbury, CT, USA). The (His)₆-fusion protein was purified by Ni²⁺-nitrilotriacetic acid agarose (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. After dialysis with PBS buffer (137 mM NaCl, 8.1 mM Na₂HPO₄·12H₂O, 2.68 mM KCl, 1.47 mM KH₂PO₄), the (His)₆-tagged SrrB protein was treated with enterokinase (Novagen, Madison, WI, USA), and the (His)₆-tag peptide upstream of the N-terminal SrrB was removed by Enterokinase Cleavage Capture Kit (Novagen) according to the manufacturer's protocol. The protein was analyzed by SDS-PAGE with 15% polyacrylamide gel. The protein concentration was determined according to the methods of Bradford using Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard.

TABLE 2 | Bacterial strains, plasmids, and oligonucleotides used in this study.

Strains/plasmids/oligonucleotides	Description	Source/References
BACTERIAL STRAINS		
<i>Streptomyces rochei</i> 7434AN4	Wild type (pSLA2-L, M, S)	Kinashi et al., 1994
<i>Streptomyces rochei</i> 51252	Ultraviolet irradiation of 7434AN4 (pSLA2-L)	Kinashi et al., 1994
<i>Streptomyces rochei</i> KA07	<i>in frame</i> deletion of <i>srrB</i> in 51252 ($\Delta srrB$)	Arakawa et al., 2007
<i>Streptomyces rochei</i> KA12	<i>in frame</i> deletion of <i>srrA</i> in 51252 ($\Delta srrA$)	Arakawa et al., 2007
<i>Streptomyces rochei</i> TS03	<i>in frame</i> deletion of <i>srrA</i> in KA07 ($\Delta srrAB$)	This study
<i>Streptomyces rochei</i> KA61	<i>in frame</i> deletion of <i>srrY</i> in 51252 ($\Delta srrY$)	Yamamoto et al., 2008
<i>Streptomyces rochei</i> KA64	<i>in frame</i> deletion of <i>srrY</i> in KA07 ($\Delta srrBY$)	This study
<i>Streptomyces rochei</i> KA20	<i>kan::srrX</i> in KA07 ($\Delta srrXB$)	Arakawa et al., 2012
<i>Escherichia coli</i> XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac F' proAB lacIqZΔ[M15 Tn10 (Tet^r)]</i>	Stratagene
<i>Escherichia coli</i> BL21(DE3)pLysS	F' <i>ompT hsdS_B (r_B m_B) gal dcm</i> (DE3) pLysS(Cam ^r)	Novagen
PLASMIDS		
Cosmid A8	38.9-kb pSLA2-L DNA (nt 106,868-145,771) cloned into SuperCos-1 at <i>Bam</i> HI site	Mochizuki et al., 2003
pKAR3004	4.3 kb <i>Pst</i> I- <i>Eco</i> 47III fragment containing <i>srrB</i> in pUC19	(Arakawa et al., 2007)
pKAR3014	3.0 kb <i>Nco</i> I- <i>Sac</i> I fragment deleted 207-bp <i>Bsp</i> EI fragment from <i>srrA</i>	Arakawa et al., 2007
pKAR3055	1.5 kb <i>Eco</i> RI- <i>Pst</i> I fragment deleted 267-bp <i>Pvu</i> II fragment from <i>srrY</i>	(Yamamoto et al., 2008)
pKAR3036	1.0 kb <i>Bgl</i> II- <i>Eco</i> RI fragment containing <i>srrB</i> in pET32b(+)	This study
pKAR4002	9.2 kb <i>Pst</i> I fragment containing <i>srrY</i> in pUC19	(Yamamoto et al., 2008)
pUC19	Cloning vector; <i>amp</i>	Takara
pET32b(+)	T7 expression vector for histidine-tagging, <i>amp</i>	Novagen
pJ8600	Integrative <i>E. coli</i> - <i>Streptomyces</i> shuttle vector, inducible <i>tipA</i> promoter, <i>apr</i> , <i>tsr</i>	Sun et al., 1999
pKAR3065	0.68 kb <i>Nde</i> I- <i>Xba</i> I PCR fragment containing <i>srrB</i> in pJ8600	This study
OLIGONUCLEOTIDES (5'-3')		
KAR7903OE	CGCAGATCTACATATGGCCATGCAGGAACGT	This study
KAR7902OE	CTAGAATTCGTACAGCTCGGCCACCATGGC	This study
SRRBf3	ACCCGCACGGCCCGTACATC	This study
SRRBr3	GTACCCCTCTTCCGCGAACA	This study
SRRYf2	GGCGTGTCTGCCTGTCTGCC	Yamamoto et al., 2008
SRRYr2	ATATCCGCCGGGGCGGTGG	Yamamoto et al., 2008
SRRYf4	CTCCCTTGTCTGTCTGTGAG	This study
SRRYr4	GCGCCCGCGGCGTCAACGAGA	Yamamoto et al., 2008
RT75-F	CAGGTTCTCGTGTGCGGTA	This study
RT75-R	GTGCGACGTACAAGCGGGACC	This study
KA82010E	CTAGGATCCGCATATGGCACAGCAGGAAC	This study
SRRAr2B	GGGGATCCCACCGACCGGAGGGCACCCG	This study
SRRBf1E	GGGGAATTCGAGCGGTGGAGACCAGGCCG	This study
SRRBr4	AGGAGCAGTTCACAGAACGC	This study
16S-357F	CCTACGGGAGGCAGCAG	Turner et al., 1999
16S-907R	CCCCGTCAATTCCTTTGAGTT	Lane 1991
KA-RT079S1	GCGAGACACCGGGAGCCAACTG	This study
KA-RT079AS1	TCGCGGAAGAGGGGTACGTGCC	This study
<i>srrB</i> -8600f1	GAACATATGCCATGCAGGAA	This study
<i>srrB</i> -8600r1	TGAAGATCTCACTGTCTGGGCTG	This study
<i>srrB</i> -GSP1	GCTGCGAACCAGCTCGAAAC	This study
RT79-R2	CGCCTTCTGTTCTCGAAGTG	This study
<i>srrB</i> -GSP3	TCCCGCCGGTGACCGCTCCGTCC	This study

Preparation of DNA Probes and Gel Shift Assay

The *srrB* probes for gel shift assay were prepared as follows. For preparation of probe B1, a 564-bp DNA fragment containing the upstream region of *srrB* was amplified using pKAR3004 as a template and primers SRRBf3 and SRRBr3 (positions -81 to +483 from TSS of *srrB*; nt 140,677-141,240 of pSLA2-L). For preparation of probe B2, a 386-bp DNA fragment containing the internal region of *srrB* was amplified using pKAR3004 as a template and primers SRRBf1E and SRRBr4 (positions +574 to +959 from TSS of *srrB*; nt 140,201-140,586 of pSLA2-L).

Probe B1 was then 3' -end labeled with [γ - 32 P]ATP (GE Healthcare) and T4 polynucleotide kinase (Toyobo). The reaction mixture contained the binding buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM dithiothreitol, 0.1 mg of bovine serum albumin and 5% glycerol), 0.5 nM labeled DNA and 2 μ M SrrA protein. SrrA protein was prepared as reported previously (Yamamoto et al., 2008). When necessary, synthetic SRB1 [(1'-R)-isomer; **Figure 1B**] (Arakawa et al., 2012) was added to the reaction mixture. For competition experiment, unlabeled probes B1 and B2 were used at a final concentration of 200 nM. The reaction mixture was incubated at 26°C for 30 min, and subjected to electrophoresis at room temperature on a native 4.5% polyacrylamide gel in 0.5 \times TBE buffer (46 mM Tris base, 46 mM boric acid, 1 mM EDTA). The 32 P-labeled DNAs were detected by autoradiography.

Preparation of *srrY* probes for gel shift assay was described previously (Yamamoto et al., 2008). To analyze the effect of SRB on the binding of SrrA and SrrB, various concentration of synthetic SRB1 [(1'-R)-isomer; **Figure 1B**] (Arakawa et al., 2012) was added to the reaction mixture. In order to evaluate the effect of endogenous metabolites in *S. rochei* and other antibiotics on the binding of SrrB, the following compounds (1 mM) were separately added to the reaction mixture; LC, LM, chlorotetracycline, kanamycin, and ampicillin.

DNase I Footprinting

The method used for DNase I footprinting analysis for the upstream region of *srrY* was described previously (Yamamoto et al., 2008). For the upstream region of *srrB*, the primer SRRBf3 was 5'-end labeled using [γ - 32 P]ATP (GE Healthcare) and T4 polynucleotide kinase (Toyobo), and then PCR reaction was performed with unlabeled primer SRRBr3 and pKAR3004 as a template to afford a 564-bp product containing the upstream region of *srrB* (positions -81 to +483 from TSS of *srrB*; nt 140,677-141,240 of pSLA2-L). Binding reaction mixture (50 μ l) contained 10 nM labeled DNA, 20 mM Tris-HCl (pH8.0), 1 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol, 0.1 mg/ml BSA, 5% glycerol, and various concentrations of SrrA. The binding reaction mixture was incubated for 30 min at 25°C, and then a mixture was treated with DNase I (Roche Diagnostics) solution [1 ng in 50 μ l of 5 mM MgCl₂ and 5 mM CaCl₂] for 2 minutes at room temperature. The reaction was terminated by 100 μ l of phenol-chloroform. The aqueous fraction containing DNAs was precipitated by ethanol and separated on a 5% polyacrylamide gel containing 6 M urea. The labeled DNAs were detected by autoradiography. Sequencing ladders were generated

by Maxam-Gilbert sequencing of the labeled DNA used for binding reaction.

Comparative Sequence Analysis

Alignment of Amino acid sequences of the pseudo-receptors including SrrB was performed by BioEdit version 7.2.5 software (<https://bioedit.software.informer.com/>) (Hall, 1999) (**Figure S1A**). Phylogenetic tree was constructed by the neighbor-joining algorithm of MEGA X version 10.1.5 software (Kumar et al., 2018) (**Figure S1B**).

RESULTS

SrrB Acts as a Negative Regulator for Lankacidin and Lankamycin Production

The gene product of *srrB* belongs to the TetR-type transcriptional regulator family proteins, which contains a helix-turn-helix DNA binding motif at the N-terminal region (**Figure S1**). We previously reported overproduction of LM and LC in the *srrB* mutant KA07 based on TLC bioautography (Arakawa et al., 2007). In this study, we performed comparative metabolite analysis of the *srrB* mutant and its parent strain 51252. As shown in **Figures 2A,B**, the *srrB* mutant KA07 accumulated larger amount of compounds **1-6** compared with the parent strain 51252. Namely, KA07 produced 6-folds of lankamycin (**1**) (**Figure 2A**) and 9.9, 25, 4.2, and 5.7-folds of lankacidin C (**2**), lankacidinol A (**4**), iso-lankacidinol (**5**), and lankacidinol (**6**), respectively (**Figure 2B**). To investigate the effect of SrrA on antibiotic production, we further analyzed two mutants, an *srrA* mutant KA12 and an *srrA-srrB* double mutant TS03 (**Figure S2**). KA12 produced about 40% of metabolites when compared with the parent, while TS03 overproduced metabolites **1-6** at the same level with the *srrB* mutant KA07 (**Figures 2A,B**). These results confirmed the following two aspects; *srrA* mutation causes a slight decrease of the metabolic titer, whereas SrrB acts as a negative regulator for lankacidin and lankamycin production in *S. rochei*.

To determine the role of *srrB* in the regulation of lankacidin and lankamycin production, we further performed time-course analysis of metabolite profile, growth curve, and transcription in the parent and three mutants (KA07, KA12, and TS03) at various time periods. As shown in **Figure 2C**, all strains grew in a similar proportion, indicating that overproduction in KA07 and TS03 was due to *srrB* mutation but not to cell growth difference. The time-course of antibiotic production was analyzed by the titer of **2**, a major product among lankacidin derivatives (**2-6**; **Figure 1A**). As shown in **Figure 2D**, **2** was detected after 18 h, and its titer at 48-h growth in the *srrB* deficient strains, KA07 and TS03, were 9.0- and 7.2-times of 51252, respectively, which agrees with the overproduction profiles in KA07 and TS03 in **Figures 2A,B**. To confirm the negative regulatory property of SrrB *in vivo*, overexpression of SrrB in *S. rochei* was carried out. The intact *srrB* gene was introduced into plasmid pIJ8600, an *E. coli-Streptomyces* shuttle plasmid with a thiostrepton-inducible *tipA* promoter, to give pKAR3065. We tested antibiotic production in the *S. rochei* 51252 recombinants containing either the empty vector pIJ8600 or the *srrB* overexpression plasmid (pKAR3065). Compared with the control recombinant *S. rochei*

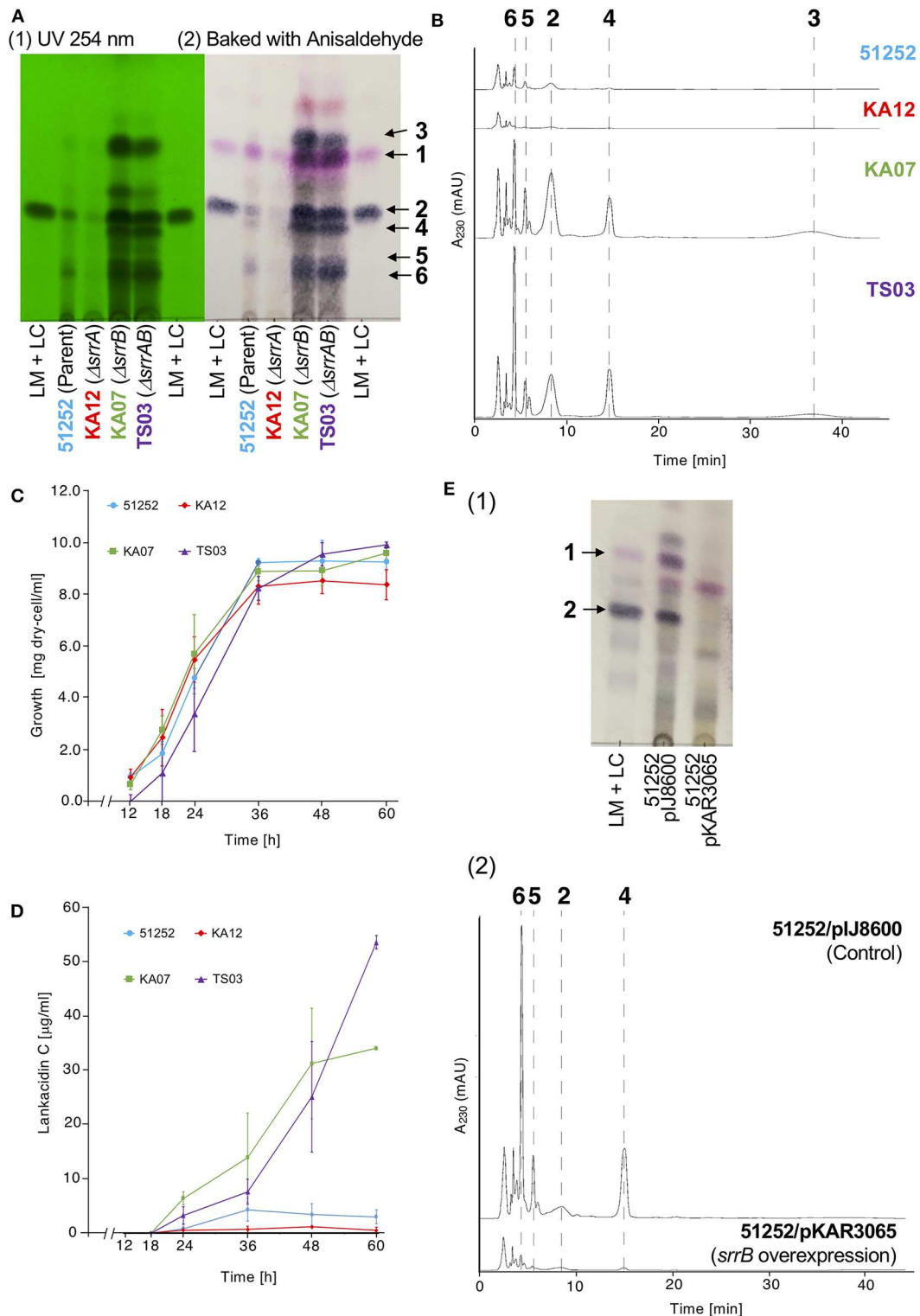


FIGURE 2 | Metabolite profiles and cell growth of four *S. rochei* strains; 51252 (parent), KA12 ($\Delta srrA$), KA07 ($\Delta srrB$), and TS03 ($\Delta srrA$ -*srrB*). **(A)** TLC analysis of the crude extract of *S. rochei* strains. All strains were grown at 28°C for 48 h. The left panel represents the TLC plate under ultraviolet irradiation (254 nm). The right panel represents the TLC plate after baking with anisaldehyde- H_2SO_4 . TLC plates were developed with chloroform-methanol = 15:1 (v/v). **(B)** HPLC analysis of metabolites produced by *S. rochei* strains. The crude extracts were applied on a COSMOSIL Cholesterol column (4.6 \times 250 mm, Nacalai Tesque) and eluted with a mixture of acetonitrile-10 mM sodium phosphate buffer (pH 8.2) (3:7, v/v) at a flow rate of 1.0 ml/min. **(C)** Time-course growth of *S. rochei* strains. Symbols represent

(Continued)

FIGURE 2 | each dry cell weight (DCW; g/l); strain 51252, blue circles and line; strain KA12, red diamonds and line; strain KA07, green squares and line; TS03, purple triangles and line. Results are representative of at least three independent experiments. **(D)** Time-course production of **2** in *S. rochei* strains. Symbols represent each production yield of **2** ($\mu\text{g/ml}$); strain 51252, blue circles and line; strain KA12, red diamonds and line; strain KA07, green squares and line; TS03, purple triangles and line. Results are representative of at least three independent experiments. **(E)** Effect of *srrB* overexpression on antibiotic production. Thiostrepton ($10\ \mu\text{g/ml}$) was added to the 24-h culture of *S. rochei* 51252 recombinants harboring either pJ8600 (control) or pKAR3065 (intact *srrB*), and then the cultures were further incubated at 28°C for 24 h. **(1)** TLC analysis of crude extracts. Crude extracts were separated by TLC [eluent; chloroform-methanol = 15:1 (v/v)] and detected by baking with anisaldehyde- H_2SO_4 . Lane 1, LM and LC standard; lane 2, recombinant harboring pJ8600 (control); lane 3, recombinant harboring pKAR3065 (intact *srrB*). **(2)** HPLC analysis of crude extracts. The crude extracts were applied on a COSMOSIL Cholesterol column (4.6 x 250 mm, Nacal Tesque) and eluted with a mixture of acetonitrile-10 mM sodium phosphate buffer (pH 8.2) (3:7, v/v) at a flow rate of 1.0 ml/min.

51252/pJ8600, the *S. rochei* 51252/pKAR3065 recombinant significantly reduced antibiotic production (**Figure 2E**). These results clearly indicated that SrrB acts as a negative regulator for antibiotic production in *S. rochei*.

In our preliminary experiment, gel shift assay indicated that SrrA and SrrB could bind to the upstream region of *srrX*, a gene responsible for SRB biosynthesis. This finding suggested that the signaling molecule receptor/pseudo-repressor repress the transcription of *srrX*. Two strains were used to evaluate the comparative yield of SRB; an *srrY* single mutant KA61 and an *srrY-srrB* double mutant KA64 (**Figure S3**), both of which are unable to produce LC or LM due to a mutation on the major activator *srrY*. The yields of SRBs were evaluated by a help of bioassay using an *srrX*-deficient mutant as described previously (Arakawa et al., 2012). One-eighth of the crude extract of KA64 contained an equivalent amount of SRBs to that of KA61 (**Figure S4**), suggesting that *srrB* negatively controls the titer of SRBs.

***srrB* and *srrY* Are Expressed Under the SRB/SrrA Regulatory System, and SrrB Then Represses *srrY* Expression at the Later Stage**

To analyze the role of *srrB* in the SRB/SrrA regulatory system in *S. rochei*, we performed comparative transcriptional analysis of the selected regulatory genes in the parent and three mutants (*srrA*, *srrB*, and *srrA-srrB*). Transcription of *srrY* in the parent appeared around 18 h and diminished after 32 h (**Figure 3A**), while that in the *srrB* mutant KA07 continued until the later stage (**Figure 3C**). On the other hand, *srrB* transcription in the parent appeared around 16 h and prolonged until the late stage of fermentation (**Figure 3A**). In the *srrA* mutant KA12, transcription of both *srrY* and *srrB* appeared at 12 h or earlier (**Figure 3B**), whereas *srrY* transcription in the *srrA-srrB* mutant TS03 was detected through all time periods (12–36 h) (**Figure 3D**). These findings together with our previous result (Yamamoto et al., 2008) showed that SRB/*srrA* regulatory system controls transcription of both *srrY* and *srrB*, and SrrB represses *srrY* transcription at the later stage.

SrrA Binds to the Promoter Region of *srrB*

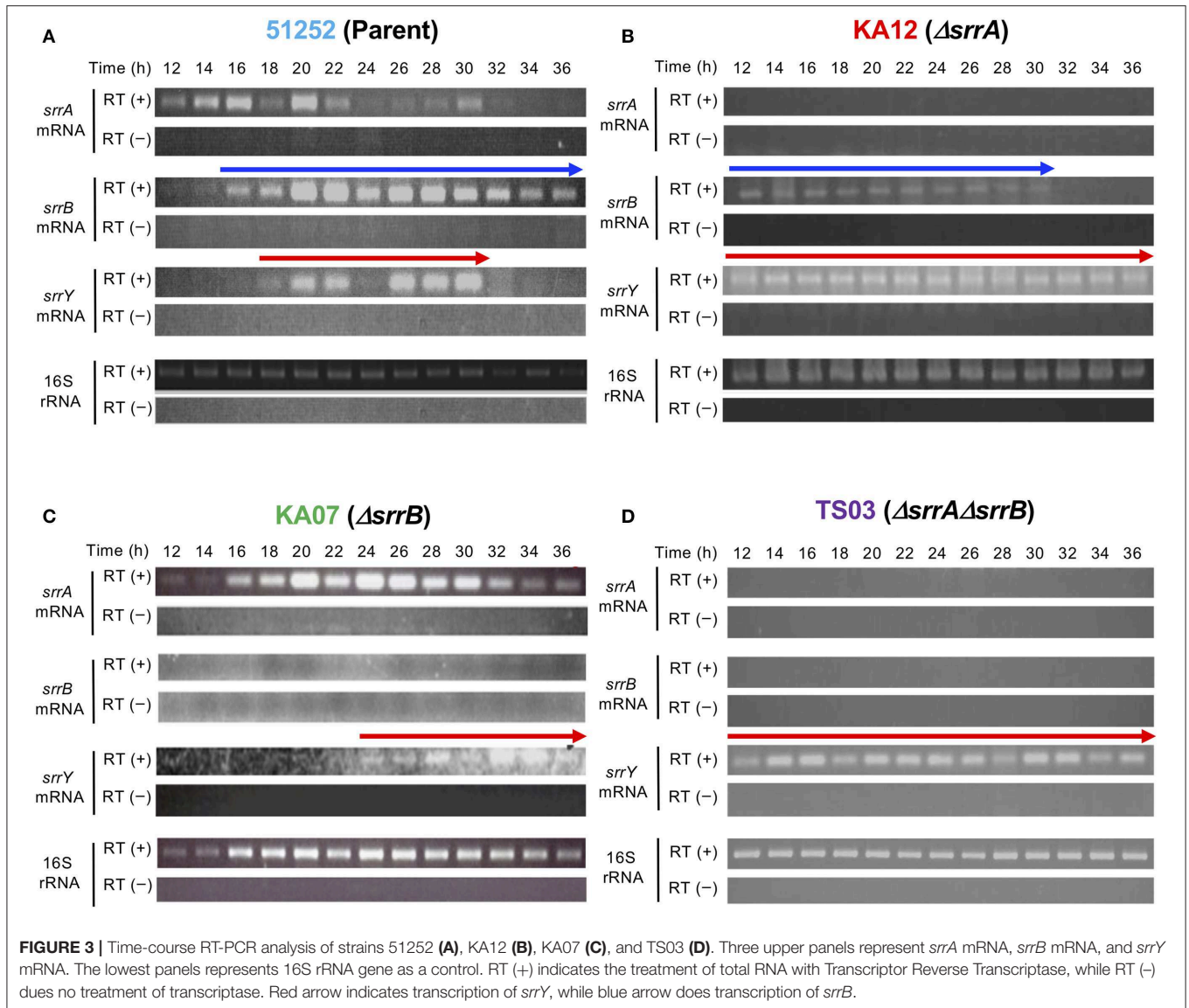
A transcriptional start point (TSS) of *srrB* was determined to be 401-bp upstream of its translational start codon by 5'-RACE (**Figure 4** and **Figure S5**). To determine whether SrrA binds to the upstream promoter region of *srrB* (*srrBp*), gel shift assay was performed using ^{32}P -labeled probe B1 (nt

140,677–141,240 of pSLA2-L) that contained the upstream region of *srrB* (**Figure 5A**). SrrA protein was overexpressed in *E. coli* as described previously (Yamamoto et al., 2008). A band shift of probe B1 was observed in the presence of SrrA protein in a concentration-dependent manner (**Figure 5B**). Competition experiments using unlabeled probes B1 and B2 (nt 140,201–140,585 of pSLA2-L) (**Figure 5C**) were performed to determine the specific binding of SrrA to the region of probe B1. A band shift disappeared in the large excess of unlabeled probe B1, whereas probe B2 gave no effect on band shift (**Figure 5C**). Addition of SRB led to dissociation of SrrA from probe B1 (**Figure 5C**), indicating that the *srrB* transcription is controlled by SRB/SrrA regulatory system.

DNase I footprinting experiment was performed to identify the SrrA binding sequence in the upstream of *srrB*. Positions –36 to –11 of the non-template strand was protected by SrrA (**Figure 5D**). The protected region overlapped with a possible *srrB* promoter containing a palindromic sequence (asterisks in **Figure 4B**), whose sequence well-matched with the SrrA-binding sequence of *srrY* (Yamamoto et al., 2008). Taking account of transcriptional analysis above mentioned, SrrA binds to the upstream regions of both *srrB* and *srrY* to repress their transcription at the early growth phase (~16 h).

SrrB Represses *srrY* Transcription at the Later Stage of Fermentation by Binding to the Promoter Region of *srrY*

To analyze whether SrrB binds to the promoter region of *srrY* (*srrYp*), gel shift assay was performed using probe Y1 (positions –452 to +100 from TSS of *srrY*) (**Figure 6A**) containing the promoter region of *srrY*, which was constructed previously (Yamamoto et al., 2008). SrrB protein was overexpressed in the *E. coli* BL21(DE3)pLysS/pKAR3036 recombinant with IPTG induction and purified by Ni-NTA agarose (**Figure S6**). The addition of SrrB protein gave a shifted band of probe Y1 in a concentration-dependent manner (**Figure 6B**). Competitive experiments (**Figure 6C**) revealed that SrrB specifically binds to probe Y1, not to probe Y2 (positions +101 to +333 from TSS of *srrY*). The pseudoreceptors hitherto studied are insensitive to endogenous signaling molecules and interact with endogenous antibiotics (Martín and Liras, 2019; Xu and Yang, 2019) (details are described in Discussion Section). We tested the effects of various signaling-molecule/antibiotics on the binding of SrrB to *srrYp* through gel shift analysis by using endogenous signaling molecule SRB, endogenous antibiotics (LC and LM),



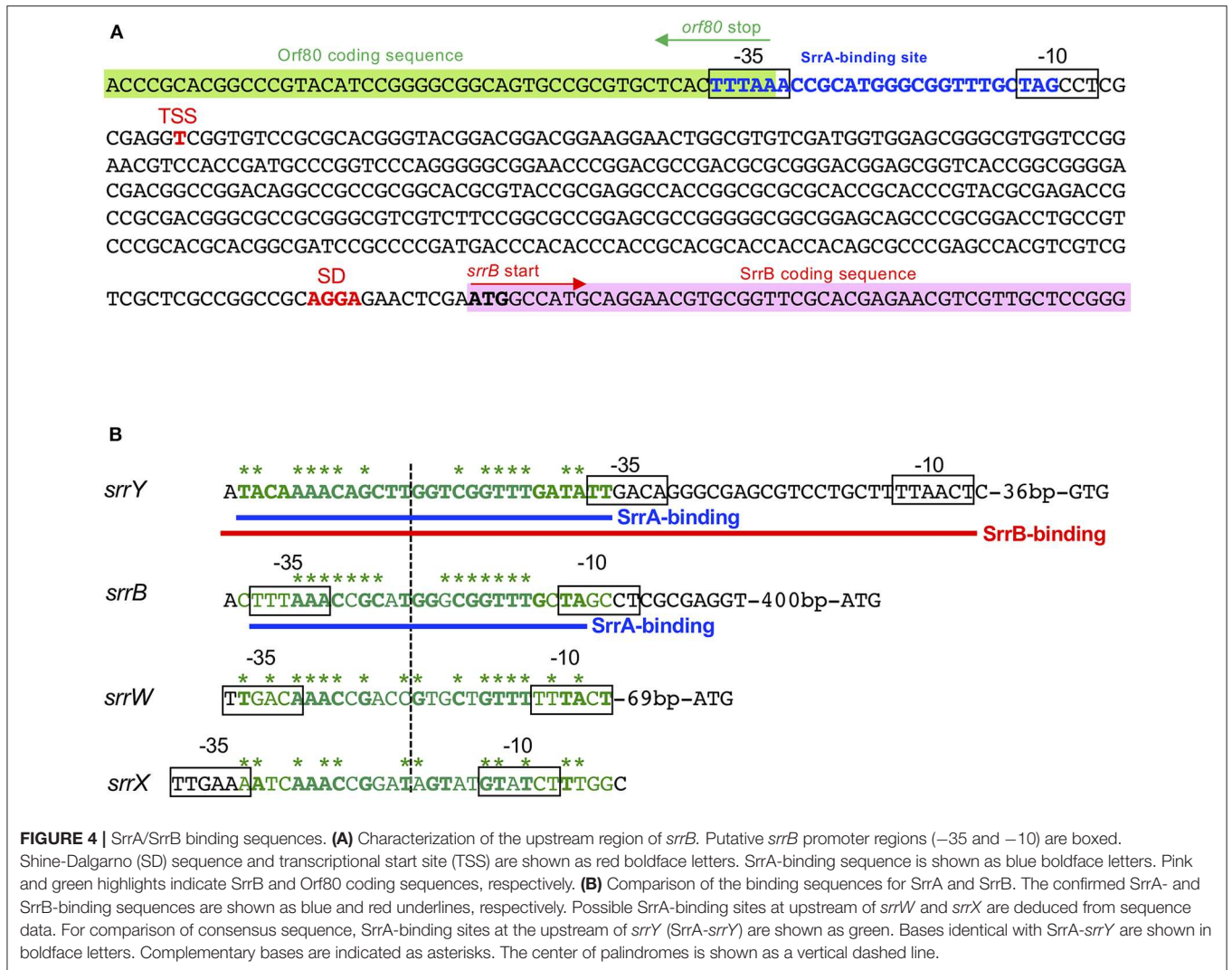
and other exogenous antibiotics (Figures 6D,E). Dissociation of SrrB from *srrYp* could be caused by SRB, however, a higher concentration of 1 mM was required (500-fold excess against SrrB protein). The sensitivity of SrrB against SRB was 50-fold lower compared with the signaling molecule receptor SrrA (Figure 6D). Dissociation of SrrB from *srrYp* was not caused by endogenous antibiotics LC and LM in *S. rochei* and neither by exogenous antibiotics (chlorotetracycline, kanamycin, ampicillin) at even 1 mM concentration (500-fold excess against SrrB protein) (Figure 6E).

We further performed DNase I footprinting experiment to identify the SrrB binding sequence in the upstream of *srrY*. As shown in Figure 6F, positions -61 to -4 of non-template strand were protected by SrrB. Although SrrB covers larger upstream region (58 bp) than SrrA does (28 bp; blue letters in Figure 6F), both SrrA and SrrB could bind to the promoter region of *srrY* (Figure 4B).

DISCUSSION

In this study, we revealed that *srrB* acts as a negative regulator by binding to the promoter region of the target gene *srrY* to repress LC and LM production in *S. rochei*. Expression of *srrB* is controlled by SRB/SrrA regulatory system.

TetR-type receptors have a conserved DNA-binding helix-turn-helix motif in the N-terminus and a ligand-binding pocket in the C-terminus (Yu et al., 2010). Particularly, the signaling molecule receptors and the pseudo-receptors constitute one of a major class of TetR-type regulators (Figure S1). The signaling molecule receptors have a helix-turn-helix DNA-binding motif in the N-terminus and a ligand-binding Trp residue in the C-terminus. It is noteworthy that the signaling molecule synthase and its cognate receptor gene pairs usually locate adjacently on the genome (Biarnes-Carrera et al., 2015), which allows us to predict signaling molecule/receptor systems



in *Streptomyces* species (Niu et al., 2016). On the other hand, the pseudo-receptors also have a conserved DNA-binding motif like the signaling molecule receptors (Figure S1A), however, their location has no relationship with the signaling molecule synthase genes. Many of them act as negative regulators for antibiotic production; for example, TylQ for tylosin production in *Streptomyces fradiae* (Stratigopoulos and Cundliffe, 2002), BarB for virginiamycin in *Streptomyces virginiae* (Matsuno et al., 2004), ScbR2 for coelimycin P-1 in *Streptomyces coelicolor* (Gottelt et al., 2010), AlpW for orange pigment kinamycin in *Streptomyces ambofaciens* (Bunet et al., 2008), and AvaR2 for avermectin in *Streptomyces avermitilis* (Zhu et al., 2016) (Table 1).

In general, the pseudo-receptors are insensitive to endogenous signaling molecules. BarB has no binding affinity to virginia butanolides in *S. virginiae* (Matsuno et al., 2004). Surprisingly, ScbR2 from *S. coelicolor* does not bind to the signaling molecules SCB1-3 but binds to two endogenous antibiotics, actinorhodin and undecylprodigiosin (Xu et al., 2010), and exogenous antibiotic jadomycin (Wang W., 2014). In *S. venezuelae*, JadR2 binds to endogenous jadomycin and chloramphenicol

as ligands, (Xu et al., 2010). Thus, ScbR2 and JadR2 bind to multiple antibiotics, and coordinate their biosynthesis (Xu et al., 2010; Zou et al., 2014). In *S. avermitilis*; AvaR2 binds to the endogenous signaling molecule avenolide, but not to oligomycin and avermectin (Zhu et al., 2016). Its mutational analysis revealed that AvaR2 plays a negative regulatory role in avermectin production and cell growth (Zhu et al., 2016). In *S. rochei*, SrrB-*srrYp* complex was disrupted by endogenous signaling molecule SRB at 1 mM concentration, although its minimum dissociation concentration for SrrB was 50-fold higher than that for SrrA, the SRB receptor. SrrB showed no binding activity to endogenous polyketide antibiotics LM or LC in *S. rochei* and neither to exogenous antibiotics including aromatic polyketide chlorotetracycline, aminoglycoside antibiotic kanamycin, and β -lactam antibiotic ampicillin even at 1 mM concentration (500-fold excess against SrrB). Thus, functions of the pseudo-receptors are variable in *Streptomyces* species.

The possible regulatory pathway in *S. rochei* is shown in Figure 7. At the early growth phase, SrrA represses transcription

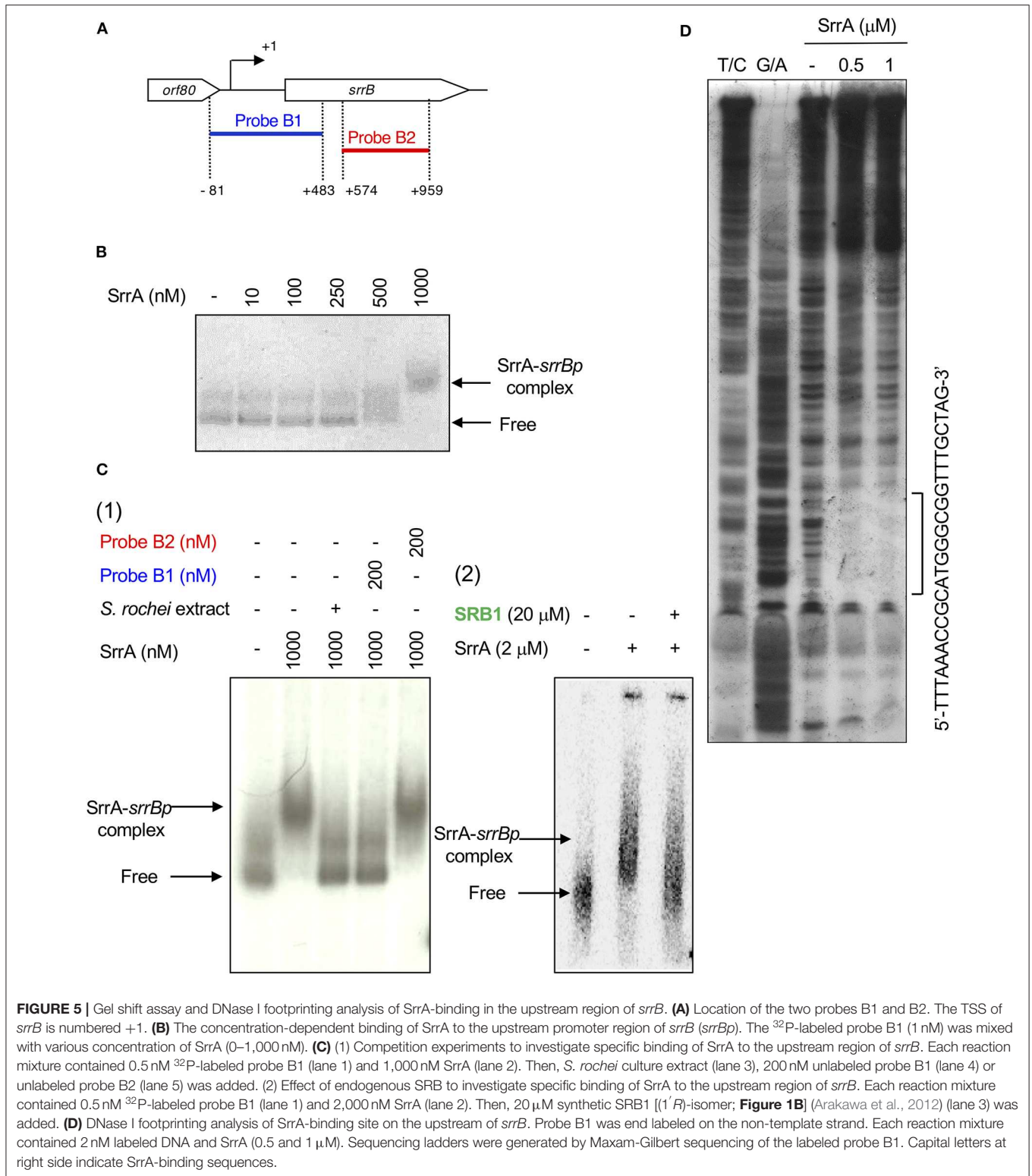


FIGURE 5 | Gel shift assay and DNase I footprinting analysis of SrrA-binding in the upstream region of *srrB*. **(A)** Location of the two probes B1 and B2. The TSS of *srrB* is numbered +1. **(B)** The concentration-dependent binding of SrrA to the upstream promoter region of *srrB* (*srrBp*). The ³²P-labeled probe B1 (1 nM) was mixed with various concentration of SrrA (0–1,000 nM). **(C)** (1) Competition experiments to investigate specific binding of SrrA to the upstream region of *srrB*. Each reaction mixture contained 0.5 nM ³²P-labeled probe B1 (lane 1) and 1,000 nM SrrA (lane 2). Then, *S. rochei* culture extract (lane 3), 200 nM unlabeled probe B1 (lane 4) or unlabeled probe B2 (lane 5) was added. (2) Effect of endogenous SRB to investigate specific binding of SrrA to the upstream region of *srrB*. Each reaction mixture contained 0.5 nM ³²P-labeled probe B1 (lane 1) and 2,000 nM SrrA (lane 2). Then, 20 μM synthetic SRB1 [(1′ *R*)-isomer; **Figure 1B**] (Arakawa et al., 2012) (lane 3) was added. **(D)** DNase I footprinting analysis of SrrA-binding site on the upstream of *srrB*. Probe B1 was end labeled on the non-template strand. Each reaction mixture contained 2 nM labeled DNA and SrrA (0.5 and 1 μM). Sequencing ladders were generated by Maxam-Gilbert sequencing of the labeled probe B1. Capital letters at right side indicate SrrA-binding sequences.

of both *srrY* and *srrB* (panel A). When SRB reaches a critical concentration, SrrA-SRB complex dissociates from both promoter regions to induce expression of *srrY* and *srrB* (panel B). Then SrrB represses *srrY* transcription at the later stage

fermentation (panel C), suggesting a transient expression of *srrY* by two receptor proteins SrrA and SrrB in *S. rochei*. A similar regulatory pathway was proposed for kinamycin production in *S. ambofaciens* (Bunet et al., 2008) although

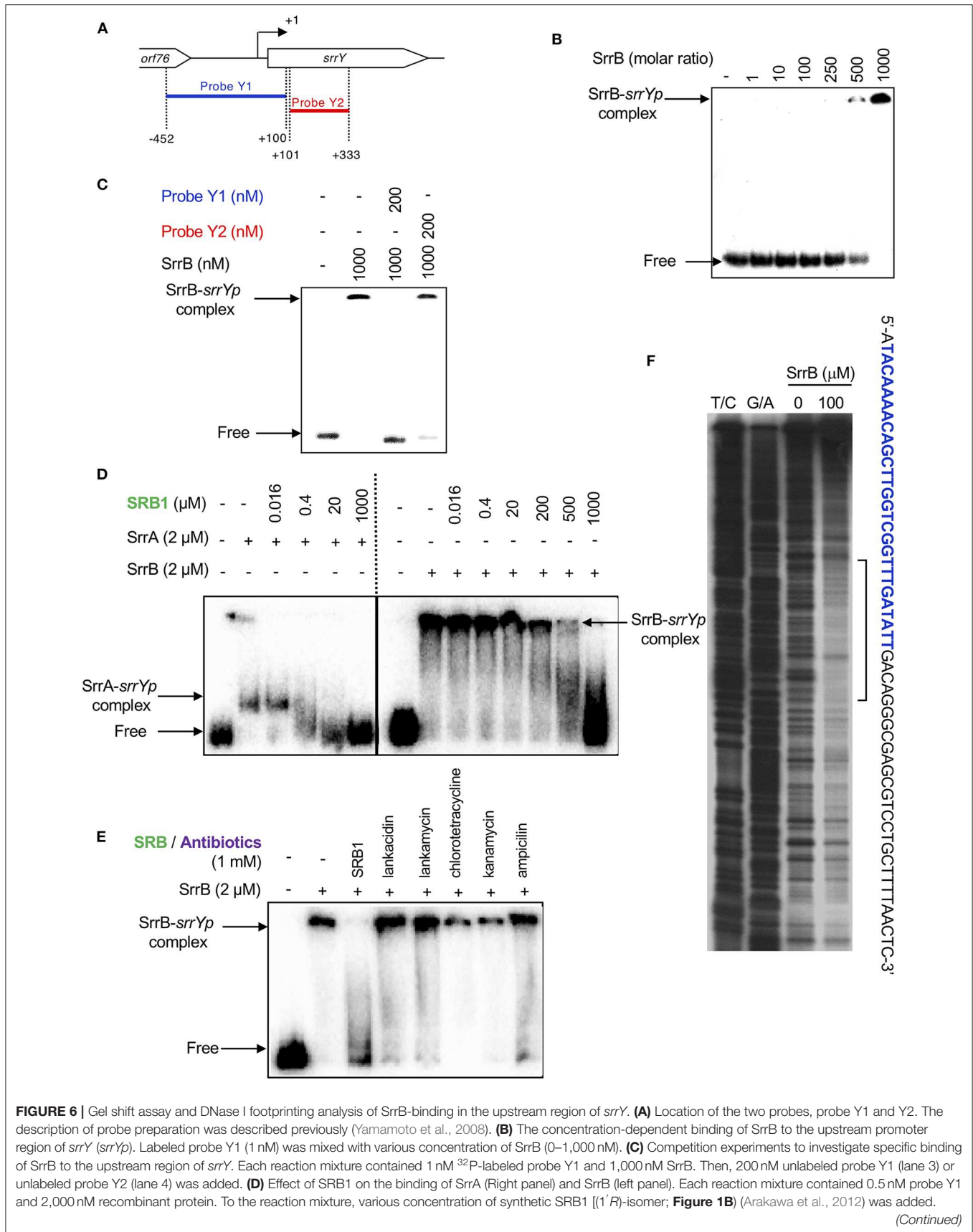
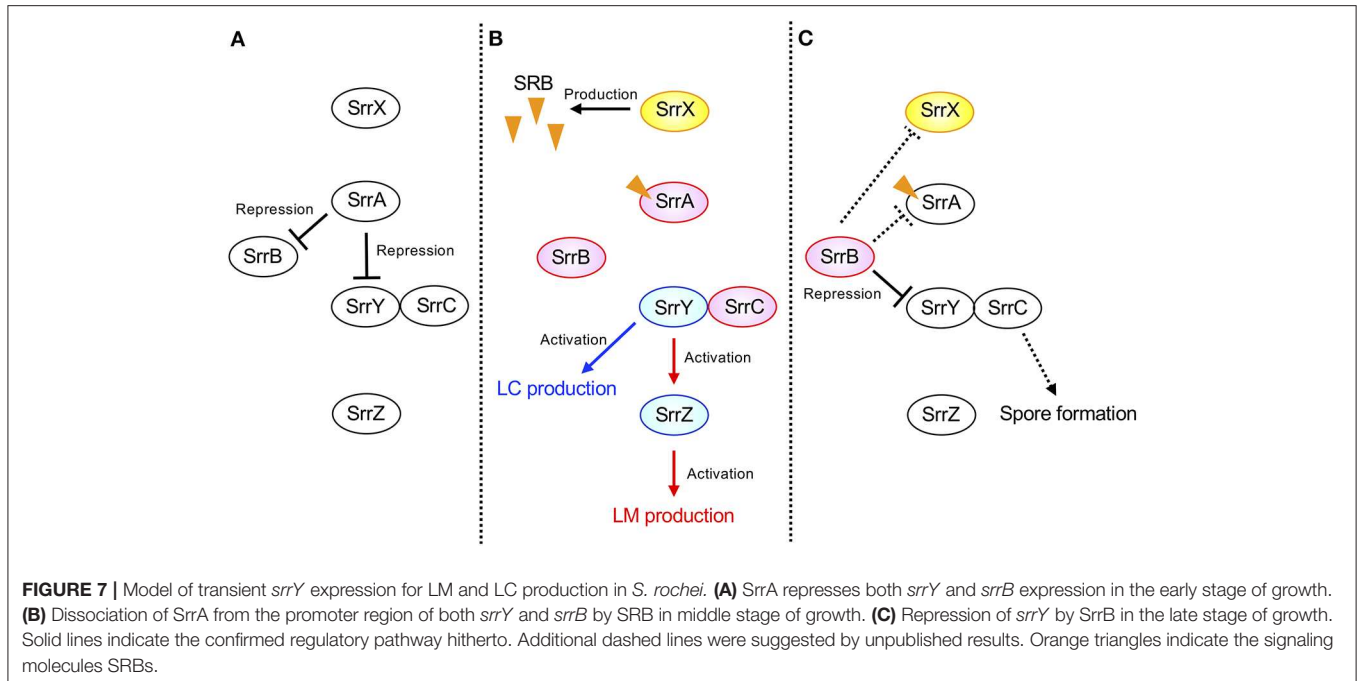


FIGURE 6 | (E) Effect of endogenous metabolites in *S. rochei* and other antibiotics on the binding of SrrB. To the same reaction mixture described for panel D, various compounds including SRB1, LC, LM, chlorotetracycline, kanamycin, and ampicillin (each 1 mM) were separately added. **(F)** DNase I footprint analysis of SrrB-binding site on the upstream of *srrY*. Probe Y1 was end labeled on the non-template strand. Each reaction mixture contained 2 nM labeled DNA and SrrB (100 nM). Sequencing ladders were generated by Maxam-Gilbert sequencing of the labeled probe Y1. Capital letters at right side indicate SrrB-binding sequences, among which blue letters indicate SrrA-binding sequences.



its ligand has not yet been identified. In the early stage of growth, the signaling molecule receptor AlpZ represses both transcription of *alpV* (an SARP-type activator gene) and *alpW* (a pseudo-receptor gene). When an unidentified ligand interacts with AlpZ, this protein dissociates from the promoter regions in both *alpV* and *alpW*, leading to kinamycin production. At the later stage of fermentation, AlpW represses *alpV* transcription again to cease kinamycin production. Another interesting features in the *S. rochei* regulatory pathway is the presence of *srrY-srrC* cistron (Figure 7). The *srrC* mutant showed no sporulation, suggesting that *srrC* acts as a positive regulator for morphological differentiation (Arakawa et al., 2007). As shown in Figure 7, *srrB* negatively regulates the transcription of both *srrY* and *srrC*, which leads to transient controls for antibiotic production and morphological differentiation, respectively.

The *srrB* mutation increased the titers of antibiotics as well as SRBs. This result well agreed with our preliminary gel shift assay that both SrrA and SrrB bind to the upstream region of SRB biosynthesis gene *srrX* (data not shown). Large excess of SRBs has no effect on antibiotic overexpression in *S. rochei* (Arakawa et al., 2012), hence, exact mechanism of the *srrX* repression by SrrB at the later stage remains to be clarified.

Manipulation of regulatory genes often causes activation of “silent” secondary metabolites (Olano et al., 2008; Zerikly and

Challis, 2009; Rutledge and Challis, 2015; Arakawa, 2018). In *S. ambofaciens*, a mutant of the pseudo-receptor gene *alpW* accumulated kinamycin (Bunet et al., 2011). A mutant of the repressor gene *ksbC* accumulated β -carboline compound kitasetaline in *Kitasatospora setae* (Aroonsri et al., 2012). To our surprise, azoxyalkene compound KA57-A accumulated in a triple knockout mutant of *S. rochei* that have mutations on two biosynthetic gene clusters for LC, LM, and *srrB* (Kunitake et al., 2015). The genome sequence of the *S. rochei* chromosome has been determined to be 8.36 Mb in size, and at least 35 secondary metabolites gene clusters are coded on the chromosome (Nindita et al., 2019). Comprehensive mutational analysis on various regulatory genes may lead to activate silent secondary metabolite gene clusters in *S. rochei*, which is in progress in our laboratory. In conclusion, we have extensively characterized the role of the pseudo-receptor SrrB for antibiotic production in *S. rochei*. Further understanding and manipulation of the regulatory system in *Streptomyces* will lead to a natural product discovery with notable biological activities.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

YM, SY, TS, HK, and KA designed the experiments. YM, SY, TS, MI, HS, YT, KI, and KA performed the experiments. YM, SY, TS, MI, HS, and KA analyzed the data. YM, SY, TS, HK, and KA wrote the manuscript with input from all of the authors. All authors approved the final version of the manuscript.

FUNDING

This work was supported by Grants-in-Aid for Scientific Research on Innovative Areas (23108515, 25108718, 17H05446, and 19H04659 to KA) from Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), Grants-in-Aid for Scientific Research (B) (16H04917 to KA) from the Japan

Society for the Promotion of Science (JSPS), and JSPS A3 Foresight Program.

ACKNOWLEDGMENTS

We would like to thank Dr. D. Kajiya and Mrs. T. Amimoto (N-BARD, Hiroshima University) for measurement of the high resolution mass spectra.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.01089/full#supplementary-material>

REFERENCES

- Aigle, B., Pang, X., Decaris, B., and Leblond, P. (2005). Involvement of AlpV, a new member of the Streptomyces antibiotic regulatory protein family, in regulation of the duplicated type II polyketide synthase alp gene cluster in *Streptomyces ambofaciens*. *J. Bacteriol.* 187, 2491–2500. doi: 10.1128/JB.187.7.2491-2500.2005
- Arakawa, K. (2018). Manipulation of metabolic pathways controlled by signaling molecules, inducers of antibiotic production, for genome mining in *Streptomyces* spp. *Antonie. Leeuwen.* 111, 743–751. doi: 10.1007/s10482-018-1052-6
- Arakawa, K., Cao, Z., Suzuki, N., and Kinashi, H. (2011). Isolation, structural elucidation, and biosynthesis of 15-norlankamycin derivatives produced by a type-II thioesterase disruptant of *Streptomyces rochei*. *Tetrahedron* 67, 5199–5205. doi: 10.1016/j.tet.2011.05.047
- Arakawa, K., Mochizuki, S., Yamada, K., Noma, T., and Kinashi, H. (2007). γ -Butyrolactone autoregulator-receptor system involved in lankacidin and lankamycin production and morphological differentiation in *Streptomyces rochei*. *Microbiology* 153, 1817–1827. doi: 10.1099/mic.0.2006/002170-0
- Arakawa, K., Tsuda, N., Taniguchi, A., and Kinashi, H. (2012). The butenolide signaling molecules SRB1 and SRB2 induce lankacidin and lankamycin production in *Streptomyces rochei*. *ChemBioChem* 13, 1447–1457. doi: 10.1002/cbic.201200149
- Aroonsri, A., Kitani, S., Hashimoto, J., Kosone, I., Izumikawa, M., Komatsu, M., et al. (2012). Pleiotropic control of secondary metabolism and morphological development by KsbC, a butyrolactone autoregulator receptor homologue in *Kitasatospora setae*. *Appl. Environ. Microbiol.* 78, 8015–8024. doi: 10.1128/AEM.02355-12
- Bate, N., Butler, A. R., Gandecha, A. R., and Cundliffe, E. (1999). Multiple regulatory genes in the tylosin biosynthetic cluster of *Streptomyces fradiae*. *Chem. Biol.* 6, 617–624. doi: 10.1016/S1074-5521(99)80113-6
- Bate, N., Stratigopoulos, G., and Cundliffe, E. (2002). Differential roles of two SARP-encoding regulatory genes during tylosin biosynthesis. *Mol. Microbiol.* 43, 449–458. doi: 10.1046/j.1365-2958.2002.02756.x
- Biarnes-Carrera, M., Breiting, R., and Takano, E. (2015). Butyrolactone signaling circuits for synthetic biology. *Curr. Opin. Chem. Biol.* 28, 91–98. doi: 10.1016/j.cbpa.2015.06.024
- Bibb, M. J. (2005). Regulation of secondary metabolism in streptomycetes. *Curr. Opin. Microbiol.* 8, 208–215. doi: 10.1016/j.mib.2005.02.016
- Bunet, R., Mendes, M. V., Rouhier, N., Pang, X., Hotel, L., Leblond, P., et al. (2008). Regulation of the synthesis of the angucyclinone antibiotic alpomycin in *Streptomyces ambofaciens* by the autoregulator receptor AlpZ and its specific ligand. *J. Bacteriol.* 190, 3293–3305. doi: 10.1128/JB.01989-07
- Bunet, R., Song, L., Mendes, M. V., Corre, C., Hotel, L., Rouhier, N., et al. (2011). Characterization and manipulation of the pathway-specific late regulator AlpW reveals *Streptomyces ambofaciens* as a new producer of kinamycins. *J. Bacteriol.* 193, 1142–1153. doi: 10.1128/JB.01269-10
- Gottelt, M., Kol, S., Gomez-Escribano, J. P., Bibb, M., and Takano, E. (2010). Deletion of a regulatory gene within the cpk gene cluster reveals novel antibacterial activity in *Streptomyces coelicolor* A3(2). *Microbiology* 156, 2343–2353. doi: 10.1099/mic.0.038281-0
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* 41, 95–98.
- Hara, O., and Beppu, T. (1982). Mutants blocked in streptomycin production in *Streptomyces griseus* - The role of A-factor. *J. Antibiot.* 35, 349–358. doi: 10.7164/antibiotics.35.349
- Horinouchi, S., and Beppu, T. (2007). Hormonal control by A-factor of morphological development and secondary metabolism in streptomycetes. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* 83, 277–295. doi: 10.2183/pjab.83.277
- Hsiao, N. H., Nakayama, S., Merlo, M. E., de Vries, M., Bunet, R., Kitani, S., et al. (2009). Analysis of two additional signaling molecules in *Streptomyces coelicolor* and the development of a butyrolactone-specific reporter system. *Chem. Biol.* 16, 951–960. doi: 10.1016/j.chembiol.2009.08.010
- Ishikawa, J., Niino, Y., and Hotta, K. (1996). Construction of pRES18 and pRES19, *Streptomyces-escherichia coli* shuttle vectors carrying multiple cloning sites. *FEMS Microbiol. Lett.* 145, 113–116. doi: 10.1016/0378-1097(96)0397-7
- Kawauchi, R., Akashi, T., Kamitani, Y., Sy, A., Wangchaisoonthorn, U., Nihira, T., et al. (2000). Identification of an AfsA homologue (BarX) from *Streptomyces virginiae* as a pleiotropic regulator controlling autoregulator biosynthesis, virginiamycin biosynthesis and virginiamycin M1 resistance. *Mol. Microbiol.* 36, 302–313. doi: 10.1046/j.1365-2958.2000.01819.x
- Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000). *Practical Streptomyces Genetic: A Laboratory Manual*. Norwich: John Innes Foundation.
- Kinashi, H., Mori, E., Hatani, A., and Nimi, O. (1994). Isolation and characterization of large linear plasmids from lankacidin-producing *Streptomyces* species. *J. Antibiot.* 47, 1447–1455. doi: 10.7164/antibiotics.47.1447
- Kinoshita, H., Ipposhi, H., Okamoto, S., Nakano, H., Nihira, T., and Yamada, Y. (1997). Butyrolactone autoregulator receptor protein (BarA) as a transcriptional regulator in *Streptomyces virginiae*. *J. Bacteriol.* 179, 6986–6993. doi: 10.1128/JB.179.22.6986-6993.1997
- Kitani, S., Miyamoto, K. T., Takamatsu, S., Herawati, E., Iguchi, H., Nishitomi, K., et al. (2011). Avenolide, a *Streptomyces* hormone controlling antibiotic production in *Streptomyces avermitilis*. *Proc.*

- Natl. Acad. Sci. U.S.A.* 108, 16410–16415. doi: 10.1073/pnas.1113908108
- Kondo, K., Higuchi, Y., Sakuda, S., Nihira, T., and Yamada, Y. (1989). New virginiae butanolides from *Streptomyces virginiae*. *J. Antibiot.* 42, 1873–1876. doi: 10.7164/antibiotics.42.1873
- Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096
- Kunitake, H., Hiramatsu, T., Kinashi, H., and Arakawa, K. (2015). Isolation and biosynthesis of an azoxyalkene compound produced by a multiple gene disruptant of streptomyces rochei. *ChemBioChem* 16, 2237–2243. doi: 10.1002/cbic.201500393
- Lane, D. J. (1991). “16S/23S rRNA sequencing,” in *Nucleic Acid Techniques in Bacterial Systematics*, eds E. Stackebrandt and M. Goodfellow (New York, NY: John Wiley and Sons), 115–175.
- Li, X., Wang, J., Li, S., Ji, J., Wang, W., and Yang, K. (2015). ScbR- and ScbR2-mediated signal transduction networks coordinate complex physiological responses in *Streptomyces coelicolor*. *Sci. Rep.* 5:14831. doi: 10.1038/srep14831
- Martín, J. F., and Liras, P. (2019). Harnessing microbiota interactions to produce bioactive metabolites: communication signals and receptor proteins. *Curr. Opin. Pharmacol.* 48, 8–16. doi: 10.1016/j.coph.2019.02.014
- Matsuno, K., Yamada, Y., Lee, C. K., and Nihira, T. (2004). Identification by gene deletion analysis of barB as a negative regulator controlling an early process of virginiamycin biosynthesis in *Streptomyces virginiae*. *Arch. Microbiol.* 181, 52–59. doi: 10.1007/s00203-003-0625-5
- Mochizuki, S., Hiratsu, K., Suwa, M., Ishii, T., Sugino, F., Yamada, K., et al. (2003). The large linear plasmid pSLA2-L of *Streptomyces rochei* has an unusually condensed gene organization for secondary metabolism. *Mol. Microbiol.* 48, 1501–1510. doi: 10.1046/j.1365-2958.2003.03523.x
- Nindita, Y., Cao, Z., Fauzi, A. A., Teshima, A., Misaki, Y., Muslimin, R., et al. (2019). The genome sequence of *Streptomyces rochei* 7434AN4, which carries a linear chromosome and three characteristic linear plasmids. *Sci. Rep.* 9:10973. doi: 10.1038/s41598-019-47406-y
- Niu, G., Chater, K. F., Tian, Y., Zhang, J., and Tan, H. (2016). Specialized metabolites regulating antibiotic biosynthesis in streptomyces spp. *FEMS Microbiol. Rev.* 40, 554–573. doi: 10.1093/femsre/fuw012
- Ohnishi, Y., Kameyama, S., Onaka, H., and Horinouchi, S. (1999). The A-factor regulatory cascade leading to streptomycin production in streptomyces griseus: identification of a target gene of the A-factor receptor. *Mol. Microbiol.* 34, 102–111. doi: 10.1046/j.1365-2958.1999.01579.x
- Ohnishi, Y., Yamazaki, H., Kato, J. Y., Tomono, A., and Horinouchi, S. (2005). AdpA, a central transcriptional regulator in the A-factor regulatory cascade that leads to morphological development and secondary metabolism in *Streptomyces griseus*. *Biosci. Biotechnol. Biochem.* 69, 431–439. doi: 10.1271/bbb.69.431
- Olano, C., Lombó, F., Méndez, C., and Salas, J. A. (2008). Improving production of bioactive secondary metabolites in actinomycetes by metabolic engineering. *Metab. Eng.* 10, 281–292. doi: 10.1016/j.ymben.2008.07.001
- Onaka, H., Ando, N., Nihira, T., Yamada, Y., Beppu, T., and Horinouchi, S. (1995). Cloning and characterization of the A-factor receptor gene from *Streptomyces griseus*. *J. Bacteriol.* 177, 6083–6092. doi: 10.1128/JB.177.21.6083-6092.1995
- Rutledge, P. J., and Challis, G. L. (2015). Discovery of microbial natural products by activation of silent biosynthetic gene clusters. *Nat. Rev. Microbiol.* 13, 509–523. doi: 10.1038/nrmicro3496
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Stratigopoulos, G., and Cundliffe, E. (2002). Expression analysis of the tylosin biosynthetic gene cluster: pivotal regulatory role of the tylQ product. *Chem. Biol.* 9, 71–78. doi: 10.1016/S1074-5521(01)00095-3
- Sun, J., Kelemen, G. H., Fernandez-Abalos, J. M., and Bibb, M. J. (1999). Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). *Microbiology* 145, 2221–2227. doi: 10.1099/00221287-145-9-2221
- Suzuki, T., Mochizuki, S., Yamamoto, S., Arakawa, K., and Kinashi, H. (2010). Regulation of lankamycin biosynthesis in streptomyces rochei by two SARP genes, srrY and srrZ. *Biosci. Biotechnol. Biochem.* 74, 819–827. doi: 10.1271/bbb.90927
- Takano, E. (2006). γ -Butyrolactones: Streptomyces signaling molecules regulating antibiotic production and differentiation. *Curr. Opin. Microbiol.* 9, 287–294. doi: 10.1016/j.mib.2006.04.003
- Takano, E., Chakaraburty, R., Nihira, T., Yamada, Y., and Bibb, M. J. (2001). A complex role for the γ -butyrolactone SCB1 in regulating antibiotic production in streptomyces coelicolor A3(2). *Mol. Microbiol.* 41, 1015–1028. doi: 10.1046/j.1365-2958.2001.02562.x
- Takano, E., Kinoshita, H., Mersinias, V., Bucca, G., Hotchkiss, G., Nihira, T., et al. (2005). A bacterial hormone (the SCB1) directly controls the expression of a pathway-specific regulatory gene in the cryptic type I polyketide biosynthetic gene cluster of *Streptomyces coelicolor*. *Mol. Microbiol.* 56, 465–479. doi: 10.1111/j.1365-2958.2005.04543.x
- Turner, S., Pryer, K. M., Miao, V. P., and Palmer, J. D. (1999). Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *J. Eukaryot. Microbiol.* 46, 327–338. doi: 10.1111/j.1550-7408.1999.tb04612.x
- Wang, J., Wang, W., Wang, L., Zhang, G., Fan, K., Tan, H., et al. (2011). A novel role of ‘pseudo’ γ -butyrolactone receptors in controlling γ -butyrolactone biosynthesis in streptomyces. *Mol. Microbiol.* 82, 236–250. doi: 10.1111/j.1365-2958.2011.07811.x
- Wang, J. B., Zhang, F., Pu, J. Y., Zhao, J., Zhao, Q. F., and Tang, G. L. (2014). Characterization of AvaR1, an autoregulator receptor that negatively controls avermectins production in a high avermectin-producing strain. *Biotechnol. Lett.* 36, 813–819. doi: 10.1007/s10529-013-1416-y
- Wang, L., Tian, X., Wang, J., Yang, H., Fan, K., Xu, G., et al. (2009). Autoregulation of antibiotic biosynthesis by binding of the end product to an atypical response regulator. *Proc. Natl. Acad. Sci. U.S.A.* 106, 8617–8622. doi: 10.1073/pnas.0900592106
- Wang, L., and Vining, L. C. (2003). Control of growth, secondary metabolism and sporulation in *Streptomyces venezuelae* ISP5230 by jadW1, a member of the afsA family of γ -butyrolactone regulatory genes. *Microbiology* 149, 1991–2004. doi: 10.1099/mic.0.26209-0
- Wang, W., Ji, J., Li, X., Wang, J., Li, S., Pan, G., et al. (2014). Angucyclines as signals modulate the behaviors of *Streptomyces coelicolor*. *Proc. Natl. Acad. Sci. U.S.A.* 111, 5688–5693. doi: 10.1073/pnas.1324253111
- Xu, G., Wang, J., Wang, L., Tian, X., Yang, H., Fan, K., et al. (2010). “Pseudo” γ -butyrolactone receptors respond to antibiotic signals to coordinate antibiotic biosynthesis. *J. Biol. Chem.* 285, 27440–27448. doi: 10.1074/jbc.M110.143081
- Xu, G., and Yang, S. (2019). Regulatory and evolutionary roles of pseudo γ -butyrolactone receptors in antibiotic biosynthesis and resistance. *Appl. Microbiol. Biotechnol.* 103, 9373–9378. doi: 10.1007/s00253-019-10219-0
- Yamamoto, S., He, Y., Arakawa, K., and Kinashi, H. (2008). γ -Butyrolactone-dependent expression of the SARP gene srrY plays a central role in the regulatory cascade leading to lankacidin and lankamycin production in *Streptomyces rochei*. *J. Bacteriol.* 190, 1308–1316. doi: 10.1128/JB.01383-07
- Yamauchi, Y., Nindita, Y., Hara, K., Umeshiro, A., Yabuuchi, Y., Suzuki, T., et al. (2018). Quinoprotein dehydrogenase functions at the final oxidation step of lankacidin biosynthesis in *Streptomyces rochei* 7434AN4. *J. Biosci. Bioeng.* 126, 145–152. doi: 10.1016/j.jbiosc.2018.03.006
- Yang, K., Han, L., and Vining, L. C. (1995). Regulation of jadomycin B production in *Streptomyces venezuelae* ISP5230: involvement of a repressor gene, jadR2. *J. Bacteriol.* 177, 6111–6117. doi: 10.1128/JB.177.21.6111-6117.1995
- Yu, Z., Reichheld, S. E., Savchenko, A., Parkinson, J., and Davidson, A. R. (2010). A comprehensive analysis of structural and sequence conservation in the TetR family transcriptional regulators. *J. Mol. Biol.* 400, 847–864. doi: 10.1016/j.jmb.2010.05.062
- Zerikly, M., and Challis, G. L. (2009). Strategies for the discovery of new natural products by genome mining. *ChemBioChem* 10, 625–633. doi: 10.1002/cbic.200800389

- Zhang, H., Shinkawa, H., Ishikawa, J., Kinashi, H., and Nimi, O. (1997). Improvement of transformation system in *Streptomyces* using a modified regeneration medium. *J. Ferment. Bioeng.* 83, 217–221. doi: 10.1016/S0922-338X(97)80982-8
- Zhu, J., Sun, D., Liu, W., Chen, Z., Li, J., and Wen, Y. (2016). AvaR2, a pseudo γ -butyrolactone receptor homologue from *Streptomyces avermitilis*, is a pleiotropic repressor of avermectin and avenolide biosynthesis and cell growth. *Mol. Microbiol.* 102, 562–578. doi: 10.1111/mmi.13479
- Zou, Z., Du, D., Zhang, Y., Zhang, J., Niu, G., and Tan, H. (2014). A γ -butyrolactone-sensing activator/repressor, JadR3, controls a regulatory mini-network for jadomycin biosynthesis. *Mol. Microbiol.* 94, 490–505. doi: 10.1111/mmi.12752

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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