Organization of the biosynthetic gene cluster for the macrolide antibiotic spiramycin in *Streptomyces ambofaciens*

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Spiramycin, a 16-membered macrolide antibiotic used in human medicine, is produced by *Streptomyces ambofaciens*; it comprises a polyketide lactone, platenolide, to which three deoxyhexose sugars are attached. In order to characterize the gene cluster governing the biosynthesis of spiramycin, several overlapping cosmids were isolated from an *S. ambofaciens* gene library, by hybridization with various probes (spiramycin resistance or biosynthetic genes, tylosin biosynthetic genes), and the sequences of their inserts were determined. Sequence analysis showed that the spiramycin biosynthetic gene cluster spanned a region of over 85 kb of contiguous DNA. In addition to the five previously described genes that encode the type I polyketide synthase involved in platenolide biosynthesis, 45 other genes have been identified. It was possible to propose a function for most of the inferred proteins in spiramycin biosynthesis, in its regulation, in resistance to the produced antibiotic or in the provision of extender units for the polyketide synthase. Two of these genes, predicted to be involved in deoxysugar biosynthesis, were inactivated by gene replacement, and the resulting mutants were unable to produce spiramycin, thus confirming their involvement in spiramycin biosynthesis. This work reveals the main features of spiramycin biosynthesis and constitutes a first step towards a detailed molecular analysis of the production of this medically important antibiotic.

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INTRODUCTION

Macrolides are a large class of natural products, many of which are effective antibacterial agents. They are mostly produced by actinobacteria belonging to the genera *Streptomyces*, *Saccharopolyspora* or *Micromonospora*. Their structures consist of a macrocyclic lactone to which one or

several sugar moieties are attached. The macrolactone is synthesized through the repeated condensation of simple carboxylic acid units catalysed by type I polyketide synthase (PKS). Macrolides bind to the large subunit of the bacterial ribosome, thereby inhibiting protein synthesis, which confers on them their antibacterial activity (Gale *et al.*, 1981; Hansen *et al.*, 2002). They also inhibit ribosome assembly in some bacteria such as *Staphylococcus aureus* (Champney & Tober, 2000). Macrolides are an old family of antibacterial agents, as erythromycin was introduced in clinical practice in the 1950s, but they still play an important role in the chemotherapy of infectious diseases. This is mostly due to the development of semi-synthetic derivatives, for instance clarithromycin, azithromycin and more recently ketolides, with improvements to features such as stability, pharmacokinetics and activity against resistant bacteria (Schnfeld & Kirst, 2002). The characterization of macrolide biosynthetic gene clusters has opened the way to rational genetic yield improvement of production strains (Stratigopoulos *et al.*, 2004) and to the development of combinatorial strategies for the synthesis of new macrolides (Butler *et al.*, 2002;

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Abbreviations: NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the regions downstream and upstream of the PKS genes are AM709783 and AM709784.

Details of the construction of pWED2 and pOSV238 are available as supplementary material with the online version of this paper.

Hutchinson & McDaniel, 2001; Katz, 1997; Long *et al.*, 2002; Melançon & Liu, 2007).

Streptomyces ambofaciens produces spiramycin, composed of a 16-membered polyketide lactone ring (platenolide) on which two amino sugars (mycaminose and forosamine) and one neutral sugar (mycarose) are attached. Spiramycin is produced as a mixture of three major compounds differing by acyl substitutions at the position of the hydroxyl group at carbon 3 (Fig. 1). Spiramycin is used in human medicine as an antibacterial agent (Smith, 1988) and also for the treatment of *Toxoplasma* infections (McCabe & Oster, 1989). Several genes that confer spiramycin resistance in heterologous hosts have been cloned from *S. ambofaciens* (Pernodet *et al.*, 1999; Richardson *et al.*, 1987), and spiramycin biosynthetic genes were shown to be located in the vicinity of one of the resistance genes, *srmB* (Richardson *et al.*, 1990). The five genes encoding the PKS were characterized (Kuhstoss *et al.*, 1996), and the product of another gene, *srmR*, was shown to be a transcriptional activator (Geistlich *et al.*, 1992). However, only partial sequence data were available, and the complete cluster has not been characterized.

The cloning, sequencing and analysis of the gene cluster for the biosynthesis of spiramycin in *S. ambofaciens* is reported here. This provides information on its gene organization and on the spiramycin biosynthetic pathway and an indication of the regulation of its expression. This work will facilitate further genetic and biochemical studies of this cluster and also give access to genes that could be helpful for the combinatorial biosynthesis of new macrolide molecules.

METHODS

Strains, plasmids and culture conditions. Strains and plasmids used in this study are described in Table 1. The construction of some of the vectors used in this work is described in the supplementary material. Standard media and culture conditions were used (Kieser *et al.*, 2000; Sambrook & Russell, 2001). The following antibiotics were added to the medium when required for selection: ampicillin

(Amp), apramycin (Apr), hygromycin B (Hyg), puromycin (Pur) and thiostrepton (Tsr). For spiramycin production, *S. ambofaciens* strains were grown in MP5 liquid medium as described previously (Pernodet *et al.*, 1993). The detection and quantification of spiramycin in culture supernatants was performed by bioassay or HPLC as described previously (Gourmelen *et al.*, 1998).

DNA manipulations. All DNA manipulations, cloning and transformation experiments in *Escherichia coli* and *S. ambofaciens* were performed according to standard protocols (Kieser *et al.*, 2000; Sambrook & Russell, 2001). Radiolabelled DNA probes were prepared with the Megaprime DNA labelling system (GE Healthcare).

Construction and screening of cosmid libraries. Two cosmid libraries of *S. ambofaciens* DNA were constructed, using the cosmid pWED1 for the strain ATCC 23877 library and pWED2 for the strain OSC2 library. OSC2 is a derivative of ATCC 23877. These two *S. ambofaciens* strains differ only by the presence of pSAM2, which is absent in OSC2; their growth, differentiation and antibiotic production abilities are indistinguishable (Raynal *et al.*, 2006). Total DNA was partially digested with *Bam*HI and fragments of 35 to 45 kb were ligated with *Bam*HI-digested cosmid vectors. *In vitro* packaging was performed with the Packagene Lambda DNA packaging system (Promega), according to the manufacturer's protocol. The phage particles were used to infect the *E. coli* SURE strain. Colony hybridization was performed on about 2000 clones with various radiolabelled probes. Several hybridizing clones were obtained, the cosmids they contained were characterized by their restriction patterns and some were chosen for sequencing and further studies. The cosmids pOS49.1, pSPM5 and pSMP7 came from the first library, constructed with pWED1. The cosmid pSPM36 came from the second library, constructed with pWED2.

DNA sequencing, sequence analysis and annotation. DNA sequencing of cosmids pSPM5 and pSPM7 was performed by standard shotgun cloning followed by automated DNA sequencing carried out on double-stranded DNA templates to obtain at least sixfold coverage. Sequencing was done by Eurogentec or Genome Express. Primer walking was used to close the gaps. Fragments from the cosmids pOS49.1 and pSPM36 were sequenced by primer walking. A software program, combining an analysis similar to that performed by Frame plot (Ishikawa & Hotta, 1999) with an analysis similar to that performed by Glimmer (Salzberg *et al.*, 1998) (C. Gerbaud, unpublished), was used to identify protein-coding regions. Sequence analysis and comparisons were performed using BLAST (Altschul *et al.*, 1997) and CD-Search (Marchler-Bauer & Bryant, 2004).

Targeted disruption of spiramycin biosynthetic genes. The construction of an *orf3* in-frame deletion mutant (strain SPM24) was described previously (Raynal *et al.*, 2006).

An in-frame *orf2* deletion mutant was also created by PCR targeting (Chaveroche *et al.*, 2000; Gust *et al.*, 2003; Yu *et al.*, 2000) followed by *in vivo* excision of the interrupting resistance cassette (Raynal *et al.*, 2006). For this purpose, a 4.5 kb *Eco*RI–*Bam*HI fragment containing *orf2* and flanking genes, from *srmG1* to *orf3*, was cloned into the plasmid pUC19, yielding pOS49.99. This plasmid was introduced into *E. coli* strain KS272 already containing pKOBEG (Chaveroche *et al.*, 2000) and expressing the λ -RED recombination system. The Apr resistance cassette *att30aac*– (Raynal *et al.*, 2006) was amplified from pOSV211 using the primers ORF2A (5'-CCCGCGCGGCAGCCTCT-CCGTGATCGAGTCCGGCGTGACCATCGCGCGCGCTTCGTTCCG-3') and ORF2B (5'-GCTCCGTGCGTCATGCAGGAAGGTGTCGTAGTCGCGGTAGATCTGCCTCTTCGTCCCGAA-3') [sequences identical to the beginning (ORF2A) and end (ORF2B) of the *orf2* coding sequence are underlined]. The resulting PCR product was used

Fig. 1. Structure of spiramycins.

Table 1. Bacterial strains and plasmids used in this study

Strain	Description	Source/reference
<i>S. ambofaciens</i>		
ATCC 23877	Wild-type <i>S. ambofaciens</i> strain	ATCC
OSC2	Derivative of <i>S. ambofaciens</i> ATCC 23877 devoid of pSAM2	Raynal <i>et al.</i> (2006)
SPM21	<i>orf2::att3Ωaac-</i> in OSC2	This work
SPM22	<i>orf2::att3</i> in OSC2	This work
SPM23	<i>orf3::att1Ωhyg-</i> in OSC2	Raynal <i>et al.</i> (2006)
SPM24	<i>orf3::att1</i> in OSC2	Raynal <i>et al.</i> (2006)
<i>E. coli</i>		
DH5 α	General cloning host strain	Promega
KS272/pKOBEG	Used for PCR-targeted mutagenesis	Chaveroche <i>et al.</i> (2000)
SURE	Host strain for cosmid libraries	Stratagene
S17.1	Host strain for conjugation from <i>E. coli</i> to <i>S. ambofaciens</i>	Simon <i>et al.</i> (1983)
Plasmid		
pWED1	Amp ^R , cosmid used for construction of <i>S. ambofaciens</i> genomic library	Gourmelen <i>et al.</i> (1998)
pWED2	Amp ^R , Pur ^R , <i>oriT</i> , cosmid used for construction of <i>S. ambofaciens</i> genomic library	This work
pUWL201	Amp ^R , Tsr ^R , <i>E. coli</i> - <i>Streptomyces</i> shuttle vector for gene expression in <i>S. ambofaciens</i> under the control of the <i>ermE*</i> promoter	Doumith <i>et al.</i> (2000)
pOSV211	Amp ^R , Apr ^R , <i>att3Ωaac-</i> cassette (Raynal <i>et al.</i> , 2006) cloned into the vector pGP704Not (Chaveroche <i>et al.</i> , 2000; Miller & Mekalanos, 1988)	This work
pOSV238	Hyg ^R , derivative of pBK-CMV (Stratagene) in which the <i>neo</i> gene has been replaced by the Ω hyg cassette	This work
pOSV508	Amp ^R , Tsr ^R , <i>E. coli</i> - <i>Streptomyces</i> shuttle plasmid expressing the Xis and Int proteins for site-specific excision of excisable cassettes	Raynal <i>et al.</i> (2006)
pOS49.1, pSPM5, pSPM7	Cosmids from the <i>S. ambofaciens</i> ATCC 23877 gene library containing part of the spiramycin cluster	This work
pSPM36	Cosmid from the <i>S. ambofaciens</i> OSC2 gene library containing part of the spiramycin cluster	This work
pOS49.99	Amp ^R , 4.5 kb <i>EcoRI</i> - <i>Bam</i> HI fragment containing <i>orf2</i> and flanking genes, cloned into pUC19	This work
pSPM17	Amp ^R , Apr ^R , <i>orf2</i> inactivation (<i>orf2::att3Ωaac-</i>) by PCR targeting into pOS49.99	This work
pSPM21	Hyg ^R , Apr ^R , insert from pSPM17 cloned into pOSV238	This work
pSPM30	Amp ^R , Tsr ^R , coding sequence of <i>orf2</i> cloned into pUWL201	This work
pSPM33	Amp ^R , Tsr ^R , coding sequence of <i>orf1a</i> from <i>S. fradiae</i> cloned into pUWL201	This work

to transform *E. coli* strain KS272 containing pKOBEG and pOS49.99. After selection for Apr resistance, clones were obtained in which most of the plasmid-borne *orf2* coding sequence has been replaced by the *att3 Ω aac-* cassette, through λ -RED-mediated recombination. The resulting plasmid was named pSPM17. The insert of pSPM17, obtained as a *EcoRI*/Klenow-*Xba*I DNA fragment, was cloned in the plasmid pOSV238 (Hyg^R), previously digested by *Bam*HI/Klenow-*Xba*I, yielding pSPM21. It was introduced into *S. ambofaciens* OSC2 via protoplast transformation and Apr selection was applied. Apr^R transformants were screened for sensitivity to Hyg, indicating a double-crossover allelic exchange. This was confirmed by PCR and Southern blot analysis. One *orf2::att3 Ω aac-* *S. ambofaciens* mutant strain was chosen for further studies and named SPM21. In order to excise the *att3 Ω aac-* cassette, the plasmid pOSV508, expressing the excisionase and the integrase from pSAM2, was introduced into strain SPM21. As a result, the Apr-resistance determinant was excised through site-specific recombination, leading to an in-frame *orf2* deletion mutant. After curing of pOSV508, the resulting strain, verified by PCR amplification and sequencing of the PCR products, was named SPM22.

Complementation of SPM22 and SPM24 mutants. The plasmid pOS49.52, a pIJ903 derivative expressing *orf3* under the control of the *ermE** promoter, was described previously (Raynal *et al.*, 2006). It was introduced into strain SPM24 by protoplast transformation.

The *orf2* coding sequence was amplified with the primers KF8 (5'-AAGCTTCCGGGTCGACTGGAACTGAACCCGAGGG-3', *Hind*III site underlined) and KF11 (5'-GGATCCGATGGTCATGTGAGGC-TCCGTGCGTCAT-3', *Bam*HI site underlined). The resulting PCR product was cloned into pUWL201 digested by *Bam*HI and *Hind*III, yielding pSPM30, in which *orf2* is expressed under the control of the *ermE** promoter. The SPM22 mutant was also complemented by *orf1a* from *Streptomyces fradiae*, the producer of tylosin. The primers KF12 (5'-AAGCTTGCAGGAGCAGCCCGATGGCGGCGAGCA-3', *Hind*III site underlined) and KF13 (5'-GGATCCCCCTGTCA-CGGGTGGCTCCTGCCGGCCCC-3', *Bam*HI site underlined) were used to amplify *orf1a* from *S. fradiae* total DNA. The resulting PCR product was cloned into pUWL201 digested by *Bam*HI and *Hind*III to obtain pSPM33. The plasmids pSPM30 and pSPM33 were introduced into strain SPM22 by protoplast transformation.

RESULTS AND DISCUSSION

Cloning and sequencing of the spiramycin biosynthetic gene cluster

Large cloned DNA fragments of the spiramycin biosynthetic gene cluster were isolated by screening *S. ambofaciens* cosmid libraries by colony hybridization with various probes. The first probe (P1; Fig. 2a) corresponded to *tylB*, a gene from the tylosin cluster in *S. fradiae* involved in the biosynthesis of mycaminose (Merson-Davies & Cundliffe, 1994), a sugar also found in the spiramycin molecule. A *tylB* orthologue is present in *S. ambofaciens*, as some cosmids from an *S. ambofaciens* library restored tylosin biosynthesis when introduced into a tylosin non-producing mutant affected in *tylB* (Richardson *et al.*, 1990). From a colony hybridizing with the probe P1, the cosmid pOS49.1 was extracted. A 3.3 kb *SacI* DNA fragment, responsible for the hybridization, was identified, subcloned and sequenced. Sequence analysis showed the presence of four coding sequences (two complete, two partial). One of them encoded a protein showing end-to-end similarity to TylB

(64 % identity). Inactivation of this coding sequence (see below) abolished spiramycin production, confirming that the gene identified was part of the spiramycin cluster.

More sequence data were generated from pOS49.1, revealing that this region was located between two genes sequenced previously, *srnX* (*orf9c*) on one side and *srnG* on the other (Geistlich *et al.*, 1992) (Fig. 2b). In order to select other cosmids, the library was probed again with three probes. Two of them were fragments of pOS49.1, each located at one end of the sequenced region (probes P2 and P3; Fig. 2a). The third one (P4; Fig. 2a) contained the *srnD* gene, a spiramycin-resistance determinant cloned previously (Pernodet *et al.*, 1999; M.-H. Blondelet-Rouault, unpublished) and part of the upstream gene. The products of *srnD* and of the upstream gene were similar to those of *mdmA* and *mdmB* from *Streptomyces mycarofaciens*, involved respectively in midecamycin resistance and biosynthesis (Hara & Hutchinson, 1992). Several cosmids hybridized with these probes, although not with all three of them, and from their restriction maps two cosmids that showed minimal overlap with pOS49.1 were selected. These

Fig. 2. Spiramycin biosynthetic gene cluster. (a) Schematic representation of a ~90 kb locus from the *S. ambofaciens* chromosome. The positions of the fragments hybridizing with the various probes used are indicated (P1 to P5). Probe P1, 1 kb *NaeI* fragment containing most of the *tylB* gene from *S. fradiae*; probe P2, 3.7 kb *BamHI*–*PstI* fragment containing part of *srnGI*, *orf1*, *orf2* and part of *orf3*; probe P3, 2 kb *PstI*–*BamHI* fragment containing *orf7* and *orf8*; probe P4, 1.8 kb *PstI*–*BamHI* fragment containing *srnD* (*orf7**); probe P5, PCR product containing part of *orf23c*, *orf24c* and part of *orf25c*. Lines below correspond to the positions of the inserts of some of the cosmids, thick lines indicating regions of the insert that have been sequenced. Hatched lines indicate the two regions sequenced previously: the PKS genes (Burgett *et al.*, 1999; Kuhstoss *et al.*, 1996) and *srnX* (*orf9c*), *srnR* (*orf10*) and *srnB* (*orf11c*) (Geistlich *et al.*, 1992). (b) Genetic organization of the spiramycin biosynthetic gene cluster. In the gene names, 'c' indicates those orientated to the left. The proposed functions of the gene products in spiramycin biosynthesis are indicated by various filling patterns.

were designated pSPM5 and pSPM7 (Fig. 2a). Sequence analysis showed that the right end of the pSPM5 insert could still be located within the spiramycin cluster. Therefore, another hybridization experiment was performed using probe P5, corresponding to the right end of the pSPM5 insert (Fig. 2a). One cosmid, named pSPM36, overlapping pSPM5 and spanning the remainder of the spiramycin cluster, was partially sequenced (Fig. 2a).

From the analysis of these sequences and their alignment with the sequences of the five PKS genes (Burgett *et al.*, 1999), it was possible to deduce the gene organization of the region. The five *srnG* genes, encoding the PKS, occupy about 40 kb. Upstream of *srnGI*, 34 ORFs (*orf1* to *orf34c*) account for about 40 kb, and downstream of *srnGV*, 11 ORFs (*orf1*c* to *orf11**) occupy about 12 kb. The inferred products of these ORFs were compared to protein databases. From these comparisons, most of the deduced protein products could be assigned putative functions in spiramycin biosynthesis (Fig. 2; Table 2).

Genes involved in the provision of extender units for the PKS, in platenolide biosynthesis and modification

The five megagenes, *srnGI* to *srnGV*, encoding the PKS have been sequenced and studied previously (Burgett *et al.*, 1999; Kuhstoss *et al.*, 1996). The organization of the modules within the five *SrmG* proteins is the same as in other known PKS involved in the biosynthesis of 16-membered macrolides, and the domain organization is similar to that of the tylosin or niddamycin PKS (McDaniel *et al.*, 2005). However, the extender units used for platenolide biosynthesis are different from those used for tylactone biosynthesis. The platenolide PKS was predicted to incorporate seven extender units: four malonyl-CoA, one ethylmalonyl-CoA, one methylmalonyl-CoA and one unknown product, incorporated by module 6 (Kuhstoss *et al.*, 1996).

The product of *orf4*c*, a crotonyl-CoA reductase, presumably contributes to the flux of ethylmalonyl-CoA. Crotonyl-CoA reductase genes are found in other polyketide biosynthetic clusters where the PKS uses ethylmalonyl extender units, such as the tylosin or concanamycin clusters (Cundliffe *et al.*, 2001; Haydock *et al.*, 2005).

Five proteins, the products of *orf5**, *orf22c*, *orf23c*, *orf24c* and *orf25c*, are similar to FkbG, FkbH, FkbI, FkbJ and FkbK, respectively, proteins that are involved in the biosynthesis of methoxymalonyl-acyl carrier protein (ACP) in *Streptomyces hygroscopicus* var. *ascomyceticus*, the producer of the macrolide FK520 (ascomycin) (Wu *et al.*, 2000). The greatest similarities are observed with proteins encoded in the geldanamycin and concanamycin clusters (Haydock *et al.*, 2005; Rascher *et al.*, 2003). Recently, sequence comparison of various acyltransferase domains incorporating methoxymalonate allowed identification of a sequence motif predicting the incorporation of this extender unit (Haydock *et al.*, 2005). The conservation

of this motif in the AT domain of module 6 in *SrmGIV*, together with the presence of the five genes involved in methoxymalonyl-ACP biosynthesis, strongly support the incorporation of methoxymalonate by module 6.

Several other gene products are predicted to be involved in lactone ring biosynthesis or modification. A type II thioesterase is encoded by *orf6*. Such enzymes play an editing role in the biosynthesis of polyketides (Butler *et al.*, 1999; Heathcote *et al.*, 2001). The product of *orf1* is a cytochrome P₄₅₀ hydroxylase, probably involved in ring hydroxylation at position C20 (Fig. 1). The deduced acyltransferase encoded by *orf6** is similar to acyltransferases involved in lactone ring acylation. Therefore, *Orf6** might be involved in ring acylation at position 3, leading to the biosynthesis of spiramycins II and III.

Another important ring modification during the early steps of spiramycin biosynthesis is that at position 9. The PKS generates platenolide I, with a keto group at position 9 (Omura *et al.*, 1979), which is later converted to platenolide II, with a hydroxyl group at C9, by a post-PKS ketoreductase. Indeed, the presence of such an activity in *S. ambofaciens* is attested by the fact that the keto group at C9 in tylactone could be modified and that forosamine could then be attached at this position by enzymes from *S. ambofaciens* (Omura *et al.*, 1983). Such post-PKS reductases have been described previously, for instance AveF from *Streptomyces avermitilis* (Ikeda *et al.*, 1999), but none of the spiramycin cluster deduced proteins presented similarity to characterized post-PKS ketoreductases. Nonetheless, the product of *orf31* shows similarity to oxidoreductases (COG0667, pfam00248) and could be a candidate to perform this reaction.

No gene encoding the phosphopantetheinyl transferase required for the post-translational modification of the PKS is present in the spiramycin cluster. A phosphopantetheinyl transferase is encoded by *alpN* in the *alp* cluster, located in the terminal inverted repeats of the *S. ambofaciens* chromosome (Pang *et al.*, 2004), but it is not known whether this enzyme is the one involved in the spiramycin PKS modification.

Genes for deoxysugar biosynthesis

The three deoxysugars present in the structure of spiramycin are also found in other macrolide molecules whose biosyntheses have been thoroughly studied. Mycaminose is found in tylosin, produced by *S. fradiae*; the genes involved in its biosynthesis have been identified and a biosynthetic pathway has been established (Cundliffe *et al.*, 2001; Melançon *et al.*, 2005). Mycarose is found in erythromycin and tylosin and its biosynthetic genes were first characterized in *Saccharopolyspora erythraea* (Gaisser *et al.*, 1997, 1998; Summers *et al.*, 1997) and *S. fradiae* (Bate *et al.*, 2000; Takahashi *et al.*, 2005). Forosamine biosynthetic genes were studied in *Saccharopolyspora spinosa*, the spinosyn producer (Waldron *et al.*, 2001; Zhao *et al.*,

Table 2. Components of the spiramycin biosynthetic gene cluster

Abbreviations: For, forosamine; Mcn, mycaminose; Mcr, mycarose. Percentages of identity/similarity refer to deduced amino acid sequence comparisons.

ORF	Product size (aa)	Best BLAST hit			Putative function	Proposed role in spiramycin biosynthesis
		Accession no. or reference	Species	Identity/similarity (%)		
<i>orf11*</i>	Partial	CAA67249	<i>Streptomyces pristinaespiralis</i>	49/61	Non-ribosomal peptide synthetase	Not involved
<i>orf10*</i>	75	CAB38589	<i>Streptomyces coelicolor</i> A3(2)	69/89	MbtH-like protein	Not involved
<i>orf9*</i>	293	CAC22118	<i>Streptomyces griseus</i>	62/72	ABC transporter	Not involved
<i>orf8*</i>	276	AAX31554	<i>Streptomyces filamentosus</i>	63/77	ATP-binding protein	Not involved
<i>orf7*c</i>	282	CAA66307	<i>Streptomyces fradiae</i>	56/72	ABC transporter transmembrane protein	Resistance
<i>orf6*</i>	414	AAA26781	<i>Streptomyces mycarofaciens</i>	76/85	rRNA methyltransferase	Lactone ring modification
<i>orf5*</i>	223	AAA26782	<i>Streptomyces mycarofaciens</i>	85/93	Acyltransferase	Provision of extender units for PKS
<i>orf4*c</i>	449	ABG67707	<i>Streptomyces mycarofaciens</i>	87/92	O-Methyltransferase	Provision of extender units for PKS
<i>orf3*c</i>	421	ABG67706	<i>Streptomyces mycarofaciens</i>	90/95	Crotonyl-CoA reductase	Sugar attachment
<i>orf2*c</i>	423	ABG67705	<i>Streptomyces mycarofaciens</i>	61/66	Glycosyltransferase	Sugar attachment
<i>orf1*c</i>	236	ABG67704	<i>Streptomyces mycarofaciens</i>	82/88	Glycosyltransferase auxiliary protein	Sugar biosynthesis (Mcn or For)
<i>srnGV</i>	1891	Burgett <i>et al.</i> (1999)			NDP-aminohexose N-dimethyltransferase	Platenolide biosynthesis
<i>srnGIV</i>	1596	Burgett <i>et al.</i> (1999)			Type I PKS	Platenolide biosynthesis
<i>srnGIII</i>	3724	Burgett <i>et al.</i> (1999)			Type I PKS	Platenolide biosynthesis
<i>srnGII</i>	2008	Burgett <i>et al.</i> (1999)			Type I PKS	Platenolide biosynthesis
<i>srnGI</i>	4552	Burgett <i>et al.</i> (1999)			Type I PKS	Platenolide biosynthesis
<i>orf1</i>	403	ABG67708	<i>Streptomyces mycarofaciens</i>	80/88	Cytochrome P ₄₅₀ hydroxylase	Lactone ring modification
<i>orf2</i>	116	Melançon <i>et al.</i> (2007)†	<i>Streptomyces fradiae</i>	58/71	NDP-hexose isomerase	Sugar biosynthesis (Mcn)
<i>orf3</i>	388	ABG67699	<i>Streptomyces mycarofaciens</i>	79/85	NDP-hexose aminotransferase	Sugar biosynthesis (Mcn)
<i>orf4</i>	302	ABG67700	<i>Streptomyces mycarofaciens</i>	83/89	NDP-glucose synthase	Sugar biosynthesis
<i>orf5</i>	345	ABG67702	<i>Streptomyces mycarofaciens</i>	84/88	NDP-glucose dehydratase	Sugar biosynthesis
<i>orf6</i>	267	ABG67701	<i>Streptomyces mycarofaciens</i>	69/78	Type II thioesterase	PKS editing
<i>orf7</i>	469	ABG67703	<i>Streptomyces mycarofaciens</i>	72/78	NDP-hexose dehydratase	Sugar biosynthesis (Mcr and For)
<i>orf8</i>	382	AAG23279	<i>Saccharopolyspora spinosa</i>	61/74	NDP-hexose aminotransferase	Sugar biosynthesis (For)
<i>orf9c</i> = <i>srnX</i>	239	CAA45052	<i>SrmX</i> (<i>S. ambofaciens</i>)		NDP-aminohexose N-dimethyltransferase	Sugar biosynthesis (Mcn or For)
<i>orf10</i> = <i>srnR</i>	655	CAA45051	<i>SrmR</i> (<i>S. ambofaciens</i>)		Regulatory protein	Regulatory protein
<i>orf11c</i> = <i>srnB</i>	550	CAA45050	<i>SrmB</i> (<i>S. ambofaciens</i>)		ABC transporter	Resistance
<i>orf12</i>	433	AAF72550	<i>Streptomyces fradiae</i>	71/83	ATP-binding protein	Sugar biosynthesis (For)
<i>orf13c</i>	481	CAA18333	<i>Streptomyces coelicolor</i> A3(2)	75/86	NDP-hexose dehydratase	Putatively involved in regulation
<i>orf14</i>	347	EAR69813	<i>Bacillus cereus</i>	37/54	GTPase	
					NDP-sugar epimerase	

Table 2. cont.

ORF	Product size (aa)	Best BLAST hit			Putative function	Proposed role in spiramycin biosynthesis
		Accession no. or reference	Species	Identity/similarity (%)		
<i>orf15c</i>	346	ABB69760	<i>Streptomyces</i> sp. Tu6071	52/62	NDP-hexose ketoreductase	Sugar biosynthesis (For)
<i>orf16</i>	412	CAA57471	<i>Streptomyces fradiae</i>	44/50	Glycosyltransferase auxiliary protein	Sugar attachment
<i>orf17</i>	423	ABB52547	<i>Streptomyces</i> sp. KCTC 0041BP	53/69	Glycosyltransferase	Sugar attachment
<i>orf18</i>	394	CAE17548	<i>Streptomyces giseus</i>	41/54	Glycosyltransferase	Sugar attachment
<i>orf19</i>	315	AAD41822	<i>Streptomyces fradiae</i>	58/70	NDP-hexose ketoreductase	Sugar biosynthesis (Mcr)
<i>orf20</i>	335	AAB84068	<i>Saccharopolyspora erythraea</i>	75/86	NDP-hexose ketoreductase	Sugar biosynthesis (Mcr)
<i>orf21c</i>	410	AAD41823	<i>Streptomyces fradiae</i>	82/88	NDP-hexose methyltransferase	Sugar biosynthesis (Mcr)
<i>orf22c</i>	371	ABI93783	<i>Streptomyces hygroscopicus</i>	71/79		Provision of extender units for PKS
<i>orf23c</i>	373	AAZ94394	<i>Streptomyces neyagawaensis</i>	68/80	Dehydrogenase	Provision of extender units for PKS
<i>orf24c</i>	103	AAZ94393	<i>Streptomyces neyagawaensis</i>	64/68	Acyl carrier protein	Provision of extender units for PKS
<i>orf25c</i>	288	ABI93786	<i>Streptomyces hygroscopicus</i>	64/72	Dehydrogenase	Provision of extender units for PKS
<i>orf26</i>	405	AAD41824	<i>Streptomyces fradiae</i>	65/75	Glycosyltransferase	Sugar attachment
<i>orf27</i>	206	AAD41825	<i>Streptomyces fradiae</i>	70/80	NDP-hexose epimerase	Sugar biosynthesis (Mcr)
<i>orf28c</i>	387	BAA32409	<i>Streptomyces thermotolerans</i>	69/77	TylR-like regulatory protein	Putatively involved in regulation
<i>orf29</i>	327	AAK19890	<i>Sorangium cellulosum</i>	31/47	Glycosidase	Putatively involved in antibiotic reactivation
<i>orf30c</i>	282	ABC28150	<i>Hahella chejuensis</i>	30/50	NDP-sugar epimerase	
<i>orf31</i>	324	AAZ55423	<i>Thermobifida fusca</i>	61/72	Oxidoreductase	Lactone ring modification
<i>orf32c</i>	503	BAC71139	<i>Streptomyces avermitilis</i>	44/57	GntR-family regulatory protein	Putatively involved in regulation
<i>orf33</i>	91	ABG98233	<i>Rhodococcus</i> sp. RHA1	56/65	Conserved hypothetical protein	Not involved
<i>orf34c</i>	Partial	CAA16189	<i>Streptomyces coelicolor</i> A3(2)	90/94	Arabinofuranosidase	Not involved

†This match was obtained with TBLASTN, as the product of *orf1a* from *S. fradiae* is not in protein databases.

2005). Sequence comparisons, particularly with proteins from *S. fradiae*, *Saccharopolyspora erythraea* and *Saccharopolyspora spinosa*, whose function in mycaminose, mycarose and forosamine biosynthesis is well characterized, enabled us to identify a potential enzyme for each of the steps of the proposed pathways (Fig. 3).

The products of *orf14* and *orf30c* are both related to NDP-sugar epimerases. However, with the proposed biosynthetic routes, no role could be found for these two proteins in the biosynthesis of spiramycin deoxysugars.

Genes for sugar attachment

Four genes, *orf3*c*, *orf17*, *orf18* and *orf26*, encode putative glycosyltransferases that could be involved in the

attachment of sugars. It was surprising to find four glycosyltransferase genes, as only three sugars are present in the spiramycin molecule. Several hypotheses can be proposed. Firstly, one of these genes might not be expressed or its product could be inactive, but the alignment of the four putative glycosyltransferases with functional glycosyltransferases did not reveal obvious differences suggesting that one of them could be inactive. Secondly, two different glycosyltransferases could perform the same reaction independently, but, to our knowledge, no such case has been described previously. Thirdly, one of the glycosyltransferases could be involved in macrolide inactivation by glycosylation and play a role in resistance towards the produced antibiotic (see below). Sequence comparisons with characterized enzymes that transfer mycaminose, mycarose or forosamine, and analyses

Fig. 3. Proposed pathways and proteins involved in the biosynthesis of the three spiramycin deoxysugars from glucose 1-phosphate.

performed with SEARCHGTr, a program for analysis of glycosyltransferases involved in glycosylation of secondary metabolites (Kamra *et al.*, 2005), did not allow us to make unambiguous predictions concerning the sugars transferred by these four putative glycosyltransferases. In addition, two genes, *orf2**c and *orf16*, encode products that belong to the recently identified family of glycosyltransferase accessory proteins, required for efficient glycosylation (Borisova *et al.*, 2004; Hong *et al.*, 2007; Melançon *et al.*, 2004). These two genes are located immediately upstream of glycosyltransferase genes, as is generally the case. Their products share 42% amino acid sequence identity. The presence of two glycosyltransferase/auxiliary protein pairs suggests that each auxiliary protein is preferentially associated with a glycosyltransferase, as shown by Hong *et al.* (2007).

Resistance genes

srmB (*orf11c*) was previously isolated from *S. ambofaciens* as a spiramycin-resistance determinant (Richardson *et al.*, 1987); it encodes the ATP-binding component of an ABC transporter and is thought to be involved in spiramycin efflux (Schoner *et al.*, 1992). *orf7** was previously isolated from *S. ambofaciens* as a spiramycin-resistance determinant

called *srmD* (Pernodet *et al.*, 1999); it encodes a methyltransferase modifying 23S rRNA (M.-H. Blondelet-Rouault; unpublished data).

Inactivation of macrolides by glycosylation has been observed in *S. ambofaciens* extracts. Two distinct glycosyltransferase activities could be identified from their antibiotic substrate profiles. The gene *gimA*, encoding the glycosyltransferase responsible for one of these activities, has been detected, and is not located within the spiramycin biosynthetic cluster (Gourmelen *et al.*, 1998). However, the gene responsible for the second activity has not yet been identified. One of the four glycosyltransferases might be responsible for this activity. Macrolide inactivation by glycosyltransferases in macrolide-producing strains has been well studied in *Streptomyces antibioticus*, the oleandomycin producer. It was demonstrated that intracellular glycosylation inactivates oleandomycin, which is subsequently reactivated, after secretion, by an extracellular glycosidase. Two glycosyltransferases, OleI and OleD, show oleandomycin-glycosylating activity but differ in the pattern of substrate specificity, OleI being much more specific for oleandomycin. A glycosidase OleR converts glycosylated oleandomycin into active oleandomycin (Quiros *et al.*, 1998). The genes *oleR* and *oleI* are located in the oleandomycin cluster, while *oleD* is located elsewhere

in the genome. As a similar resistance mechanism might operate in *S. ambofaciens*, we searched for an *oleR* orthologue, but none was found in the cluster. However, the deduced product of *orf29* had a putative glycosylhydrolase domain (pfam02156). Furthermore, analysis of the Orf29 sequence by SignalP (Nielsen *et al.*, 1997) showed that this protein, having a predicted signal sequence of 30 amino acids, was probably excreted. It might therefore be involved in the reactivation of glycosylated spiramycin.

The two genes *orf9** and *orf8** encode an ABC transporter. To test their possible involvement in spiramycin resistance, these two genes were expressed in the spiramycin-sensitive host *Streptomyces lividans* OS456 (Pernodet *et al.*, 1996), but no resistant phenotype was observed (data not shown). Therefore, these genes are probably not involved in spiramycin resistance and might belong to the uncharacterized non-ribosomal peptide synthetase (NRPS) cluster flanking the spiramycin cluster.

Regulatory genes

The gene *srnR* (*orf10*) has been shown previously to encode a transcriptional activator; its inactivation abolished spiramycin production (Geistlich *et al.*, 1992). Besides *srnR*, three putative regulatory genes are present: *orf13c*, *orf28c* and *orf32c*. The deduced product of *orf13c* is a putative GTPase belonging to the HflX family. The cellular functions of proteins from this family remain elusive (Brown, 2005). *tylV*, an orthologue of *orf13c*, is present in the tylosin cluster, where it might play a regulatory role in tylosin biosynthesis (Stratigopoulos *et al.*, 2004). The deduced product of *orf28c* showed high similarity to AcyB2, an activator of carbomycin biosynthesis from *Streptomyces thermotolerans* (Arisawa *et al.*, 1993), and to TylR, a pathway-specific transcriptional activator of tylosin biosynthesis in *S. fradiae* (Bate *et al.*, 1999; Stratigopoulos *et al.*, 2004). The gene *orf32c* encodes a putative transcriptional regulator from the GntR family. It should be noted that neither *SrnR* nor any of the putative regulators belong to those families of regulatory proteins that are commonly involved in the regulation of secondary metabolism, such as SARP, LAL or γ -butyrolactone-binding proteins (Bibb, 2005).

Limits of the cluster and genes of unknown function

On one side, the resistance gene *srnD* probably marks the limit of the spiramycin cluster, as the genes *orf8** and *orf9** are not involved in spiramycin resistance and as the other genes *orf10** and *orf11** encode an MbtH-like protein and an NRPS, respectively, two proteins for which no role in spiramycin biosynthesis could be proposed. The presence of these last two genes suggests that another secondary metabolite biosynthetic gene cluster, involving an NRPS, is adjacent to the spiramycin cluster. The most likely functions for most of the genes from *orf7*c* to *orf28c* are

either antibiotic biosynthesis or resistance or regulation of secondary metabolism. After *orf28c*, the limit is not easily predictable; *orf29* might be involved in the reactivation of glycosylated spiramycin, no role could be proposed for *orf30c*, *orf31* could encode a post-PKS reductase and *orf32c* a transcriptional regulator. The two genes *orf33* and *orf34c* are probably not part of the spiramycin cluster.

Inactivation of *orf3* and *orf2*

The gene *orf3* encodes an NDP-hexose aminotransferase probably involved in mycaminose biosynthesis. The inactivation of *orf3* was performed early in the course of this study, to verify that the first genes isolated were indeed part of the spiramycin cluster. Inactivation of this gene by in-frame deletion using an excisable cassette resulted in a spiramycin non-producing mutant (strain SPM24); spiramycin production could be restored by expression of the cloned *orf3* gene in this mutant, as reported previously (Raynal *et al.*, 2006). Spiramycin biosynthesis could also be restored by expressing the *tylB* gene from *S. fradiae* in strain SPM24 (data not shown). As TylB is the NDP-hexose aminotransferase involved in mycaminose biosynthesis, this complementation experiment thus supports the involvement of Orf3 in mycaminose rather than in forosamine biosynthesis. Moreover, strain SPM24 was shown to produce platenolide aglycone, as expected if Orf3 is involved in mycaminose biosynthesis (data not shown).

The gene *orf2* encodes an NDP-hexose isomerase and is highly similar to *orf1a* from *S. fradiae*, whose role in mycaminose biosynthesis was demonstrated recently (Melançon *et al.*, 2005, 2007). This gene was inactivated by in-frame deletion using an excisable cassette and the resulting mutant (*orf2::att3*) was named SPM22. This mutant was unable to synthesize spiramycin, but production was restored by expression of *orf2* (under the control of the *ermE** promoter; plasmid pSPM30) or by expression of *orf1a* from *S. fradiae* (under the control of the *ermE** promoter; plasmid pSPM33) (data not shown). It is interesting to note that the product of *orf2* is also similar (46 % identity, 59 % similarity) to that of SAMR0473, a gene clustered with other deoxyhexose biosynthetic genes, and with large type I PKS genes, in the right arm of the *S. ambofaciens* chromosome (Choulet *et al.*, 2006). Nothing is known about the expression of this other PKS I cluster and the product synthesized, but SAMR0473 obviously could not complement *orf2* deletion.

Concluding remarks

The analysis of the spiramycin biosynthetic gene cluster from *S. ambofaciens* ATCC 23877 provides information on the origin of the precursors used by the PKS, the biosynthetic route for the deoxysugars and putative regulatory genes. For many of the biosynthetic genes, the closest orthologues are found in the midecamycin and tylosin clusters, in agreement with the chemical similarities

of the macrolide molecules synthesized. Despite this high similarity at the protein level, the gene organization in the spiramycin cluster is strictly identical to that of the tylosin cluster only for the PKS genes and the seven genes upstream (*orf1* to *orf7* in the spiramycin cluster). From the partial data available on the midecamycin cluster (Cong & Piepersberg, 2007), the gene organization seems to be conserved between the spiramycin and midecamycin clusters for the seven genes downstream of the PKS and for the seven genes upstream (with the exception of an inversion that affects two genes). The complex regulation of tylosin biosynthesis has been studied thoroughly in *S. fradiae* (Bignell *et al.*, 2007; and references therein), but the similarity with *S. ambofaciens* is probably limited, as only *tylR*, one of the multiple genes controlling tylosin biosynthesis, has an orthologue (*orf10*) in the spiramycin cluster. In addition to the question of regulation, this work also raised questions about the genes whose products are involved in the attachment of deoxysugars and about the existence of a mechanism for inactivation/reactivation of the produced antibiotic. The present determination of the sequence and organization of the spiramycin gene cluster will allow such questions to be addressed via targeted mutations.

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