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Regulation of the Biosynthesis of the Macrolide Antibiotic Spiramycin in *Streptomyces ambofaciens*▽†

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Streptomyces ambofaciens **synthesizes the macrolide antibiotic spiramycin. The biosynthetic gene cluster for spiramycin has been characterized for *S. ambofaciens*. In addition to the regulatory gene *srmR* (*srm22*), previously identified (M. Geistlich et al., Mol. Microbiol. 6:2019-2029, 1992), three putative regulatory genes had been identified by sequence analysis. Gene expression analysis and gene inactivation experiments showed that only one of these three genes, *srm40*, plays a major role in the regulation of spiramycin biosynthesis. The disruption of *srm22* or *srm40* eliminated spiramycin production while their overexpression increased spiramycin production. Expression analysis was performed by reverse transcription-PCR (RT-PCR) for all the genes of the cluster in the wild-type strain and in the *srm22* (*srmR*) and *srm40* deletion mutants. The results from the expression analysis, together with the ones from the complementation experiments, indicated that Srm22 is required for *srm40* expression, Srm40 being a pathway-specific activator that controls most, if not all, of the spiramycin biosynthetic genes.**

Streptomyces species are Gram-positive, soil-inhabiting, filamentous bacteria that undergo a complex process of morphological differentiation and produce a great variety of secondary metabolites, including antibiotics with important applications in human medicine and in agriculture. These secondary metabolites are synthesized by complex pathways that utilize primary metabolites as building blocks. The genes required for the biosynthesis of a particular compound are generally clustered, and their expression is coregulated. Secondary metabolite biosynthesis is often activated in a growth phase-dependent manner and is genetically controlled at several levels (5). Pleiotropic regulatory genes can control the onset of production of several secondary metabolites produced by one *Streptomyces* strain. In some cases, these pleiotropic regulators also have an influence on morphological differentiation (12, 38, 39). For most of the secondary metabolite biosynthetic pathways, there is a specific level of control exerted by pathway-specific regulatory proteins. These regulatory proteins are encoded by genes generally located within the biosynthetic gene cluster. Many of these regulatory proteins belong to the SARP (*Streptomyces* antibiotic regulatory protein) family of DNA-binding proteins (46). But regulatory proteins belonging to other families, such as the LAL family (large ATP-binding regulators of the LuxR family) (18) and families comprising two-component histidine kinase and response regulator pairs (1), LysR-like regulators (17), TetR regulators (9), and γ-butyrolactone receptors (44), could also play a role as pathway-specific regulators. In addition, proteins that do not belong to large recognized families have been found to regulate the expression of biosynthetic genes (20). The expression of biosynthetic gene clusters could be regulated by a single pathway-specific regulator, for instance, PikD, which belongs to a LAL family and is a positive regulator for the pikromycin biosynthetic gene cluster in *Streptomyces venezuelae* (47). But a complex regulatory cascade involving several regulatory proteins encoded by genes in the cluster could also control antibiotic biosynthesis, as for tylosin biosynthesis in *Streptomyces fradiae* (15) or alpomycin biosynthesis in *Streptomyces ambofaciens* (2, 9).

The macrolide antibiotic spiramycin is produced by *S. ambofaciens* (35). The spiramycin molecule consists of a polyketide lactone ring (platenolide) synthesized by a type I polyketide synthase (PKS), to which three deoxyhexoses (mycaminose, forosamine, and mycarose, in that order) are attached successively (Fig. 1A). The entire spiramycin biosynthetic gene cluster has previously been cloned and sequenced (20, 25, 28) (Fig. 1B). The transcriptional activator SrmR (Srm22), encoded by a gene within the spiramycin biosynthetic gene cluster of *S. ambofaciens*, has been shown to be required for the transcription of at least two of the genes involved in spiramycin biosynthesis, *srmGI* (*srm12*) and *srmX* (*srm21*) (20). Three other putative regulatory genes located within the spiramycin biosynthetic gene cluster have been identified by sequence analysis (25). Neither Srm22 (SrmR) nor any of the putative regulators belong to an identified family of proteins involved in the pathway-specific regulation of antibiotic production. In this study, we demonstrated that two of these genes are not involved in the regulation of spiramycin biosynthesis but that the transcription of the biosynthetic genes is con-

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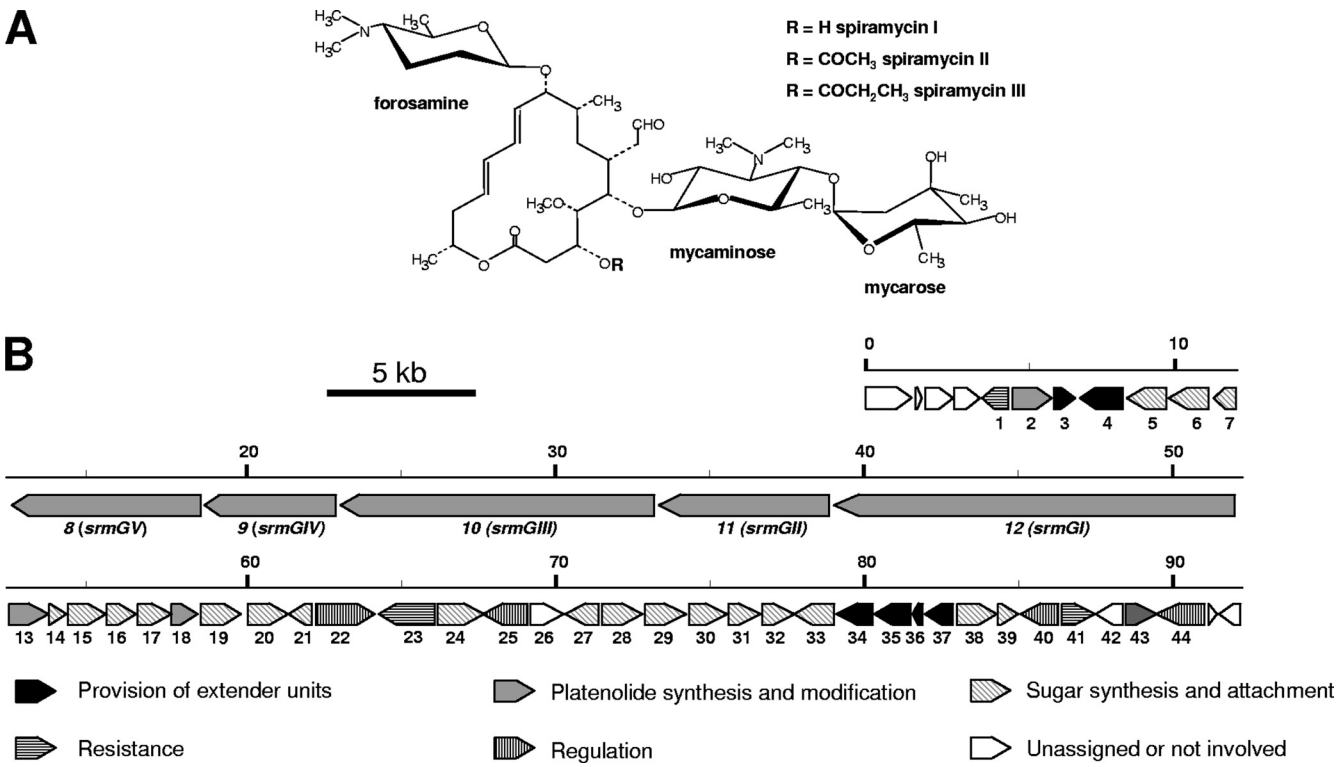

FIG. 1. Structure of spiramycins and genetic organization of the spiramycin biosynthetic gene cluster. (A) Structure of spiramycins. (B) Genetic organization of the spiramycin biosynthetic gene cluster. The proposed functions of the gene products in spiramycin biosynthesis are indicated by various filling patterns.

trolled by Srm22 (SrmR) and the newly identified Srm40 regulator.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. All strains and plasmids used in this study are described in Table 1. Standard media and culture conditions were used (27, 40). The following antibiotics were incorporated in the medium when required for selection: ampicillin (Amp), thiostrepton (Tsr), apramycin (Apr), hygromycin B (Hyg), puromycin (Pur), and kanamycin (Kan). For spiramycin production, *S. ambofaciens* strains were grown in MP5 liquid medium as previously described (34). The detection and quantification of spiramycin were performed by bioassay and high-performance liquid chromatography (HPLC) as previously described (21). Spiramycins I, II, and III were used as standards for quantification by HPLC. The nomenclature used for the genes of the spiramycin biosynthetic gene cluster is different from the one published earlier (25) but is the one used by Nguyen et al. (32).

DNA manipulation and bacterial transformation. DNA extraction and manipulation and transformation of *Escherichia coli* and *Streptomyces* were performed according to standard protocols (27, 40).

Isolation of total RNA from *Streptomyces* mycelium. Mycelium was separated from fermentation broth and was washed with diethyl pyrocarbonate (DEPC)-treated water by vacuum filtration through glass fiber filters. The mycelium was rapidly collected, and about 0.4 g of mycelium was added to the mixture of 0.4 g glass beads from Sigma (106 μm and finer; acid washed), 400 μl RNase-free water, 500 μl acid phenol, and 400 μl macaloid. The mixture was shaken twice in a Fast Prep machine from Bio 101 (Savant) (force, 6.5; 30 s) and was then centrifuged. The supernatant obtained was extracted by acid phenol and then precipitated overnight with NaCl (0.2 M final concentration) and 1 volume of isopropanol. Nucleic acid preparations were treated with DNase I (DNA-free kit; Ambion), according to the manufacturer's instructions.

Gene expression analysis by RT-PCR. Reverse transcription-PCR (RT-PCR) was performed with a Qiagen OneStep RT-PCR kit using 1 μg of total RNAs as a template. The conditions were as follows: for cDNA synthesis, 50°C for 30 min, followed by 95°C for 15 min, and for amplification, 5 cycles at 97°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by 20 cycles at 97°C for 1 min, 58°C for 1 min, and 72°C for 1 min. Primers (20- to 22-mers; average melting temperature [T_m], 65°C to 70°C) (see the supplemental material) were designed to generate PCR products of approximately 400 bp, except for *srm9* (*srmGIV*), *srm36*, and *hrdB*, where specific primers amplified fragments of 317 bp, 151 bp, and 177 bp, respectively. The gene *hrdB*, encoding the major sigma factor, was used as a control, as it is expressed at a constant level (11). All RT-PCR products were purified with s QIAquick PCR purification kit (Qiagen). With each pair of primers, negative-control experiments were carried out in the absence of reverse transcriptase (with DNA polymerase alone) to confirm that the amplified products were derived from RNA templates and not from chromosomal DNA, which might contaminate RNA preparations.

Targeted disruption of *srm22*, *srm25*, and *srm40*. The copy of *srm22* (*srmR*) was inactivated by gene replacement with a copy of the gene disrupted by the Ω*hyg* hygromycin resistance cassette. A DNA fragment internal to the *srm22* coding sequence was amplified by PCR using the SRMR1 and SRMR2 primers (see the supplemental material). The 1.5-kb PCR product was inserted into the vector pCR2.1, leading to plasmid pOS49.32. pOS49.32 was then digested by BstEII and treated by the Klenow enzyme. The cassette Ω*hyg* (7) was obtained as a BamHI fragment and then blunt ended by treatment with the Klenow enzyme. The hygromycin resistance cassette Ω*hyg* was introduced in the digested plasmid pOS49.32, leading to plasmid pOS49.43, in which the genes *srm22* and *hyg* are in the same orientation. Plasmid pOS49.43 was finally digested by Asp718I and XbaI and the insert fragment, blunt ended by Klenow treatment, and cloned into the plasmid pOJ260, previously digested by EcoRV, yielding pOS49.50. *S. ambofaciens* protoplasts were then transformed by alkali-denatured pOS49.50 DNA according to the method of Oh and Chater (33). Hyg^r clones were selected; among them, Apr^s clones were screened. The Apr^s clones probably resulted from a double recombination event leading to the replacement of *srm22* by *srm22*::Ω*hyg*. This was checked by Southern analysis (data not shown). One such clone (*srm22*::Ω*hyg*) was retained for further analysis and named SPM249.

The disruptions of *srm25* and *srm40* were obtained by PCR targeting (13, 22, 48). For the inactivation of *srm25*, a 15.1-kb fragment from pSPM5 containing *srm25* and flanking genes was first cloned into pMBL18, yielding pSPM502, and

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
ATCC 23877	Wild-type *S. ambofaciens* strain	ATCC
OSC2	Derivative *of S. ambofaciens* ATCC 23877 devoid of pSAM2	36
SPM249	*srm22*::Ω*hyg* in *S. ambofaciens* ATCC 23877	This work
SPM508	Δ*srm25*::*att3*-Ω*aac* in *S. ambofaciens* OSC2	This work
SPM107	Δ*srm40*::*att3-aac* in *S. ambofaciens* OSC2	This work
DH5α	*E. coli* general cloning host strain	Promega
S17.1	*E. coli* strain for conjugation from *E. coli* to *S. ambofaciens*	41
DY330	*E. coli* strain used for PCR-targeted mutagenesis	48
KS272/pKOBEG	*E. coli* strain used for PCR-targeted mutagenesis	13
Plasmids		
pGEMT-Easy	Amp^r; *E. coli* vector for cloning PCR products	Promega
pCR2.1	Amp^r; *E. coli* vector for cloning PCR products	Invitrogen
pMBL18	Amp^r; *E. coli* cloning vector	31
pOJ260	Apr^r; replicative vector in *E. coli*; nonreplicative in *Streptomyces*; used for gene disruption in *S. ambofaciens*	6
pUWL201	Amp^r Tsr^r; *E. coli-Streptomyces* shuttle vector for gene expression in *S. ambofaciens* under the control of *ermE*p*	19
pOSV211	Amp^r Apr^r; *att3*-Ω*aac* cassette (36) cloned into the vector pGP704*Not* (13, 30), the template for PCR amplification of *att3-aac*	This work
pOSV234	Amp^r Apr^r; *att3-aac* cassette (34) cloned into the vector pGP704*Not* (13, 30), the template for PCR amplification of *att3-aac*	32
pOSV238	Hyg^r; *E. coli* vector derived from pBK-CMV (Stratagene) by replacing the *neo* gene with the *hyg* gene	25
pOS49.32	Amp^r Kan^r; internal fragment of *srm22* cloned into pRC2.1	This work
pOS49.43	Amp^r Kan^r; derived from pOS49.32; Ω*hyg* into *srm22*	This work
pOS49.50	Apr^r Hyg^r; insert from pOS49.43 cloned into pOJ260	This work
pSPM5	Amp^r; cosmid (vector pWED1) from the *S. ambofaciens* gene library containing part of the spiramycin cluster	25
pSPM36	Amp^r Pur^r; cosmid (vector pWED2) from the *S. ambofaciens* gene library containing part of the spiramycin cluster	25
pSPM75	Amp^r Tsr^r; coding sequence of *srm40* cloned into pUWL201	This work
pSPM107	Amp^r Apr^r Pur^r; *srm40* inactivation (Δ*srm40*::*att3-aac)* by PCR targeting into pSPM36	This work
pSPM502	Amp^r; 15.1-kb BglII-AseI/Klenow fragment from pSPM5 cloned into pMBL18 digested by BamHI/Klenow	This work
pSPM504	Hyg^r; 15.1-kb insert from pSPM502 cloned into pOSV238	This work
pSPM508	Apr^r Hyg^r; *srm25* inactivation (Δ*srm25*::*att3*-Ω*aac)* by PCR targeting into pSPM504	This work
pSPM520	Amp^r; fragment of 2 kb containing the short form of the *srm22* coding sequence cloned into pGEM-T Easy	This work
pSPM521	Amp^r; fragment of 2.2 kb containing the long form of the *srm22* coding sequence cloned into pGEM-T Easy	This work
pSPM522	Amp^r; fragment of 2.6 kb containing the long form of the *srm22* coding sequence and its promoter cloned into pGEM-T Easy	This work
pSPM523	Amp^r Tsr^r; insert from pSPM520 (HindIII-BamHI fragment) cloned into pUWL201	This work
pSPM524	Amp^r Tsr^r; insert from pSPM521 (HindIII-BamHI fragment) cloned into pUWL201	This work
pSPM525	Amp^r Tsr^r; insert from pSPM522 (HindIII-BamHI fragment) cloned into pUWL201	This work
pSPM527	Amp^r; pSPM521 with a 4-bp insertion at the XhoI restriction site	This work
pSPM528	Amp^r Tsr^r; insert from pSPM527 (HindIII-BamHI fragment) cloned into pUWL201	This work

then the insert of pSPM502 was cloned into pOSV238, yielding pSPM504. The excisable cassette *att3*-Ω*aac* (36) was amplified using pOSV211 as a template and the primer set EDR3/EDR4 (see the supplemental material). Electrocompetent KS272/pKOBEG cells containing the plasmid pSPM504 were transformed with the purified PCR product. Apr^r clones in which most of the *srm25* coding sequence had been replaced by the *att3*-Ω*aac* cassette through λ-RED recombination were obtained. The resulting plasmid, pSPM508, was introduced into *S. ambofaciens* OSC2 via protoplast transformation, and apramycin selection was applied. Apr^r transformants were screened for sensitivity to hygromycin, indicating a double-crossover allelic exchange in *Streptomyces*. This was confirmed by PCR and Southern blot analysis (data not shown). One clone with the expected structure for *srm25* inactivation (Δ*srm25*::*att3*-Ω*aac*) was retained and named SPM508.

For the inactivation of *srm40*, the excisable cassette *att3-aac* was amplified using pOSV234 as a template and the primer set KF32/KF33 (see the supplemental material). The resulting PCR product was used to transform the *E. coli* strain DY330 (48) containing the cosmid pSPM36, which carries the target gene. Apr^r clones in which most of the *srm40* coding sequence had been replaced by the *att3-aac* cassette through λ-RED recombination were obtained. The resulting recombinant cosmid, pSPM107, was introduced into *S. ambofaciens* OSC2 via protoplast transformation, and apramycin selection was applied. Apr^r transformants were screened for sensitivity to puromycin, indicating a double-crossover allelic exchange. This was confirmed by PCR amplification and Southern blot analysis (data not shown). One Δ*srm40*::*att3-aac S. ambofaciens* mutant strain was retained and named SPM107.

Construction of plasmids for expression of *srm22* and *srm40*. For the expression of *srm22*, three plasmids were constructed, containing the different forms of *srm22*. These forms were obtained by PCR using chromosomal DNA from *S. ambofaciens* OSC2 as a template and oligonucleotides, containing either BamHI or HindIII restriction sites, as primers (see the supplemental material). Oligonucleotides EDR39 and EDR42 were used to amplify the short form of *srm22*, starting with the ATG codon proposed by Geistlich et al. (20). The resulting 2-kb PCR product was cloned into the plasmid pGEMT-Easy, leading to the plasmid pSPM520. Oligonucleotides EDR40 and EDR42 were used to amplify the long

form of the *srm22* gene, starting from the most upstream ATG codon. The resulting 2.2-kb PCR product was inserted into the plasmid pGEMT-Easy, yielding the plasmid pSPM521. Oligonucleotides EDR41 and EDR42 were used to amplify the long form of *srm22* together with its own promoter region. The resulting 2.6-kb PCR product was introduced into the plasmid pGEMT-Easy, yielding the plasmid pSPM522. The three plasmids pSPM520, pSPM521, and pSPM522 were digested by BamHI and HindIII, and their inserts containing the various forms of the *srm22* gene were cloned into pUWL201 digested by BamHI and HindIII, leading to the plasmids pSPM523, pSPM524, and pSPM525, respectively. In all cases, the various forms of *srm22* are under the control of the *ermE*p* promoter.

A plasmid containing a frameshift mutation in the region between the start codon proposed by Geistlich et al. (20) and the most upstream start codon was constructed. This plasmid, pSPM527, was obtained by digestion of plasmid pSPM521 with XhoI, treatment with the Klenow enzyme, and self-ligation. The insert BamHI-HindIII of pSPM527 was then inserted into the plasmid pUWL201 cut with the same enzymes, yielding the plasmid pSPM528.

For the expression of *srm40*, the *srm40* coding sequence was amplified with the primers KF30 and KF31 (see the supplemental material). The resulting 1.5-kb PCR product was cloned into pUWL201 digested by BamHI and HindIII, yielding pSPM75, in which *srm40* is expressed under the control of the *ermE*p* promoter.

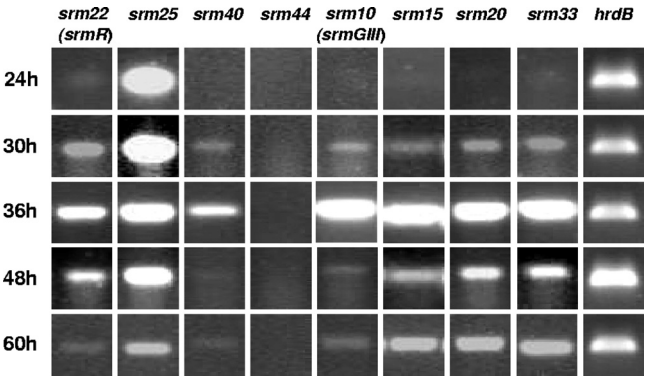

FIG. 2. Gene expression analysis of some genes from the spiramycin biosynthetic gene cluster. Gene expression was analyzed by RT-PCR in OSC2 at 24 h, 30 h, 36 h, 48 h, and 60 h. The transcript of *hrdB* was used as a control.

RESULTS

Putative regulatory genes present in the spiramycin biosynthetic gene cluster. The gene cluster directing spiramycin biosynthesis is presented in Fig. 1. The gene *srmR* (*srm22*) has previously been shown to encode an activator required for transcription from the *srmGI* (*srm12*) and *srmX* (*srm21*) promoters (20). Besides *srm22*, the sequence analysis of the spiramycin cluster revealed the presence of three other putative regulatory genes: *srm25*, *srm40*, and *srm44*.

The deduced product of *srm25* (465 amino acid residues) showed high sequence similarity to members of the HflX subfamily of GTPases. Proteins from this family act as molecular switches, modulating diverse cellular processes in response to conformational changes induced by GTP hydrolysis. Their exact role remains sometimes elusive, but members of the HflX family have been shown to have pleiotropic action (8). In particular, Srm25 resembles the product of *tylV* from the tylosin biosynthetic gene cluster (63% identity and 76% similarity). The gene *tylV* also encodes a putative GTPase and might play a regulatory role in tylosin biosynthesis, as the inactivation of this gene was associated with reduced tylosin production (16, 42).

The deduced product of *srm40* (387 amino acid residues) showed high sequence similarity to the product of *acyB2* (69% identity and 77% similarity) from *Streptomyces thermotolerans*, the producer of carbomycin (3), and to the product of *tylR* (43% identity and 57% similarity) from *S. fradiae* (4). AcyB2 is required for carbomycin production and is supposed to play a regulatory role. TylR has been identified as a pathway-specific activator of tylosin production, directly controlling the expression of most of the biosynthetic genes (4, 42).

The deduced product of *srm44* (503 amino acid residues) belongs to the GntR family of transcriptional regulators and more precisely to the MocR subfamily of transcriptional regulators containing a DNA-binding helix-turn-helix domain and an aminotransferase domain (COG1167) (37). Recent results (H. C. Nguyen, E. Darbon, S. Lautru, and J.-L. Pernodet, unpublished data) have shown that the genes *srm42* and *srm43* were not involved in spiramycin biosynthesis. Therefore, the fact that *srm44* belongs to the spiramycin gene cluster is questionable.

Time course of the expression of spiramycin biosynthetic genes and putative regulatory genes. Under the culture conditions used in liquid medium, spiramycin is detectable after 40 to 48 h of cultivation and the spiramycin concentration continues to increase at least until 72 h. To correlate this observation with the expression of biosynthetic and putative regulatory genes, the expression of the regulatory gene *srm22* (*srmR*), of the three putative regulatory genes (*srm25*, *srm40*, and *srm44*), and of four biosynthetic genes (*srm10* [*srmGIII*], *srm15*, *srm20*, and *srm33*) was studied at different cultivation time points by RT-PCR for the strain *S. ambofaciens* OSC2. The gene *srm10* encodes one of the proteins of the PKS involved in the biosynthesis of the platenolide (10, 28). The products of *srm15*, *srm20*, and *srm33* were proposed to be involved in the biosynthesis of mycaminose, forosamine, and mycarose, respectively (25). Total RNAs were prepared from OSC2 after growth for 24 h, 30 h, 36 h, 48 h, and 60 h, i.e., before and after the onset of spiramycin production, and used as a template for gene expression analysis by RT-PCR. The results of these experiments are presented in Fig. 2.

For the biosynthetic genes, the transcripts were not detected at 24 h, they were detected at a low level at 30 h, they seemed to be very abundant at 36 h, and then they decreased but were still detected after 48 h and 60 h of cultivation. This is consistent with the time course of spiramycin production. For the regulatory gene *srm22*, the transcripts were present in very small amounts at 24 h. Transcription seemed to increase, to reach a maximum at 36 h, and then to decrease. This is in agreement with the idea that Srm22 is an activator required for the expression of the biosynthetic genes (C. M. Farnet, A. Staffa, and X. Yang, U.S. patent application US 2003/0113874 A1). No RT-PCR products were detected for the putative regulatory gene *srm44*, suggesting that it does not play a major role in the regulation of spiramycin biosynthesis. The putative regulatory gene *srm25* was always abundantly transcribed, even if its transcription seemed to decrease with time. For the third putative regulatory gene, *srm40*, the transcripts were not detected at 24 h, but they were detected at 30 h, relatively abundant at 36 h, and then detected in smaller amounts at 48 and

TABLE 2. Spiramycin production by various strains

Strain	Characteristic(s)	Spiramycin concn (μM/g [DCW] ± SD)[a]
OSC2	Wild type	75.2 ± 2.5
SPM249	*srm22*::Ω*hyg*	ND
SPM107	*srm40*::*att3-aac*	ND
SPM508	*srm25*::*att3*-Ω*aac*	60.6 ± 1.4
SPM249 (pUWL201)	*srm22*::Ω*hyg* harboring the empty cloning vector	ND
SPM249 (pSPM523)	*srm22*::Ω*hyg*; overexpression of the short form of *srm22*	ND
SPM249 (pSPM524)	*srm22*::Ω*hyg*; overexpression of the long form of *srm22*	47.3 ± 1.3
SPM249 (pSPM528)	*srm22*::Ω*hyg*; overexpression of the long form of *srm22* with a frameshift mutation	ND
SPM249 (pSPM525)	*srm22*::Ω*hyg*; overexpression of the long form of *srm22*; presence of the *srm22* promoter region	132.5 ± 5.9
SPM107 (pUWL201)	*srm40*::*att3-aac* harboring the empty cloning vector	ND
SPM107 (pSPM75)	*srm40*::*att3-aac*; overexpression of *srm40*	135.2 ± 6.9
OSC2 (pUWL201)	Wild-type strain harboring the empty cloning vector	120.8 ± 6.8
OSC2 (pSPM525)	Wild-type strain; overexpression of the long form of *srm22*; presence of the *srm22* promoter region	168.1 ± 4.3
OSC2 (pSPM75)	Wild-type strain; overexpression of *srm40*	288.4 ± 8.6
SPM249 (pSPM75)	*srm22*::Ω*hyg*; overexpression of *srm40*	273.5 ± 9.3
SPM107 (pSPM525)	*srm40*::*att3-aac*; overexpression of the long form of *srm22*; presence of the *srm22* promoter region	ND

[a] DCW, dry cell weight; ND, not detectable.

60 h. Its transcription pattern was quite similar to those of the biosynthetic genes.

These experiments showed that four biosynthetic genes involved in the synthesis of the different components of the spiramycin molecule, the polyketide macrolactone and the three sugars, had similar transcription patterns and were all abundantly transcribed at 36 h, i.e., a few hours before the detection of spiramycin in the culture medium. The expression profiles of *srm22* and *srm40* are compatible with a regulatory role for these genes. From the expression profile of *srm25*, it is difficult to draw evidence concerning its putative regulatory role. The fact that no transcript could be detected for *srm44* is barely compatible with a regulatory role for this gene in spiramycin production. As other experiments showed that *srm42* and *srm43* were not involved in spiramycin biosynthesis (Nguyen et al., unpublished), we therefore considered that *srm44* was probably not part of the spiramycin gene cluster and its role was not further studied.

Test of the involvement of the putative regulatory genes in the regulation of spiramycin biosynthesis. In order to probe their involvement in the regulation of spiramycin biosynthesis, the putative regulatory genes *srm25* and *srm40* were inactivated by PCR targeting, followed by gene replacement. The resulting mutant strains were called SPM508 (Δ*srm25*::*att3*-Ω*aac*) and SPM107 (Δ*srm40*::*att3-aac*). Inactivation of *srm22* was also performed by replacing in the *S. ambofaciens* chromosome the wild-type gene with a copy of the gene interrupted by the cassette Ω*hyg*. The resulting strain was called SPM249 (*srm22*::Ω*hyg*). These mutant strains were cultivated under the condition of spiramycin production. The culture supernatants were analyzed for spiramycin production, and the results are presented in Table 2.

As expected, the strain SPM249 (*srm22*::Ω*hyg*) was unable to produce spiramycin, as observed by Geistlich et al. for the *srmR* (*srm22*) mutant strain that they studied (20). The mutant strain SPM107 (Δ*srm40*::*att3-aac*) was also unable to produce spiramycin. This indicated that *srm40* is also a major regulatory gene, essential for spiramycin biosynthesis. For the mutant strain SPM508 (Δ*srm25*::*att3*-Ω*aac*), spiramycin production was still observed. The level of spiramycin produced by SPM508 was slightly lower than that produced by the wild-type strain, and the difference in spiramycin production is statistically significant (Student test; $P = 0.0009$); this might indicate a minor role for *srm25* in regulation but could also be due to variability in the level of spiramycin production between different clones. The roles of the genes *srm22* and *srm40* in regulation were further studied.

Overexpression of *srm22* and *srm40* in the *S. ambofaciens* mutant and wild-type strains. To confirm that the inactivation of *srm22* and *srm40* was the sole reason for the loss of spiramycin production in SPM249 and SPM107, the genes *srm22* and *srm40* were introduced and expressed into the corresponding mutant strains. The vector used was pUWL201, a multicopy plasmid in which the genes can be expressed under the control of the strong constitutive promoter *ermE*p*. For *srm22*, Geistlich et al. (20) had mapped the transcription start point, and they proposed for the *srm22* coding sequence an initiation codon located 442 bp downstream from the transcription start point. A close examination of the sequence revealed that it was possible to extend the coding frame by 151 bp upstream of this initiation codon and to start from another ATG codon. To identify the codon used for translation initiation, several constructions were made in pUWL201. They contained the short form of *srm22* (beginning 39 bp upstream of the ATG codon predicted by Geistlich et al. [20]) (pSPM523), the long form of *srm22* (beginning 38 bp upstream of the most upstream ATG codon) (pSPM524), or the long form of *srm22* into which a +1 frameshift mutation was introduced by adding 4 base pairs at a position located between the most upstream start codon and the start codon proposed by Geistlich et al. (20) (pSPM528). The plasmids pSPM523, pSPM524, and pSPM528 were introduced into the *srm22* disruption mutant SPM249. pSPM523, carrying the short form of *srm22*, did not restore spiramycin production; pSPM524, carrying the long form of *srm22*, restored production, but its derivative with the frameshift muta-

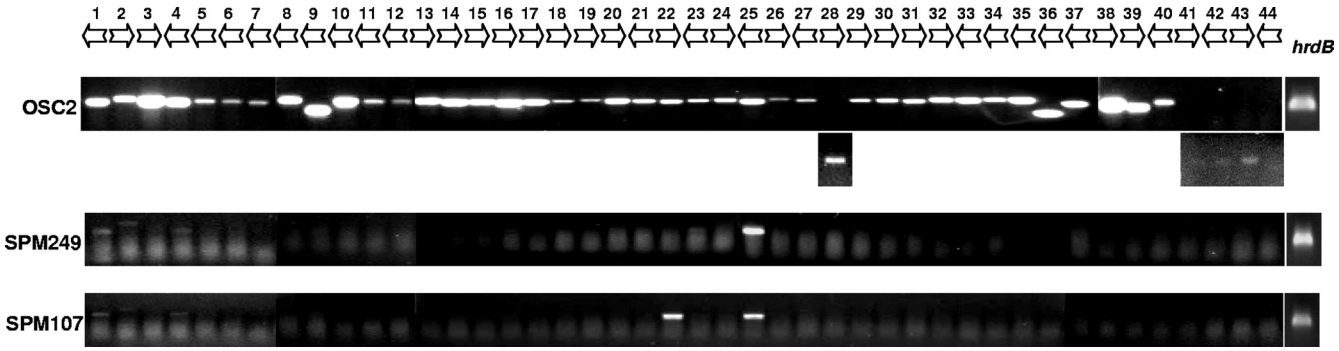

FIG. 3. Gene expression analysis of all the genes from the spiramycin biosynthetic gene cluster. Transcripts from three strains were analyzed by RT-PCR: OSC2, the *srm22*-disrupted strain (SPM249), and the *srm40*-disrupted strain (SPM107). Total RNAs were extracted from all strains after 36 h of cultivation. Twenty-five cycles of PCR were routinely employed; whenever this generated no product, analysis was repeated at 27 cycles to detect low-level transcripts. The transcript of *hrdB* was used as a control.

tion, pSPM528, did not restore the production (Table 2). This indicated that the most upstream initiation codon is most probably the one used for *srm22* expression.

The level of spiramycin production obtained when pSPM524 was introduced into SPM249 (the *srm22* disruption mutant) was not as good as the one obtained with the wild-type strain. Another construction was made, in which the long form of *srm22*, together with its promoter region (beginning 144 bp upstream of the transcription start point), was cloned into pUWL201. When this plasmid, pSPM525, was introduced into SPM249, it restored spiramycin production and increased it by 1.8-fold compared to the level for the wild-type strain (Table 2).

For the overexpression of *srm40*, a single construction, pSPM75, was made, in which a DNA fragment, containing the complete *srm40* coding region and beginning 92 bp upstream of the initiation codon, was cloned into pUWL201 under the control of *ermE*p*. When the plasmid pSPM75, expressing Srm40, was introduced into the *srm40* deletion mutant (strain SPM107), it restored and increased 1.8-fold the production of spiramycin (Table 2).

The plasmids pSPM525 and pSPM75 were also introduced into the wild-type strain OSC2, where they increased spiramycin production by factors of 2.2 and 3.8, respectively (Table 2). These results indicate that Srm22 (SrmR) and Srm40 are required for spiramycin biosynthesis and that they act as activators.

Gene transcription analysis of the strains OSC2, SPM249, and SPM107. The transcription of 44 genes from the region of the spiramycin biosynthetic cluster was analyzed by RT-PCR in *S. ambofaciens* OSC2. Total RNA was extracted after 36 h of cultivation, as the time course of biosynthetic gene expression suggested that these genes were abundantly transcribed at this time (Fig. 2). We normally used 25 cycles to detect transcripts. Whenever 25 cycles did not yield a product, analysis was repeated at 27 cycles to distinguish low-level transcription (positive at 27 cycles and negative at 25 cycles). The results of the expression analysis by RT-PCR are presented in Fig. 3.

In the wild-type strain OSC2, all genes studied were transcribed at 36 h, with the exception of *srm44*, which was silent, as previously observed at all time points (Fig. 2). For detection of the transcripts of *srm28*, *srm41*, *srm42*, and *srm43*, 27 PCR cycles were required to obtain a detectable signal.

To identify the genes whose transcription is controlled by Srm22 or Srm40, the same type of analysis was performed with the mutant strains SPM249 and SPM107 with inactivation of *srm22* and *srm40*, respectively. In the *srm22* mutant, SPM249, all genes tested were switched off, except *srm25*, for which a band was clearly visible, and *srm1*, *srm2*, and *srm4*, for which very faint bands were observed. In the *srm40* mutant, SPM107, *srm25* was expressed, and *srm22* was also expressed, but no amplification was observed for most of the genes, and very faint bands were observed for a few of them (*srm1*, *srm2*, and *srm4*).

Taken together, these results indicated that most of the genes of the spiramycin cluster are not transcribed when Srm22 or Srm40 is absent. The transcription of the few genes which did not appear totally dependent on Srm22 or Srm40 was nevertheless decreased in the absence of these activators. These results also suggest that *srm22* is not dependent on Srm40 for its transcription but that *srm40* requires Srm22 to be transcribed. This is in agreement with the time course of their expression, *srm22* being transcribed before *srm40* (Fig. 2).

Hierarchy of the regulators. The results of the transcription analysis suggested that Srm22 might activate the transcription of *srm40*, whose product, in its turn, might activate the transcription of most, if not all, of the genes involved in spiramycin biosynthesis. To confirm the role and the hierarchy of Srm22 and Srm40 in the regulation of spiramycin production, the *srm40* and *srm22* genes were overexpressed in the *srm22*- and *srm40*-disrupted mutants, respectively. For this purpose, pSPM525 (overexpressing Srm22) and pSPM75 (overexpressing Srm40) were introduced into the SPM107 (deletion of *srm40*) and SPM249 (disruption of *srm22*) mutant strains, respectively. The results of spiramycin production are presented in Table 2. Spiramycin was produced when *srm40* was overexpressed in the *srm22* mutant. However, no spiramycin was detected when *srm22* was overexpressed in the *srm40* mutant. This showed that *srm22* requires the presence of *srm40* to exert its activator role on spiramycin biosynthesis.

Taken together, the results of the expression analysis involving the phenotypes of the *srm22* and *srm40* mutants and the

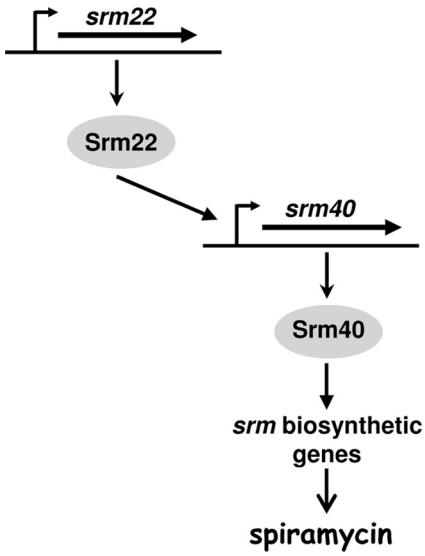

FIG. 4. Proposed model for the regulation of spiramycin biosynthesis in *S. ambofaciens*.

possibilities of restoring spiramycin production in these mutants led us to propose that Srm22 positively controls the transcription of the *srm40* gene. Srm40 positively controls the transcription of most, if not all, of the genes implicated in spiramycin biosynthesis. This regulatory cascade is schematically presented in Fig. 4.

DISCUSSION

In this work, we have shown that two genes of the spiramycin biosynthetic gene cluster, *srm22* (*srmR*) and *srm40*, encode regulatory proteins activating the expression of spiramycin biosynthetic genes. These two activators act at the transcription level.

Concerning Srm22 (SrmR), our results indicate that it is longer than was predicted by Geistlich et al. (20) and is composed of 650 amino acids. The work of Geistlich et al. (20) demonstrated a regulatory role for *srm22* (*srmR*). At that time, SrmR (Srm22) did not present any significant similarity to proteins in databases and these authors proposed that this protein could be the prototype of a new class of regulatory proteins. Now, numerous homologues of Srm22 are present in databases, and most of them are found in actinobacteria. The N-terminal part of Srm22 presents some similarity with the GAF domain. This domain is present in phytochromes and cGMP-specific phosphodiesterases, and proteins with a GAF domain are frequently involved in signal transduction. The C-terminal part of Srm22 presents similarity with protein from the COG2508 family. These proteins are thought to be involved in the regulation of secondary metabolism. The protein most similar to SrmR is CdaR (39% identity and 54% similarity) from *Streptomyces coelicolor*. The *cdaR* gene product is known to positively regulate genes for the biosynthesis of the calcium-dependent antibiotic (CDA), and its activity might be modulated by phosphorylation (26). In addition, CdaR expression was shown to be repressed by AbsA2 and activated by ppGpp (24, 29). This might provide indications for the study of the regulation of Srm22 expression.

The regulator Srm40 is a protein of 387 amino acids in which no known conserved domain was detected. Srm40 is highly similar to AcyB2 from *S. thermotolerans* and to TylR from *S. fradiae*. These two proteins act as activators of the production of the macrolide antibiotics carbomycin in *S. thermotolerans* and tylosin in *S. fradiae*, respectively.

The gene *srm44* is most probably located outside the cluster. Other experiments showed that the inactivation of *srm42* or *srm43* had no effect on spiramycin biosynthesis (Nguyen et al., unpublished), suggesting that they were not part of the cluster. No transcription of *srm44* was observed at different times during growth of *S. ambofaciens*. Therefore, this gene is probably not playing a major role in the regulation of spiramycin biosynthesis, although we cannot exclude that it might play a role under growth conditions that have not been explored in this work. The case of *srm25* is intriguing, as this gene is located among biosynthetic genes, but its expression is not regulated as one of the biosynthetic genes: it was found to be transcribed at all time points studied, and its expression is independent from Srm22 and Srm40. A homologue of *srm25*, *tylV*, is present in the tylosin cluster in *S. fradiae*. The transcription of *tylV* is activated by TylS, a regulator of tylosin biosynthesis (42), but the role of TylV is not clear and TylV is not part of the regulatory cascade controlling tylosin biosynthesis (15). The inactivation of *tylV* decreases tylosin production in *S. fradiae* (16). Similarly, a slight decrease of spiramycin production was observed for the strain SPM508, in which *srm25* is deleted. However, this could be due to variability in the level of spiramycin production between different clones, and this is not sufficient for a major role in the regulation of spiramycin biosynthesis to be attributed to Srm25.

During growth in the spiramycin production medium, the transcript of *srm22* is the first to be detected, and then the transcripts of *srm40* and of the biosynthetic genes are detected. All regulatory and biosynthetic genes are transcribed at 36 h of cultivation, before spiramycin could be detected in the production medium. Then, the transcription of the regulatory and biosynthetic genes decreases, but transcripts are nevertheless detected at 60 h. The timing of their transcription and the results of complementation experiments showed that the two activators Srm22 and Srm40 act in cascade, Srm22 activating the transcription of *srm40* and Srm40 activating the transcription of the spiramycin biosynthetic genes. However, we could not rule out the possibility that one or more genes in the spiramycin cluster also directly require Srm22 (in addition to Srm40) for their expression.

The involvement of two pathway-specific activators in the regulatory cascade controlling spiramycin biosynthesis is reminiscent of the regulation of prodiginin biosynthesis in *S. coelicolor* A3(2). In this strain, RedZ activates the transcription of *redD*; RedD activates the expression of the *red* structural genes (23, 45). The involvement of several activators might offer multiple opportunities for the input of diverse regulatory signals. For example, it should be noted that the rare codon UUA is present in the *srm22* transcript as in the *redZ* transcript, providing an opportunity for translational regulation (12).

Homologues of these two genes, *srm22* and *srm40*, were found associated in other actinobacterial species. In *Strepto-*

myces eurythermus, in the gene cluster directing the biosynthesis of the macrolide antibiotic angolamycin (GenBank accession no. EU232693), the products of the convergent *orf5* and *orf6* genes are similar to Srm22 (38% identity and 51% similarity) and Srm40 (45% identity and 60% similarity), respectively. In *Streptomyces mycarofaciens*, in the gene cluster directing the biosynthesis of the macrolide antibiotic midecamycin (N. Midoh, S. Hoshiko, and T. Murakami, U.S. patent application 2006/0121577 A1) (GenBank accession no. BD420675), the products of the convergent *orf27* and *orf28* genes are similar to Srm40 (66% identity and 74% similarity) and Srm22 (74% identity and 82% similarity), respectively. In *Micromonospora carbonacea*, in the cluster directing the biosynthesis of the macrolide antibiotic rosaramicin (Farnet et al., U.S. patent application US 2003/0113874 A1) (GenBank accession no. AX697977), the products of the convergent *orf14* and *orf15* genes are similar to Srm22 (40% identity and 54% similarity) and Srm40 (46% identity and 59% similarity), respectively. The presence of genes encoding these two regulators in various macrolide biosynthetic gene clusters suggests that the cascade of regulation in which they participate might be conserved in all these actinobacterial species. In addition, homologues of Srm22 and Srm40 are encoded by neighbor genes in the genomes of *Streptomyces* sp. strain C (genes SSNG_03326 and SSNG_03327), of *Streptomyces* sp. strain Mg1 (genes SSAG_07397 and SSAG_07398), and of *Micromonospora* sp. strain M42 (genes MCBG_00153 and MCBG_00159).

The spiramycin biosynthetic genes are related to those involved in the biosynthesis of erythromycin or tylosin, two macrolides for which the biosynthesis has been extensively studied. However, the regulatory mechanisms controlling the expression of these biosynthetic clusters are very different. No regulatory gene was found in the erythromycin biosynthetic gene cluster. Recently, however, an activator of erythromycin biosynthesis was characterized (14). It is encoded by the *bldD* gene, located 1.5 Mb away from the biosynthetic gene cluster. Comparison of BldD expression levels in the wild-type and the erythromycin-overproducing strains of *S. erythraea* suggests that during strain improvement, mutations were introduced in genes that regulate BldD expression (14).

In contrast to the erythromycin cluster, the tylosin biosynthetic gene cluster from *S. fradiae* contains multiple regulatory genes encoding γ-butyrolactone-binding protein homologues (TylP and TylQ), SARP regulators (TylS and TylT), and putative regulators which do not belong to large families of regulatory proteins (TylR and TylU). TylT is not essential for tylosin production, but detailed studies of the regulation of tylosin biosynthesis have shown that the products of the other five regulatory genes are involved in a complex regulatory cascade (for a review, see reference 15). At the bottom of this cascade is TylR, which directly activates the transcription of the tylosin biosynthetic genes. During the empirical strain improvement program for enhancing tylosin production by *S. fradiae*, only one of the five regulatory genes, *tylQ* was altered, having undergone a single point mutation that inactivated its product (43). As TylQ is a repressor of *tylR* expression, this mutation was highly beneficial for tylosin production. Interestingly, another single-nucleotide mutation affecting *tylQ* was also observed in an independent strain improvement program by genome shuffling (49).

The situation encountered for the regulation of spiramycin biosynthesis is much simpler than that encountered for tylosin biosynthesis. However, it should be noted that similar regulators, TylR and Srm40, presenting 43% sequence identity, are located at the bottoms of both regulatory cascades and are involved in the activation of the transcription of the biosynthetic genes. In *S. ambofaciens*, further studies are required for knowledge of how the expression of *srm22* is regulated and how the two regulatory proteins activate the transcription of their target genes. It might also be of interest to know if the regulatory genes have been altered during the strain improvement program that led to the overproducing strain used for industrial spiramycin production.

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