**INTRODUCTION**

The filamentous Gram-positive soil bacteria of the genus *Streptomyces* are characterized by three distinct properties: linear chromosome, complex morphological differentiation and ability to produce varieties of secondary metabolites including antibiotics. Antibiotic biosynthetic genes in *Streptomyces* species usually form a condensed gene cluster on the chromosome. However, several giant linear plasmids encoding an antibiotic gene cluster(s) have been isolated: SCP1 in *Streptomyces coelicolor* A3(2) carries the biosynthetic gene cluster for methylenomycin (Bentley et al., 2004; Chater & Bruton, 1985; Kinashi et al., 1987; Redenbach et al., 1998), pPZG103 in *Streptomyces rimosus* has that for oxytetracycline (Gravius et al., 1999; Pandza et al., 1998) and pSLA2-L in *Streptomyces rochei* has those for lankacidin and lankamycin (Arakawa et al., 2005, 2006; Mochizuki et al., 2003; Suwa et al., 2000).

*S. rochei* strain 7434AN4 contains three large linear plasmids (pSLA2-L, -M and -S) (Kinashi et al., 1994, 1998) and produces two structurally unrelated polyketide antibiotics, the 17-membered macrolide lankacidin and the 14-membered macrocyclic lankamycin (Fig. 1a). We have been studying the function of the largest linear plasmid pSLA2-L in antibiotic production, and finally determined its 210,614 bp nucleotide sequence (Mochizuki et al., 2003). It was revealed that pSLA2-L contains an unusually condensed gene organization for secondary metabolism (Fig. 1b); two type I PKS gene clusters for lankacidin (*lkc*, *orf14–orf18*) and lankamycin (*lkm*, *orf24–orf53*), a cryptic type II PKS gene cluster (*roc*, *orf62–orf70*) and a carotenoid biosynthetic gene cluster (*crt*, *orf104–orf110*). In addition, many regulatory genes were identified on pSLA2-L, including all the homologues in the A-factor regulatory cascade in *Streptomyces griseus* (Horinouchi, 2002; Ohnishi et al., 1999). Namely, *orf85*, *orf116* and *orf3* are similar to *afsA* (Horinouchi et al., 1984; Lezhava et al., 1997), *adpA* (Ohnishi et al., 2005) and *strR* (Beyer et al., 1996), respectively. Six *tetR* family receptor genes (Ramos et al., 2005) were also found on pSLA2-L, three of which have considerable similarity to the γ-butyrolactone receptor gene, *arpA*, in *S. griseus* (Onaka et al., 1995).

A γ-butyrolactone autoregulator-receptor system is well known to regulate antibiotic production and/or morphological differentiation in streptomycetes (Bibb, 2005;...
In particular, the A-factor regulatory system has been extensively studied and its hierarchical regulatory cascade was proposed (Ohnishi et al., 1999). afxA is involved in the synthesis of A-factor, a \( \gamma \)-butyrolactone autoregulator (Hara & Beppu, 1982; Khokhlov et al., 1973), which binds to ArpA, a repressor-type receptor, and releases it from the promoter region of \( \text{adpA} \). The derepressed and produced AdpA protein, a global transcriptional activator, in turn binds to the promoter of \( \text{strR} \), a positive regulator gene for streptomycin biosynthesis. Search for additional AdpA-binding DNA sequences identified an ECF sigma factor gene (Yamazaki et al., 2000), a metalloendopeptidase gene (Kato et al., 2002) and many additional genes, which make an AdpA regulon functioning for secondary metabolism and morphological development (Ohnishi et al., 2005). Following the \( \text{afsA-arpA} \) system, similar \( \gamma \)-butyrolactone receptor systems have been found in streptomycetes, for example, the \( \text{barX-barA} \) system in \( \text{Streptomyces virginiae} \) (Kawachi et al., 2000), \( \text{farX-farA} \) in \( \text{Streptomyces lavendulae} \) (Kitani et al., 2001) and \( \text{scbA-scbR} \) in \( \text{S. coelicolor} \) A3(2) (Takano et al., 2001). However, their effects on antibiotic production and morphological differentiation are different from species to species.

The presence of many regulatory genes on pSLA2-L suggests that a similar \( \gamma \)-butyrolactone autoregulator-receptor system may also function in \( \text{S. rochei} \) 7434AN4.

In this study, we constructed single mutants of the \( \text{afsA} \) homologue \( \text{srrX} \) and three receptor gene homologues, \( \text{orf74} \) (\( \text{srrC} \)), \( \text{orf79} \) (\( \text{srrB} \)) and \( \text{orf82} \) (\( \text{srrA} \)), and double mutants with a combination of \( \text{srrX} \) and each of three receptor gene homologues. The antibiotic productivity and mycelium morphology of these mutants revealed complex functions of the \( \gamma \)-butyrolactone receptor system in \( \text{S. rochei} \) 7434AN4. The construction and phenotype of the \( \text{srrX} \) mutant KY85 were preliminarily reported previously (Mochizuki et al., 2003).

**METHODS**

**Bacterial strains, media and DNA manipulation.** \( \text{Streptomyces rochei} \) wild-type strain 7434AN4, strain 51252, which carries only pSLA2-L, and strain 3-44, which carries no plasmids, were described previously (Kinashi et al., 1994). Regulatory mutants were constructed as described below and are listed in Table 1. (A full list of strains and plasmids is given in Table S1, available as supplementary data with the online version of this paper.) YM medium (0.4 % yeast extract, 1.0 % malt extract and 0.4 % glucose, pH 7.3) was used for antibiotic production and spore formation, and TSB medium (Tryptic Soy Broth, 30 g l\(^{-1}\)) for bioassay. For spore formation, \( \text{S. rochei} \) strains were grown on YM agar plates at 28 °C for 7 days.

DNA manipulations for \( \text{Streptomyces} \) (Kieser et al., 2000) and \( \text{Escherichia coli} \) (Sambrook et al., 1989) were carried out according to standard procedures. For DNA isolation, \( \text{Streptomyces} \) strains were

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**Fig. 1.** Chemical structures of lankacidin C and lankamycin (a) and gene organization of the linear plasmid pSLA2-L (b). 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, \( \text{orf4-orf18} \)), lankamycin (lkM, \( \text{orf24-orf53} \)), an unknown type-II polyketide compound (roc, \( \text{orf62-orf70} \)) and carotenoid (crt, \( \text{orf104-orf110} \)) are indicated by different shadings, and regulatory genes are drawn as black boxes.
Table 1. *Streptomyces rochei* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Properties</th>
<th>Source/ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>7434AN4</td>
<td>pSLA2-L⁺, pSLA2-M⁺, pSLA2-S⁺</td>
<td>Kinashi et al. (1994)</td>
</tr>
<tr>
<td>51252</td>
<td>pSLA2-L⁺, pSLA2-M⁺, pSLA2-S⁺</td>
<td>Kinashi et al. (1994)</td>
</tr>
<tr>
<td>3-44</td>
<td>pSLA2-L⁺, pSLA2-M⁺, pSLA2-S⁺</td>
<td>Kinashi et al. (1994)</td>
</tr>
<tr>
<td>KY85</td>
<td>srrX (orf85) : kan</td>
<td>Mochizuki et al. (2003)</td>
</tr>
<tr>
<td>KA07</td>
<td>499 bp BstWI fragment containing srrB (orf79) deleted</td>
<td>This study</td>
</tr>
<tr>
<td>KA12</td>
<td>207 bp BspEI fragment containing srrA (orf82) deleted</td>
<td>This study</td>
</tr>
<tr>
<td>KA16</td>
<td>EcoNI site eliminated from srrC (orf74) (filled in)</td>
<td>This study</td>
</tr>
<tr>
<td>KA20</td>
<td>srrX : kan 499 bp BstWI fragment containing srrB deleted</td>
<td>This study</td>
</tr>
<tr>
<td>KA21</td>
<td>srrX : kan 207 bp BspEI fragment containing srrA deleted</td>
<td>This study</td>
</tr>
<tr>
<td>KA22</td>
<td>srrX : kan EcoNI site eliminated from srrC (filled in)</td>
<td>This study</td>
</tr>
</tbody>
</table>

Disruption of the srrX, srrA, srrB and srrC genes

Disruption of srrX (orf85). The targeting plasmid pKY85-3 for srrX disruption was constructed as follows, and its structure is shown in the supplementary data available with the online version of this paper. A 2.5 kb PvuIII–EcoRI fragment containing the srrX gene was cloned into pUC19 predigested with Smal and EcoRI to give pKY85-1. Into the NrdI site in the middle of srrX (nt 146102 of pSLA2-L) was inserted a 1.2 kb Smal fragment containing a kanamycin resistance gene cassette from pUC4-KIXX (Barany, 1985) to give pKY85-2. The vector part of pKY85-2 was replaced by pRES18, an *E. coli* Streptomyces shuttle vector (Ishikawa et al., 1996), to afford a targeting plasmid, pKY85-3 (Fig. S1 in supplementary data).

Disruption of srrA (orf82). A 3.2 kb Ncol–SacI fragment containing the srrA gene was cloned into pSET-B (Invitrogen) to give pKAR3001. This plasmid was digested with BamHI and EcoRI, and the insert was recloned into pUC19 to give pKAR3012. This plasmid was digested with BspEI and self-ligated, which removed 207 bp of DNA (nt 143309–143304 of pSLA2-L) containing the 5′ end and upstream region of srrA to afford pKAR3013. The vector part of pKAR3013 was replaced by pRES18 to give a targeting plasmid, pKAR3014 (Fig. S2).

Disruption of srrB (orf79). A 4.3 kb PstI–Eco47III fragment containing the srrB gene was cloned into pUC19 predigested with PstI and Smal to give pKAR3004. This plasmid was digested with BstWI and self-ligated, which removed 498 bp of DNA (nt 140448–140945 of pSLA2-L) containing the 5′ end and upstream region of srrB to afford pKAR3006. The vector part of pKAR3006 was replaced by pRES18 to give a targeting plasmid, pKAR3008 (Fig. S3).

Disruption of srrC (orf74). A 3.5 kb ApaI fragment containing the srrC gene was inserted into the ApaI site of pUC57-2, a pUC57- (Fermentas)-derived vector without a BamHI site, to give pKAR3015. A 0.8 kb BamHI–Ncol fragment of pKAR3015-2 was subcloned into pSET-B predigested with the same enzymes to afford pKAR3016. The EcoNI site in the middle of srrC was eliminated by filling-in and self-ligating reactions to give pKAR3017. This plasmid was digested with BstXI and NcoI, and the fragment containing a frame-shifted srrC gene (the A residue at nt 13334 of pSLA2-L was duplicated) was returned to pKAR3015-2 to give pKAR3018. The vector part of pKAR3018 was replaced by pRES18 to afford a targeting plasmid, pKAR3019 (Fig. S4).

Construction of gene disruptants. The targeting plasmid pKY85-3 was transformed into protoplasts of *S. rochei* 51252, and thiostrepton- and kanamycin-resistant transformants were obtained. Among these transformants, single-crossover (plasmid-integrated) strains were selected by Southern hybridization analysis. One selected strain was serially grown in liquid YEME medium containing 10 μg kanamycin ml⁻¹ to facilitate a second crossover. Finally, kanamycin-resistant and thiostrepton-sensitive colonies were selected as double-crossover mutants to give the srrX mutant KY85. Other mutants were prepared by essentially the same method using the targeting plasmids constructed above. When the targeting plasmids did not contain a kanamycin cassette, the second crossover was induced by serially culturing in YEME medium without kanamycin.

Extraction and bioassay of antibiotics. *S. rochei* strains were cultured in YM liquid medium in Sakaguchi flasks on a reciprocating machine at 28 °C for 3 days. In all liquid cultures, 1 vol. % of a fully grown preculture was inoculated into liquid medium. The broth filtrates were extracted with ethyl acetate, concentrated and subjected to thin-layer chromatography (TLC) with chloroform-methanol (15 : 1, v/v). The antibiotic activities were assayed by bioautography as follows. A bioassay plate was contacted with the developed TLC plate for 30 min and incubated at 28 °C overnight. The bioassay plate was composed of two layers: the bottom layer contained TSB medium containing 1.5 % agar, while the top layer contained TSB-agar (0.8 %) supplemented with an overnight culture of the indicator organism, *Micrococcus luteus*.

Feeding experiments with γ-butyrolactone fraction. A 3.8 kb EcoRI–BamHI fragment containing an intact srrX gene with its promoter region was cloned into pSET132 (Bierman et al., 1992), to give pNT12. This plasmid was transformed into *S. rochei* strain 3-44 and resulting apramycin-resistant colonies were selected. One colony was cultivated in YM liquid medium (100 ml) for 24 h in the presence of apramycin (25 μg ml⁻¹) and the supernatant was extracted with ethyl acetate. The crude extract was dissolved in ethanol (1 ml) and added to a culture (100 ml) of strain KY85 at 24 h and the fermentation was stopped and analysed at 60 h.
Scanning electron microscopy (SEM). The surface morphology of S. rochei strain 51252 and its regulatory mutants was observed by SEM after growth on YM agar plates for 7 days. For the preparation of specimens, agar plugs were fixed with 1 % osmium tetroxide solution for 12 h and then dehydrated by lyophilization. The specimens were subsequently sputter-coated with platinum (2 nm) and examined by a JEOL JSM-5900 scanning electron microscope.

Complementation of srrA and srrC mutations. A 1.8 kb Mscl-Stul fragment, which contained an intact srrA gene with its promoter region, was cloned into pRES18 to give plasmid pKAR3052. This plasmid was transformed into mutant KA12 to complement the srrA mutation. A 1.2 kb FspI fragment containing an intact srrC with its promoter region was cloned into pRES18 and the obtained plasmid pKAR3050 was used to complement the srrC mutant KA16. Strains were grown on YM agar plates containing 10 μg thiostrepton ml⁻¹ to maintain plasmids.

RESULTS

Positive function of the afsA homologue (srrX, orf85) in antibiotic production

Complete nucleotide sequencing of pSLA2-L revealed many biosynthetic regulatory genes on this plasmid, among which orf85 shows high similarity (38 % identity in amino acid sequences) to afsA, involved in A-factor biosynthesis (Mochizuki et al., 2003). The orf85 gene (named srrX, Streptomyces rochei regulatory gene X) encodes a neutral protein (pl 6.85) with 331 amino acids and a molecular mass of 36.8 kDa. The SrrX protein is most similar to BarX (46 % identity), which is involved in the biosynthesis of virginiae butanolides in S. virginiae, leading to virginiamycin production (Kawachi et al., 2000).

To reveal the function of srrX in S. rochei 7434AN4, its disruptant was constructed from strain 51252, which carries only the largest linear plasmid pSLA2-L. The srrX disruptant KY85 did not produce lankacidin or lankamycin (Fig. 2a, lane IV), but sporulated well (Fig. 2b). In this respect, the γ-butyrolactone in S. rochei was superficially similar to γ-butyrolactones in many Streptomyces species, which showed a positive effect on antibiotic production and no effect on spore formation. However, this was not the case (we will return to this point later).

Next, a plasmid containing an intact srrX gene with its own promoter region was introduced into S. rochei strain 3-44, which has lost all three of the linear plasmids, pSLA2-L, -M and -S (Kinashi et al., 1994). The ethyl acetate extract from strain 3-44 containing an srrX gene restored the production of both lankacidin and lankamycin when added to the srrX mutant KY85 (Fig. 2a, lane XI), while that from strain 3-44 carrying a control plasmid did not (lane XII). These results indicated two things: (i) the srrX gene was enough for γ-butyrolactone synthesis in strain 3-44 and therefore materials for synthesis may be produced by primary metabolism coded on the chromosome and (ii) only a γ-butyrolactone fraction was enough for restoration of antibiotic production in mutant KY85 and the SrrX protein was not necessary for this event.

SrrA (Orf82) is a γ-butyrolactone receptor

Six tetR family receptor genes are present on pSLA2-L as a candidate for the γ-butyrolactone receptor gene, orf74, orf79, orf82, orf92, orf99 and orf126 (Fig. 1b), among which the gene products of the former three show considerable similarity to the known γ-butyrolactone receptor proteins such as ArpA, BarA and ScbR (Fig. 3a). All the γ-butyrolactone receptor proteins contain a helix–turn–helix DNA-binding motif near the N terminus and a tryptophan (W) residue in the middle, for example at the 119 position in ArpA. The former was important for binding of ArpA to the promoter of adpA, while the latter was crucial for binding of A-factor to ArpA (Sugiyama et al., 1998).

Orf82 (named SrrA) is the most probable candidate, because its pl value (6.07) is similar to those of the typical γ-butyrolactone receptors (ArpA, 5.14; BarA, 5.08; FarA, 5.29). In S. griseus, the afsA-arpA double mutant produced streptomyacin, because the global activator gene adpA was not repressed under arpA mutation without A-factor. Therefore, we introduced a second mutation into srrA in the srrX mutant KY85, and found that the srrX-srrA double mutant (strain KA21) recovered an ability to produce both lankacidin and lankamycin (Fig. 2a, lane VI). Thus, we concluded that srrA encodes a γ-butyrolactone receptor protein.

SrrA, a weakly acidic protein (pl 6.07) with 222 amino acids and a molecular mass of 23.8 kDa, is most similar (57 % identity) to TarA, involved in nikkomycin synthesis in Streptomyces tendae (Engel et al., 2001). Based on whole amino acid sequences, a phylogenetic tree was constructed for typical γ-butyrolactone receptors and their homologues. As shown in Fig. 3(b), the γ-butyrolactone receptors including SrrA form a major group separated from two other groups, containing Orf79 (SrrB) and Orf74 (SrrC), respectively (see later).

Negative function of srrX in spore formation

Disruption of srrX was of interest, because mutation of the γ-butyrolactone receptor genes displayed different effects on antibiotic production and morphological differentiation in different species. The srrA mutant KA12 produced lankacidin and lankamycin (Fig. 2a, lane IX), but failed to sporulate (Fig. 2b). On the other hand, the srrX-srrA double mutant KA21 formed spores normally as did the parent strain 51252 and the srrX mutant KY85 (Fig. 2b). To analyse morphological differentiation more precisely, surface-grown colonies were observed by SEM. In mutant KA12 (srrA), elongation of aerial mycelium stopped at an early stage (Fig. 4c), while mutant KA21 (srrX-srrA) produced spiral-shaped filaments with septation (Fig. 4d), as did strains 51252 (Fig. 4a) and KY85 (Fig. 4b). To confirm the function of the srrA gene in spore formation, complementation experiments were carried out. When an intact srrA gene with its promoter region was introduced into the srrA mutant KA12, partial complementation was observed.
observed. Namely, although visible changes were not observed on plate cultures (data not shown), spiral-shaped filaments with spores were detected by SEM analysis (Fig. 5a). On the other hand, the control plasmid pRES18 did not show any effects (Fig. 5b). Thus, the srrX gene showed a positive effect on antibiotic production and a negative effect on spore formation, while srrA reversed both effects of srrX. This is believed to be the first paper reporting a negative function of the afsA homologues in morphological differentiation. However, we have not succeeded in detecting an inhibitory activity of a γ-butyrolactone fraction on spore formation, when applied onto the surface of several S. rochei strains (data not shown).

**SrrC (Orf74) is a positive regulator in spore formation**

To elucidate the function of other γ-butyrolactone receptor gene homologues, srrC (orf74) and srrB (orf79), we also constructed their single and double mutants with a combination of srrX. The srrC (orf74) disruptant KA16 produced lankacidin and lankamycin normally (Fig. 2a,

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Fig. 2. Analysis of antibiotic production (a) and spore formation (b) of *S. rochei* 51252 and its regulatory mutants. (a) Antibiotic production was analysed by bioautography. I, lankamycin (LM); II, lankacidin C (LC); III, strain 51252; IV, strain KY85 (srrX); V, strain KA20 (srrX-srrB); VI, strain KA21 (srrX-srrA); VII, strain KA22 (srrX-srrC); VIII, strain KA07 (srrB); IX, strain KA12 (srrA); X, strain KA16 (srrC); XI, strain KY85 (srrX) fed with an extract from strain 3-44 carrying an intact srrX plasmid pNT12; XII, strain KY85 fed with an extract from strain 3-44 carrying the control plasmid pSET152. (b) Strains were grown on YM agar plates for 7 days; the picture was taken from the surface side.
lane X), but failed to sporulate (Fig. 2b). On the other hand, the srrX-srrC double mutant KA22 did not produce antibiotics (Fig. 2a, lane VII) but sporulated well (Fig. 2b). SEM analysis revealed that aerial mycelium elongates without coiling or septation in the srrC mutant (Fig. 4g). Furthermore, the srrC mutant frequently showed collapsed hyphae, which were not observed in the parent and srrX-srrC strains. Thus, morphological differentiation stopped at a later stage in this mutant than the srrA mutant. As shown in Fig. 5(c), the defective phenotype of the srrC mutant was also partially complemented by introduction of an intact srrC gene with its promoter region.

The SrrC protein is a basic protein (pI 9.26) with 213 amino acids and a molecular mass of 22.8 kDa. SrrC shows relatively low similarity to other ω-butyrolactone receptor proteins and is most similar (36% identity) to MmfR, involved in methylenomycin synthesis in S. coelicolor A3(2) (Bentley et al., 2004) (Fig. 3b). The SrrC protein also shows considerable similarity (33% identity) to CprA in S. coelicolor A3(2). Disruption of cprA caused a similar phenotype to srrC disruption: the cprA mutant displayed a delayed aerial mycelium formation, but produced actinorhodin and undecylprodigiosin (Onaka et al., 1998). The cprA gene was required for expression of the

![Fig. 3. Partial amino acid alignment (a) and phylogenetic tree (b) of ω-butyrolactone receptor proteins and their homologues. (a) Only two regions containing a helix-turn-helix motif and a specific tryptophan residue (W) are compared. TyIP (AAD40801, S. fradiae), SpbR (AAK07686, S. pristinaespiralis), FarA (BA21859, S. lavendulae), ScbR (NP630365, S. coelicolor A3(2)), SrrA (NP851504, S. rochei), TarA (AAF06981, S. tendae), BarA (BA06981, S. virginiensis), ArpA (BA236282, S. griseus), SrrC (NP851496, S. rochei), TyQ (AAD40803, S. fradiae), SrbB (NP851501, S. rochei), BarB (BA23612, S. virginiensis) and MmrB (CAC36768, S. coelicolor A3(2)). Highly conserved amino acid residues are marked below the alignment as identical (asterisk), well conserved (colon), or partially conserved (period). (b) Phylogenetic analysis was performed by CLUSTALW (http://www.ddbj.nig.ac.jp/search/clustalw-j.html), and the unrooted tree was drawn with the TreeView32 software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).]
Fig. 4. SEMs of surface-grown colonies of S. rochei 51252 and various regulatory mutants. (a) Strain 51252 (parent), (b) strain KY85 (srrX), (c) strain KA12 (srrA), (d) strain KA21 (srrX-srrA), (e) strain KA07 (srrB), (f) strain KA20 (srrX-srrB), (g) strain KA16 (srrC) and (h) strain KA22 (srrX-srrC). Scale bars, 5 μm.

Fig. 5. Complementation experiments of the srrA and srrC mutants. Spore formation in the srrA mutant KA12 was partially restored by plasmid pKAR3052 (a), which carried a functional srrA gene, but was not by the control plasmid pRES18 (b). Similarly, the defect of the srrC mutant KA16 was partially complemented by pKAR3050 (c) but not by pRES18 (d). All the strains were grown on YM agar plates containing 10 μg thiostrepton ml⁻¹. Scale bars, 5 μm.
**ramCSAB operon**, which contributed to the onset of aerial growth (O’Connor et al., 2002).

**SrrB (Orf79) is a negative regulator in antibiotic production**

The *srrB* mutation (strain KA07) caused an increased production of both lankacidin and lankamycin (Fig. 2a, lane VIII), while the spore formation was slightly delayed (Fig. 2b). On the other hand, the *srrX-srrB* double mutant displayed the same phenotype as the *srrX* mutant; it did not produce lankacidin or lankamycin (Fig. 2a, lane V) and sporulated well (Fig. 2b). SEM analysis showed that morphological differentiation proceeds normally in both mutants (Figs 4e, f). Thus, SrrB is a negative regulator only for antibiotic production.

Different from the typical γ-butyrolactone receptors, SrrB is an extremely basic protein (pI 11.2) with 228 amino acids and a molecular mass of 25 kDa. SrrB is similar to TylQ (49% identity) involved in tylosin production in *Streptomyces fradiae* (Stratigopoulos & Cundliffe, 2002) and BarB (42%) involved in virginiamycin synthesis in S. virginiae (Matsumo et al., 2004) (Fig. 3b). Although the basicities of BarB (pI 10.2) and TylQ (pI 6.4) are quite different, both proteins are negative regulators in antibiotic production. As shown in Fig. 3(b), SrrC and SrrB are grouped into different phylogenetic branches from the typical γ-butyrolactone receptors including SrrA, which suggests their different evolutionary history.

**DISCUSSION**

In this work, we constructed various disruptants of four regulatory genes coded on the linear plasmid pSLA2-L in *S. rochei* 7434AN4: the *afsA* homologue *srrX* (orf85) and three γ-butyrolactone receptor gene homologues, *srrA* (orf82), *srrB* (orf79) and *srrC* (orf74). All of these genes are located on the right side of the cryptic *roc* cluster (orf62–orf70), far from the biosynthetic gene clusters for lankacidin (orf4–orf18) and lankamycin (orf24–orf53) (Fig. 1b). Phenotypes of these disruptants revealed that *srrX* and *srrA* play a central role in the γ-butyrolactone receptor system. The *srrX* gene had a positive function in the production of lankacidin and lankamycin and a negative function in morphological differentiation.

Concerning the function of AfsA homologues, there was an apparent contradiction between AfsA in *S. griseus* and BarX in *S. virginiae*. The former was reported as an A-factor biosynthetic enzyme that condensed a glycerol derivative and a short-chain β-keto acid (Ando et al., 1997), while the latter was identified as a regulatory protein that stabilized binding of the receptor BarA to the target site, and addition of virginiae butanolides did not restore virginiamycin production in the *barX* mutant (Kawachi et al., 2000). Recently, another homologue, ScbA in *S. coelicolor*, was reported to possess both functions (Takano et al., 2005). In this respect, SrrX is similar to AfsA, because antibiotic production was restored in the *srrX* mutant by addition of a γ-butyrolactone fraction without the SrrX protein. However, we cannot exclude the possibility that the SrrX protein has a regulatory function in spore formation, because we have not detected a direct inhibitory activity of a γ-butyrolactone fraction in this event. Isolation of *S. rochei* γ-butyrolactone (SRB) is in progress in our laboratory.

γ-Butyrolactone receptors have been classified into the following three groups based on the phenotypes of their disruptants. The first group includes the *arpA* mutant of *S. griseus*, which showed an early and increased production of both streptomycin and spores (Miyake et al., 1990), because in this mutant the A-factor-dependent activator gene, *adpA*, is expressed earlier due to the absence of the repressor ArpA. The *tylP* mutant of *S. fradiae* is included in this group, which showed increased tylosin production and sporulated significantly earlier than the wild-type strain (Stratigopoulos et al., 2002). The second group includes many *Streptomyces* species, where disruption of the receptor genes negatively affected antibiotic production, but gave no effect on spore formation. The *barA* mutant of *S. virginiae* (Nakano et al., 1998), the *farA* mutant of *S. lavendulace* (Kitani et al., 2001) and the *scbR* mutant of *S. coelicolor* A3(2) (Takano et al., 2001) produced no or small amounts of antibiotics, but sporulated normally. The third group includes *Streptomyces pristinaespiralis*, where disruption of the receptor gene *spbR* resulted in severe defects in growth, morphological differentiation, pristinamycin biosynthesis and even expression of a secreted superoxide dismutase (Folcher et al., 2001).

To these three groups, *S. rochei* 7434AN4 added the fourth phenotype, where disruption of the receptor gene *srrA* did not affect the production of lankacidin or lankamycin, but ceased spore formation. The *srrA* gene had totally opposite effects of *srrX*, namely a negative effect on antibiotic production and a positive effect on spore formation. Thus, the *srrX-srrA* system functions similarly with the *afsA-arpA* system in antibiotic production, but conversely in morphological differentiation.

The question why disruption of the γ-butyrolactone receptor genes shows such different phenotypes has not been answered. We have been preoccupied with the clear and simple A-factor regulatory cascade, where A-factor has only positive effects on both antibiotic production and spore formation through the function of AdpA, which is a single target of the γ-butyrolactone receptor ArpA (Kato et al., 2004). However, based on accumulated data and the results of this study, we can point out the following aspects of the γ-butyrolactone-receptor systems in *Streptomyces*.

(i) γ-Butyrolactones may have a negative function in some phenomena, as shown in spore formation in *S. rochei*.

(ii) It is likely that more than one γ-butyrolactone receptor is present in one strain. Multiple receptors that repress or activate different regulatory genes with a different function...
could generate complex functions. Onaka et al. (1998) isolated two arpA homologues, cprA and cprB, from S. coelicolor A3(2), disruption of which gave different effects on antibiotic production and spore formation. Thus, it is possible that CprA and CprB function as ω-butyrolactone receptors as well as ScrB in S. coelicolor A3(2), although both are basic proteins (pI 9.8 and pI 10.0), different from typical ω-butyrolactone receptors. Our results also suggest the possibility that SrrC is a second ω-butyrolactone receptor functioning for only morphological differentiation. In contrast, only one afsA homologue has been found in all Streptomyces species including S. coelicolor A3(2) (Bentley et al., 2002) and Streptomyces avermitilis (Ikeda et al., 2003). Nor did S. rochei chromosomal DNA give any hybridizing signals when probed by the srrX DNA even in low stringency (data not shown).

(iii) ω-Butyrolactone receptor proteins may function at different hierarchical levels, as exemplified by the global transcriptional activator AdpA in S. griseus and several pathway-specific regulatory proteins in other Streptomyces species.

(iv) Accumulated data suggest that SARP family regulatory genes (Wietzorrek & Bibb, 1997) are the main targets of ω-butyrolactone receptors. In S. fradiae, the receptor protein TyIP repressed the expression of the SARP gene tylS (Stratigopoulos et al., 2002), which in turn resulted in the repression of tylR, encoding a pathway-specific activator in tylosin biosynthesis (Stratigopoulos et al., 2004). In S. pristinae spiralis, the receptor SpbR was bound to the promoter of the SARP gene papR1 and regulated pristinamycin synthesis (Folcher et al., 2001). The receptor ScrB in S. coelicolor A3(2) was directly bound to two upstream regions of kasO, a pathway-specific SARP gene for a cryptic type I polyketide gene cluster, and repressed its transcription (Takano et al., 2005). We have recently obtained data suggesting that one (orf75–srrY) of the three SARP genes (orf55, orf71 and orf75) located on pSLA2-L is a target of the receptor SrrA (unpublished result).

In addition, the srrB gene makes the regulatory system in S. rochei 7434AN4 more complex. The SrrB disruptant produced a large amount of lankacidin and lankamycin. Double mutants of srrB and each of the two P450-dependent hydroxylase genes (orf26 and orf37) accumulated even biosynthetic intermediates, which we used to study the lankamycin biosynthetic pathway (Arakawa et al., 2006). Thus, SrrB is a negative regulator in biosynthesis of both lankacidin and lankamycin. From several aspects, SrrB is similar to tylQ, which also functions negatively in tylosin production in S. fradiae. Overexpression of tylQ ceased the transcription of most of the tylosin biosynthetic genes, while disruption of tylQ caused their constitutive expression (Stratigopoulos & Cundliffe, 2002). It was shown that TylQ exhibits its negative function by repressing the transcription of the SARP gene tylS.

We have reported here the first step of our project to reveal the entire picture of the regulatory cascade in S. rochei 7434AN4. In this strain, the afsA homologue srrX and the three ω-butyrolactone receptor gene homologues (srrA, srrB and srrC) together with the SARP family genes (srrY, orf71 and orf55), all of which are located on the giant linear plasmid pSLA2-L (Fig. 1b), may form a complex regulatory cascade. To reach the final goal, extensive studies including transcriptional analysis are in progress in our laboratory.

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