Regulation of Lankamycin Biosynthesis in *Streptomyces rochei* by Two SARP Genes, *srrY* and *srrZ*

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Transcription and complementation experiments were carried out to analyze the regulatory hierarchy of two *Streptomyces* antibiotic regulatory protein (SARP) genes, *srrY* and *srrZ*, in the γ-butyrolactone (GB)-dependent regulatory cascade in *Streptomyces rochei* 7434AN4. The *srrY* gene was transcribed in the *srrZ* mutant, while the *srrZ* gene was not in the *srrY* mutant. The SrrY protein was specifically bound to the promoter region of *srrZ*, where a possible SARP binding sequence was identified 26 bp upstream of the −10 sequence. Deletion of the repeat sequences from this region abolished its SrrY binding activity. In addition, complementation of *srrZ* restored lankamycin production in the *srrY* mutant. All of these results confirm that the SARP gene *srrY* directly regulates expression of the second SARP gene *srrZ* in a positive manner. This study gave the first confirmation of direct regulation of two SARP genes in the GB-dependent regulatory cascade in *Streptomyces*.

Key words: *Streptomyces*; antibiotic; *Streptomyces* antibiotic regulatory protein (SARP); biosynthesis; regulatory cascade

The gram-positive filamentous bacterial genus *Streptomyces* is characterized by its ability to produce a wide variety of secondary metabolites and by a complex process of morphological differentiation. In many *Streptomyces* species, γ-butyrolactone (GB) signaling molecules control secondary metabolism and morphological differentiation. The best-studied GB molecule is A-factor, an antibiotic regulatory protein (SARP) family regulatory genes. At the absence of A-factor, the A-factor receptor ArpA is a master switch for secondary metabolism in streptomycetes. This family of regulatory proteins is characterized by the presence of an OmpR-like DNA-binding domain, and are typified by ActII-ORF4 for actinorhodin biosynthesis in *Streptomyces coelicolor*, RedD for undecylprodigiosin in *S. coelicolor*, DnrI for daunorubicin in *Streptomyces peucetius*, CcaR for cephamycin and clavulanic acid in *Streptomyces clavuligerus*, and TylS for tylosin in *Streptomyces fradiae*. In particular, the ActII-ORF4 and DnrI proteins have been shown experimentally to bind specifically to the promoter regions of the corresponding biosynthetic genes that contain similar SARP binding sequences.

*Streptomyces rochei* strain 7434AN4 carries three linear plasmids, pSLA2-L, -M, and -S, and produces two structurally unrelated polyketide antibiotics, lankacidin (LC) and lankamycin (LM). This strain carries three additional biosynthetic gene clusters for a cryptic type-II polyketide compound (roc) and a carotenoid (crt), and a carotenoid (orc). Remarkably, many regulatory genes were also identified on pSLA2-L, including a GB synthetic gene (*srrX*), six *tetR*-type repressor genes (*srrA*, *srrB*, *srrC*, *srrD*, *srrE*, and *srrF*), and three SARP family regulatory genes (*srrY*, *srrZ*, and *srrW*).

We have found that *srrX* and *srrA* constitute the GB/receptor system in *S. rochei*. It is noteworthy that *srrX* shows a positive effect on antibiotic production and a negative effect on morphological differentiation. Its receptor gene, *srrA*, reversed both effects of *srrX*. Mutational analysis of the repressor genes, *srrB* and *srrC*, revealed that *srrB* has a negative effect on LC and LM production, and that *srrC* has a positive effect on spore formation. In addition, extensive transcriptional analysis revealed that the SARP gene, *srrY*, is the target of *SrrA*, and that the GB signaling cascade goes from *srrX* through *srrA* to *srrY*, leading to LC and LM production, but the signaling pathway downstream of *srrY* has not been clarified.

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Abbreviations: LM, lankamycin; LC, lankacidin; SARP, *Streptomyces* antibiotic regulatory protein; GB, γ-butyrolactone; TSS, transcriptional start site
In this study, we investigated the relationship between srrY and the second SARP gene srrZ by in vivo transcriptional analysis, gel shift assay, and complementation experiments, the results of which indicated that srrZ is directly controlled by srrY in the regulatory cascade.

Materials and Methods

Bacterial strains, plasmids, and DNA manipulation. S. rochei strain 51252, carrying only linear plasmid pSLA2-L, was used as the parent strain. All the strains and plasmids used in this study are listed in Table 1. Streptomyces strains were grown in YM medium (0.4% yeast extract, 1.0% malt extract, 0.4% 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) or apramycin (25 μg/ml) when necessary. For protoplast preparation and overexpression of the (His)6-SrrY protein, Streptomyces strains were grown in YEME medium. Protoplasts were regenerated on R1M plates. DNA manipulations for E. coli and Streptomyces were performed according to the standard protocols. PCR amplification was done on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) with KOD-Plus-DNA polymerase (Toyobo, Osaka). The oligonucleotides used in this study are listed in Table 2.

RNA preparation. S. rochei strains were cultured at 28 °C for 24 h in 100 ml of YM liquid medium in Sakaguchi flasks. Total RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA), as described previously. Trace amounts of DNA were removed completely with RQ1 RNase-Free DNase (Promega, Madison, WI). The concentration of the purified RNA was determined by UV absorbance at 260 nm.

S1 nuclease protection assay. In the S1 nuclease protection assay, the srrY probe containing a 226-bp fragment (positions −213 to +13 from the TSS of srrY) was amplified using template cosmid A8 and primers, SRRYr4 and SRRYf2, where the asterisk indicates the primer 5'-end labeled with [γ-32P]ATP (GE Healthcare, Buckinghamshire, UK) and T4 polynucleotide kinase (Toyobo). The srrZ probe containing a 510-bp fragment (positions −445 to +65 from the TSS of

![Chemical Structures of Lankamycin (1), Lankacidin C (2), 8-Deoxylankanolide (3), Lankacidinol A (4), Iso-Lankacidinol (5), and Lankacidinol (6).](image)

Me, methyl; Ac, acetyl.

![Gene Organization of the Large Linear Plasmid pSLA2-L.](image)

Each gene is shown as a box above or under the DNA line based on their direction of translation (right or left). The biosynthetic gene clusters for lankacidin (lkc, orf4-orf18), lankamycin (lkm, orf24-orf53), an unknown type-II polyketide compound (roc, orf62-orf70), and carotenoid (crt, orf104-orf110) are indicated by different shadings, and regulatory genes are drawn as black boxes.

In this study, we investigated the relationship between srrY and the second SARP gene srrZ by in vivo transcriptional analysis, gel shift assay, and complementation experiments, the results of which indicated that srrZ is directly controlled by srrY in the regulatory cascade.
Regulation of Lankamycin Biosynthesis in *S. rochei*

### Table 1. Strains and Plasmids Used in This Study

<table>
<thead>
<tr>
<th>Strains/Plasmids</th>
<th>Description</th>
<th>Source/References</th>
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<tbody>
<tr>
<td><strong>Bacterial Strains</strong></td>
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<tr>
<td><em>S. rochei</em> 7434AN4</td>
<td>Wild type (pSLA2-L, M, S)</td>
<td>Ref. 13</td>
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<tr>
<td><em>S. rochei</em> 51252</td>
<td>pSLA2-L</td>
<td>Ref. 13</td>
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<tr>
<td><em>S. rochei</em> KY71</td>
<td>srrZ::kan</td>
<td>Ref. 15</td>
</tr>
<tr>
<td><em>S. rochei</em> KY75</td>
<td>srrY::kan</td>
<td>Ref. 15</td>
</tr>
<tr>
<td><em>S. lividans</em> TK64</td>
<td>Actinorhodin, undecylprodigiosin, spe-2, pro-2, str-6</td>
<td>Ref. 17</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>Cosmid A8</td>
<td>38.9 kb BamHI fragment (nt 106,868–145,771 of pSLA2-L) in SuperCos-I</td>
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<td>pHAR4002</td>
<td>9.2 kb PstI fragment containing srrY in pUC19</td>
<td>Ref. 15</td>
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<tr>
<td>pIJ4123</td>
<td>high-copy-number vector in Streptomyces, inducible ttpA promoter, kan, tsr</td>
<td>Ref. 20</td>
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<tr>
<td>pKAR3058</td>
<td>0.85 kb Ndel-BamHI PCR fragment (primers, ORF75-OE-F and ORF75-OE-R; template, pKAR4002) containing srrY in pIJ4123</td>
<td>This study</td>
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<tr>
<td>pHSA81</td>
<td>Constitutive expression vector in Streptomyces</td>
<td>M. Kobayashi</td>
</tr>
<tr>
<td>pKAR3036H</td>
<td>0.8 kb Ndel-BglII PCR fragment (primers, 4123-Ase-F and ORF75-OE-R2; template, pKAR3058) containing srrY and histidine-tag sequence in pHSA81</td>
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<td>pUC19</td>
<td>Cloning vector; amp</td>
<td>Takara</td>
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<td>pSUmtz</td>
<td>0.3 kb EcoRI-PstI PCR fragment (primers, su71-fw1 and su71-mt-r2; template, cosmid A8) and 0.3 kb BamHI-PstI PCR fragment (primers, su71-rv1 and su71-mt-r2; template, cosmid A8) in pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pIJ8600</td>
<td>Integrative <em>E. coli</em>-Streptomyces shuttle vector, inducible ttpA promoter, apr, tsr</td>
<td>Ref. 22</td>
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<td>pKAR3049</td>
<td>1.2 kb Ndel-BanHI PCR fragment (primers, KAR-750E01 and KAR-750E03; template, pKAR4002) containing srrY in pIJ8600</td>
<td>Ref. 15</td>
</tr>
<tr>
<td>pMOE71</td>
<td>0.85 kb Ndel-XhoI PCR fragment (primers, 71ox_start2 and 71ox_stop2; template, cosmid A8) containing srrY in pIJ8600</td>
<td>This study</td>
</tr>
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### Table 2. Oligonucleotides Used in This Study

<table>
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<th>Primer name</th>
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<tr>
<td>SRRY1</td>
<td>CTCCCCCTGTGCTGCTGCGAG</td>
</tr>
<tr>
<td>SRRY4</td>
<td>GGCGCCGCGGCCTACCGGAGA</td>
</tr>
<tr>
<td>SRRZ2</td>
<td>CTCTCAGGATGCGCGCGACAC</td>
</tr>
<tr>
<td>SRRZ3</td>
<td>GAGATGGTCCTCGCTGCGAC</td>
</tr>
<tr>
<td>ORF75-OE-F</td>
<td>ATCTGAGCTCTATGACGATCGA</td>
</tr>
<tr>
<td>ORF75-OE-R</td>
<td>TCTAGAATCTCACGGCAGCAG</td>
</tr>
<tr>
<td>pSUmtZ</td>
<td>0.3 kb EcoRI-PstI PCR fragment (primers, su71-fw1 and su71-mt-r2; template, cosmid A8) and 0.3 kb BamHI-PstI PCR fragment (primers, su71-rv1 and su71-mt-r2; template, cosmid A8) in pUC19</td>
</tr>
<tr>
<td>pIJ8600</td>
<td>Integrative <em>E. coli</em>-Streptomyces shuttle vector, inducible ttpA promoter, apr, tsr</td>
</tr>
<tr>
<td>pKAR3049</td>
<td>1.2 kb Ndel-BanHI PCR fragment (primers, KAR-750E01 and KAR-750E03; template, pKAR4002) containing srrY in pIJ8600</td>
</tr>
<tr>
<td>pMOE71</td>
<td>0.85 kb Ndel-XhoI PCR fragment (primers, 71ox_start2 and 71ox_stop2; template, cosmid A8) containing srrY in pIJ8600</td>
</tr>
</tbody>
</table>

srrZ was similarly amplified using primers, SRRZ3* and SRRZ3*. The methods for the S1 nucleosome protection assay were described previously. (25) Sequencing ladders were generated by T7 Sequencing Kit (USB Corporation, Cleveland, OH).

Preparation of the (His)_6-SrrY protein. The srrY coding sequence was PCR amplified using the template cosmids A8 and primers, ORF75-OE-F and ORF75-OE-R. The amplified DNA fragment was digested with *NdeI* and *BamHI* and cloned into pIJ4123, a thiostrepton-inducible Streptomyces vector, (20) to obtain pKAR3058. Using pKAR3058 as template DNA, srrY carrying an N-terminal histidine-tag sequence was PCR amplified with primers, 4123-Ase-F and ORF75-OE-R. The amplified fragment was digested with *Asel* and *BglII*, and the result was cloned into the *NdeI* and *BamHI* restriction sites in pHS81, a constitutive expression vector, to obtain pKAR3063H. *Streptomyces lividans* TK64 harboring pKAR3063H, was cultivated in YE medium containing 10 μg/ml of thiostrepton at 28°C for 4 d. The (His)_6-SrrY protein was affinity purified with Ni²⁺-nitrilotriacetic acid agarose (Qiagen GmbH, Hilden, Germany). The protein was analyzed by SDS–PAGE with 15% polyacrylamide gel. The protein concentration was determined according to the method of Bradford, (21) with bovine serum albumin as standard.

Preparation of DNA probes and gel shift assay. A 466-bp DNA fragment containing the upstream region of srrZ was amplified using the template cosmids A8 and primers, TS-71fw and orf71/72-r. The PCR product was digested with *BglII* and gel-purified to give a 336-bp DNA fragment Z1 (positions –274 to +62 from the TSS of srrZ). A 459-bp DNA fragment containing the internal region of srrZ was amplified using cosmids A8 and primers, srrZ-internal-f and srrZ-internal-r. The PCR product was digested with *AprI* to give a 331-bp DNA fragment Z2 (positions +289 to +620). A 304-bp PCR fragment was amplified using cosmids A8 and primers, su71-rv1 and su71-mt-r2. A 293-bp DNA fragment was amplified using cosmids A8 and primers, su71-rv1 and su71-mt-r2. The former fragment was digested with *EcoRI* and *PstI*, and the latter with *BamHI* and *PstI*. The two fragments obtained were cloned together into pUC19 predigested with *EcoRI* and *PstI* to give pSUmtZ. Using pSUmtZ as a template, a 459-bp DNA fragment was amplified with primers, TS-71fw and su71-rv1. The PCR product was digested with *BglII* to give the 252-bp DNA fragment Z3, in which the direct repeat sequence (positions –108 to –24) was deleted from fragment Z1. DNA fragments Z1–Z3 were 3’-end labeled with [α-32P]dCTP (Perkin Elmer, Waltham, MA) and Klenow fragment (Takara, Kyoto) for gel shift assay.

The binding reaction mixture (20 μl) contained 1 mΜ labeled DNA and 1 μg (His)_6-SrrY in the binding buffer (20 mΜ Tris–HCl pH 8.0, 100 mΜ NaCl, 10 mΜ MgCl₂, 1 mΜ dithiothreitol, 0.1 mΜ of bovine serum albumin, and 5% glycerol). In the competition assay, unlabeled DNA competitor Z1 was added to the reaction mixture at a final concentration of 25 mΜ. The reaction mixture was incubated for 30 min at 25°C and subjected to electrophoresis at room temperature on a native 4.5% polyacrylamide gel in 0.5 × TBE buffer (46 mΜ Tris base, 46 mΜ boric acid, 1 mΜ EDTA). Labeled DNA was detected by autoradiography.

Complementation experiments. The srrY expression plasmid pKAR3049 was constructed previously. (15) The srrZ gene was amplified using cosmids A8 and primers, 71ox_start2 and 71ox_stop2. The resulting PCR product was digested with *NdeI* and *XhoI* and cloned into pIJ8600, an *E. coli*-Streptomyces shuttle vector carrying a ttpA promoter, (22) to obtain pMOE71.
Strains KY75 (srrY::kan) and KY71 (srrZ::kan) were transformed with pKAR3049 or pMOE71 in all of the four combinations. Transformants were cultured for 24 h at 28 °C in YM liquid medium with 25 μg/ml of apramycin, and then thiostrepton was added to induce srrY or srrZ expression. After cultivation for an additional 48 h, the broth filtrate was extracted twice with equal volumes of ethyl acetate. The combined organic phase was dried with Na2SO4, and concentrated in vacuo to obtain crude extracts.

Isolation of metabolites and structural analysis. The crude extracts were purified by Sephadex LH-20 chromatography (1 × 40 cm. GE Healthcare) with methanol, and then by silica gel chromatography with chloroform-methanol (80:1 to 10:1, v/v). NMR spectra were recorded on a JEOL LA-500 spectrometer equipped with a field gradient accessory. Chloroform- and methanol-d4 were used as solvents. Chemical shifts were recorded in δ value based on the solvent signals (δc = 77.0 in CDCl3, δc = 49.0 in CD3OD, and δh = 3.30 in residual CH3OH) or an internal standard, tetramethylsilane (δh = 0). High-resolution positive-FAB-MS spectra were measured on a JEOL SX-102A mass spectrometer.


Results

The SARP gene srrY positively regulates the second SARP gene srrZ.

We have constructed disruptants of the regulatory genes coded on linear plasmid pSLA2-L, including the three SARP genes, srrY, srrZ and srrW, and orf3 (named srrR), a homolog of the pathway-specific activator gene strR for streptomycin biosynthesis in S. griseus. Strain KY75 (srrY mutant) did not produce LM or LC, strain KY71 (srrZ mutant) produced only LC, and strain TK01 (srrW mutant) and strain FS3 (srrR mutant) produced both LC and LM.15,21 These results indicate that srrY is involved in the production of LC and LM, and srrZ in the production of LM, and that srrW and srrR are not involved in either antibiotic production. In addition, it was also possible that srrZ functions under the control of srrY in the regulatory cascade.

To examine the regulatory hierarchy of the two SARP genes srrY and srrZ, we carried out transcriptional analysis by low-resolution S1 nuclelease protection assay. Total RNAs were isolated from mutants, KY75 and KY71, and from the parent strain 51252 at 24 h of growth in YM liquid medium. As shown in Fig. 3, srrY mRNA was detected in the srrZ mutant at a level comparable to the parent strain 51252, suggesting that the transcription of srrY was not affected by the srrZ mutation. On the other hand, srrZ mRNA was undetectable in the srrY mutant. In both cases, the mRNAs of the disrupted genes were detected, because the kanamycin resistance cassette was inserted into the middle of the genes. These results indicate that expression of the second SARP gene srrZ is positively regulated by srrY, a target gene of the GB receptor SrrA.

Characterization of the promoter region of srrZ.
To analyze the interaction between the SrrY protein and the promoter region of srrZ, first we determined the transcriptional start site (TSS) of srrZ by high-resolution S1 nuclelease protection assay, and identified it 52 bp upstream of the translational start codon of srrZ (Fig. 4A). Immediately upstream of the TSS, a possible −10 sequence (TACGGT) was identified (Fig. 4B). It was similar to the consensus −10 sequence (TAGRRT, R = A or G) of streptomyces Esr2-like promoters.24 However, the −35 sequence (consensus TTGACR) was not found at the corresponding region of the TSS of srrZ.

It has been determined that the SARP family activators ActII-ORF4 and DnrI are specifically bound to regions containing heptameric direct repeats, usually located 8 bp upstream of the −10 sequence (Fig. 4C).11,13 As shown in Fig. 4B, heptameric direct repeats (DR1, DR2, and DR3) with 4-bp spacers (TGGAGTG-GGGC-TCAACG-GCGG-TGGAGGT) were identified 26 bp upstream of the −10 sequence of srrZ. Although the repeat sequences were located farther than usual from the TSS, their pitch (11 bp) exactly coincided with the helix conformation of DNA, which confirms binding of the SARP protein SrrY to this region.

SrrY specifically bound to heptameric direct repeats upstream of srrZ.

The srrY gene was cloned into various E. coli expression vectors to obtain purified SrrY protein suitable for gel shift assay, but no soluble protein was obtained from E. coli, although a number of expression vectors and culture conditions were tested. Hence, the srrY gene was cloned into streptomycete expression vectors, including pJl412, a thiostrepton-inducible vector,20 and pHSA81, a constitutive expression vector (M. Kobayashi, personal communication), and overexpressed in S. lividans TK64. Among these, the recombinant harboring a pHSA81 derivative, pKAR3063H, produced N-terminal His-tagged SrrY as a soluble protein. As shown in Fig. 5A, the (His)6-SrrY protein was purified to homogeneity by Ni2+ affinity chromatography.

To test whether the purified SrrY protein would interact with the promoter of srrZ, a gel shift assay was performed. When 32P-labeled probe Z1 (Fig. 5B, positions −274 to +62 from the TSS of srrZ) was used, a
shifted band appeared with the addition of SrrY (Fig. 5C, lane 2). The binding specificity was examined by a competition experiment using unlabeled DNA as competitor. The shifted band disappeared with the addition of unlabeled probe Z1 (Fig. 5C, lane 3). When probe Z2, containing the srrZ internal region (Fig. 5B, positions +289 to +620), was used, the shifted band was not observed (Fig. 5C, lane 4). These results indicate that SrrY was bound specifically to the promoter of srrZ.

To test the role of the direct repeats upstream of srrZ, we constructed probe Z3 (Fig. 5B), in which the direct repeat sequence (positions /C0+108 to /C0+24) was deleted. The deleted probe, Z3, apparently lost SrrY-binding activity (Fig. 5C, lane 5), indicating that heptameric direct repeats with 4-bp spacers are the target for SrrY binding, which in turn can activate the second SARP gene srrZ, leading to LM production.

Effects of srrY and srrZ overexpression on antibiotic production in the srrY and srrZ mutants

The hierarchy of srrY and srrZ was confirmed by the analysis of gene expression in the mutants and the specific binding of SrrY to the promoter of srrZ. To examine this hierarchy in metabolite production, we analyzed the effects of overexpression of srrY and srrZ in the srrY and srrZ mutants in all of the four combinations. Both the srrY and srrZ genes were cloned into plasmid pIJ8600 under the control of a thiostrepton-inducible tipA promoter.22 First, we analyzed the effects of overexpression of srrY and srrZ in the original mutants. The introduction of srrY into the srrY mutant restored both LC (2) and LM (1) production (Fig. 6, lane 6), and srrZ restored LM production in the srrZ mutant (Fig. 6, lane 4). The latter recombinant accumulated LM at a higher yield than the parent strain, 51252. These results further confirm that SrrY positively regulates both LC and LM production, and that SrrZ positively regulates LM production.

Next, the effects of overexpression of srrY and srrZ were analyzed by cross combinations. The expression of srrY in the srrZ mutant had no effect on LM production, although the yield of LC much increased (Fig. 6, lane 3). On the other hand, the expression of srrZ in the srrY mutant restored LM production in some extent (Fig. 6,
The disruptant of the arcanosyl transferase gene, lkmL, Rf value, this compound was identified as 8-deoxylan-
2
baked after spraying with anisaldehyde-H
3
lane 7), confirming the regulatory pathway from srrY to srrZ. To our surprise, the latter recombinant
accumulated four additional metabolites that are not
usually produced by strain 51252. These four metabo-
lites (3–6) were isolated and elucidated by MS and NMR
analysis.

Since compound 3 showed a green spot on TLC
(Rf = 0.40, solvent; CHCl3–MeOH = 20 : 1) when
baked after spraying with anisaldehyde-H2SO4, it is
not seen well in Fig. 6 (lane 7). The molecular formula
of 3 was determined to be C25H14O7 by HR-FAB-MS
measurement. Based on the NMR data of 3 as well as its
Rf value, this compound was identified as 8-deoxylan-
kanolide (3, Fig. 1), which was previously isolated from
the disruptant of the arcanosyl transferase gene, lkmL.25)

Compounds 4–6 showed violet spots on TLC
(Rf = 0.36, 0.24, 0.20 in CHCl3–MeOH = 20 : 1) when
baked with anisaldehyde-H2SO4. The assignments of
1H- and 13C-NMR are summarized in Supplemental
Table S1. All the connectivities of protons and carbons
were established by COSY, HMQC, and HMBC spectra.
The spectral data for compound 4 (molecular formula,
C25H37NO9) completely agreed with those of lankaci-
dinol A (4, Fig. 1), a minor metabolite of S. rochei
7434AN4.25) Compound 6 showed a molecular formula
of C28H35NO7, 42 mass units smaller than lankacidinol
A (4), suggesting hydrolysis of the C-7 acetoxy group.
Consistently with this, the H-7 proton signal was
upfield-shifted from δ 5.43 to δ 4.20. The other signals
of 6 were almost identical to those of lankacidinol A (4).
From these data, compound 6 was determined to be
lankacidinol (6, Fig. 1), which was previously isolated
from S. rochei var. volubilis.26)

The molecular formula (C25H18NO7) of compound 5
was identical to that of lankacidinol (6). All of the
proton signals of 6 were observed in 5. However, the
H-5 proton signal was upfield-shifted from δ 4.54 to
δ 3.59, and the H-4 signal was downfield-shifted from
δ 2.39 to δ 3.03. Based on these data, compound 5 was
deduced to be iso-lankacidinol (5, Fig. 1),27) a C-5 epimer
of lankacidinol (6). The accumulation of these
metabolites suggests that overexpression of srrZ acti-
vates some of the lankacidin biosynthetic genes in place
of srrY, leading to synthesis of the lankacidin skeleton.

Discussion

In this study we carried out transcription and comple-
mentation experiments to analyze the regulatory
hierarchy of two SARP genes, srrY and srrZ, in the GB-
dependent regulatory cascade in S. rochei. The srrY
gene was transcribed in the srrZ mutant, while the srrZ
The downstream genes regulated by srrZ have not been identified, because comprehensive RT-PCR analysis was difficult due to low expression of the biosynthetic structural genes. However, preliminary analysis showed that in the srrZ mutant, the expression of lkmAI, the first PKS gene to synthesize the lankamycin skeleton, was not detected (unpublished results). In addition, overexpression of srrZ in the srrY mutant accumulated aglycon 8-deoxylankanolide (3) without post-PKS modifications including two glycosylation steps (Fig. 6, lane 7). Based on these results, we speculate that srrZ positively regulates lkmAl expression and therefore the lkmAl gene was tentatively located below srrZ in this cascade.

Of many GB-dependent regulatory cascades, the A-factor regulatory cascade in S. griseus has been the most extensively studied. In this cascade, disruption of A-factor receptor gene arpA accelerated both streptomycin production and spore formation, because its target gene adpA activates many genes involved in antibiotic production and morphological differentiation, making the huge AdpA regulon. However, the effects of disruption of the GB receptor genes are different from species to species. This reflects the diversity of the target regulatory genes as well as their downstream genes in Streptomyces, but the complete GB-dependent regulatory cascade ending at antibiotic production has not been clarified in any case except for that of A-factor.

The regulatory genes involved in typical GB-dependent regulatory cascades in Streptomyces are listed in Table 3. In several cases, second tetR-type repressor genes are involved, for example, barB in Streptomyces virginiae, tylQ in S. fradiae, alpW in Streptomyces ambofaciens, and srrB in S. rochei. Differently from the low pI value characteristic of GB receptors, the second repressors have a high pI value. Accumulated data indicate that transcription of second repressor genes occurred at a late stage of growth, their disruption increased antibiotic production, and the GB receptors were bound to their promoter region. In addition, the second repressors in turn were bound to the promoter of the same target gene of the GB receptors. Consequently, a model was proposed to explain transient antibiotic production in Streptomyces: antibiotic production is repressed by the GB receptor at the early stage, is derepressed by the GB molecule at the middle stage, and

![Fig. 6. Effects of srrY and srrZ Overexpression on Antibiotic Production in the srrY and srrZ Mutants.](image)

Crude extracts of the cultured broths of the recombinants were analyzed by TLC. Lane 1, S. rochei 51252 (parent); lane 2, KY71 (srrZ::kan) with pD6600 (control); lane 3, KY71 with pKAR3049 (intact srrY); lane 4, KY71 with pMOE71 (intact srrZ); lane 5, KY75 (srrY::kan) with pD6600; lane 6, KY75 with pKAR3049; lane 7, KY75 with pMOE71.

![Fig. 7. Postulated γ-Butyrolactone-dependent Regulatory Cascade for LC and LM Production in S. rochei.](image)

The signaling pathways, confirmed previously and in this study, are shown by solid lines. The pathways drawn by broken lines have not been confirmed, but are suggested by unpublished and preliminary results. →, activation; ←, inhibition.
is repressed again by the second repressor at the late stage.30) However, the GB synthesis gene has not been identified in the tyl cascade in S. fraudiae or the alp cascade in S. ambofaciens. Moreover, the resistance of the GB fraction to alkaline treatment suggests that an autoregulator without a γ-butyrolactone structure is involved in the alp regulatory system.30) In fact, signaling molecules with a furan ring were isolated from S. coelicolor, which induced methylenomycin production in place of GB molecules.31) All of these results again indicate the diversity of regulatory systems for secondary metabolism in Streptomyces.

Leading studies of the interaction between the SARP family activators, ActII-ORF411 and DnrI,12) with their target genes have identified heptameric direct repeats with 4-bp spacers 8 bp upstream of the −10 sequence. In addition, extensive mutational analysis of the AfsR-afsS system in S. coelicolor indicates that the 8-bp distance from the −10 sequence is critical to make a ternary complex of the promoter of afsS/the SARP AfsR/RNA polymerase.32) The upstream region of srrZ also contains three heptameric direct repeats (DR1, DR2, and DR3), which are similar to the consensus sequence (5’TTCAGGT-3’). However, their distance from the −10 sequence is farther (26 bp) than usual, which suggests that the 8-bp distance is not always essential for binding of SARP activators.

The SARP genes are usually located inside the antibiotic biosynthetic clusters, which help efficient regulation of their own cluster. In contrast, the two SARP genes, srrY and srrZ, together with other regulatory genes (srrX, srrA, srrB, and srrC), all of which are located on linear plasmid pSLA2-L, constitute a regulatory cluster far from the lkm and lkc clusters (Fig. 2).14) Such organization might have been generated on pSLA2-L by horizontal transfer and rearrangement in evolutionary history under pressure to efficiently regulate multiple gene clusters.

Overexpression of srrZ in the srrY mutant restored LM production in some extent. In addition, this recombinant accumulated lankamycin aglycon 8-deoxyxylanolide (3) and three possible biosynthetic intermediates of lankacidin C (2): lankacidinol A (4), iso-lankacidinol (5), and lankacidinol (6) (Fig. 6, lane 7). As described above, we speculate that srrZ positively regulates the expression of lkmAI, a PKS gene for lankamycin synthesis. Accumulation of 8-deoxyxylanolide (3) in this recombinant suggests that srrZ activates PKS genes rather than post-PKS modifying enzyme genes. The latter genes might be activated by the SrrY protein directly or indirectly. In this connection, we identified repeat sequences with 1-bp spacers (GGGATTG-CCTAGCCG-TGAGCTT) 47 bp upstream of the translational start codon of lkmAI, the role of which should be analyzed.

A similar mechanism might work in lankacidin biosynthesis. Overexpression of srrZ in the srrY mutant accumulated three possible intermediates (4, 5, 6) of lankacidin biosynthesis. Therefore, SrrZ can substitute for SrrY to activate PKS genes for the lankacidin skeleton, but cannot do so to activate modifying enzyme genes necessary for the final steps to convert lankacidinol A (4) to lankacidin C (2). On the other hand, overexpression of srrY in the srrZ mutant failed to restore LM production (Fig. 6, lane 3), indicating that SrrY cannot substitute for SrrZ.

To our knowledge, this study gives the first evidence of direct regulation of two SARP genes (srrY and srrZ) in the GB-dependent regulatory cascade in Streptomyces. To determine the entire regulatory cascade for antibiotic production and spore formation in S. rochei 7434AN4, transcriptional analysis of other regulatory genes as well as structural genes is in progress. The results will be published in due course.

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References

Regulation of Lankamycin Biosynthesis in *S. rochei*