Genetic Organization of the Biosynthetic Gene Cluster for the Antitumor Angucycline Oviedomycin in *Streptomyces antibioticus* ATCC 11891

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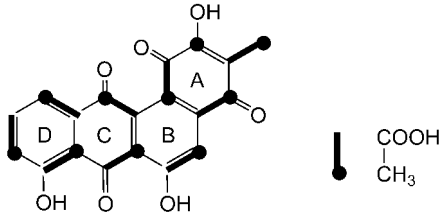
The oviedomycin biosynthetic gene cluster from Streptomyces antibioticus *ATCC 11891 has been sequenced and characterized. It contains all the necessary genes for oviedomycin biosynthesis, together with several genes for the generation of malonyl-CoA extender units. Production of this unusual angucyclinone in its natural host occurs only in solid cultures in parallel with aerial mycelium and spore formation. A mutant that did not produce oviedomycin was generated by disruption of the* β*-ketoacyl synthase gene* ovmK. *No other physiological process in the mutant appears to be affected; this rules out a direct relationship between oviedomycin production and cell differentiation in* S. antibioticus.

Introduction

Among the actinomycetes, members of the genus *Streptomyces* account for the biosynthesis of approximately two-thirds of all known bioactive secondary metabolites. They are Gram-positive bacteria, forming filamentous mycelium and spores during their life cycles, and their main habitat is the soil. One of the most important families of bioactive compounds produced by the streptomycetes is the polyketides. Polyketides are complex natural products and comprise one of the most important and structurally diverse families of pharmaceutical natural products. There are about 10000 known polyketides, some of which have pharmaceutical applications in many therapeutic areas such as antibiotics (erythromycin, tetracyclines) and antitumor (doxorubicin), antifungal (amphotericin B), immunosuppressant (FK506) and cholesterol-lowering agents (lovastatin, pravastatin), and also as important compounds in different areas of agriculture, such as insecticides (spinosyn). Information arising from analysis of the two so far freely available fully sequenced streptomycete genomes, *Streptomyces coelicolor*[1] and *Streptomyces avermitilis*[2] has revealed that the ability of these organisms (and very probably others) to synthesize bioactive natural products may be greater than was previously thought. In *S. avermitilis*, 30 secondary-metabolite gene clusters were predicted, of which eight clusters code for type I modular polyketides and two clusters for type II aromatic polyketides.[2] In *S. coelicolor*, 23 clusters encoding for secondary metabolites were identified, of which three code for type I polyketides and two for iterative type II polyketides.[1] Sequencing of other streptomycete genomes will probably extend the number of secondary metabolite clusters in these organisms and reveal new insights into the chemical diversity and great potential of the actinomycetes for the biosynthesis of secondary metabolites.

Streptomyces antibioticus ATCC 11891 is the producer of the macrolide antibiotic oleandomycin. The oleandomycin cluster has been fully sequenced and characterized.[3–8] This strain is not known to produce any other secondary metabolites, although other strains of this species are known to produce other antibiotics such as dactinomycin (actinomycin D[9]), antimycin A[10] or simocyclinone.[11] In the course of extensive work in our laboratory on the *S. antibioticus* ATCC 11891 strain, we observed the existence of different hybridization bands on its chromosomal DNA when the *actI* and *actIII* regions of the actinorhodin polyketide synthase were used as probes. Since we were interested in finding novel clusters for the biosynthesis of previously unknown secondary metabolites, we decided to analyse the *actI/actIII*-hybridizing clones for the production of novel polyketides through heterologous expression. As a result, we identified the novel and unusual angucyclinone polyketide oviedomycin (Scheme 1). The structural elucidation of oviedomycin has been reported previously.[12] Here we report the complete nucleotide sequence and organization of the oviedomycin gene cluster (*ovm* cluster) and the generation of a nonproducer mutant in *S. antibioticus*.



Scheme 1. *Structure of oviedomycin. Carbon atoms presumably derived from acetate during polyketide biosynthesis are highlighted.*

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 DOI: 10.1002/cbic.200400073

Results and Discussion

We have previously reported[12] the isolation of several cosmid clones from a chromosomal library from *S. antibioticus* that specifically hybridized with the *actI* and *actIII* regions of *S. coelicolor*.[13–15] Heterologous expression of some of these cosmids into *S. albus* J1074 and *S. lividans* TK21 resulted in the production of a novel natural nonglycosylated angucyclinone, oviedomycin[12] (Scheme 1).

Sequence and organization of the oviedomycin gene cluster

A region of 24720 nt comprising two overlapping cosAB3 and cosAB4 cosmid clones was sequenced and analysed for the presence of open reading frames (ORFs) by use of the UWGCG software package and BLAST program for comparison with proteins in databases. The sequence has been deposited at the EMBL Nucleotide Sequence Database under accession number AJ632203. Sequence analysis revealed 22 complete ORFs and two incomplete ORFs (Figure 1). The G+C content of this DNA region was 72.8%, in accordance with the typical *Streptomyces* G+C bias content. The proposed functions of each ORF and its closest homologues are shown in Table 1.

Genes involved in the biosynthesis of the polyketide moiety

Most sequenced angucycline gene clusters have a conserved gene organization core composed of an oxygenase, a cyclase, α- and β-ketoacyl synthases, a C_9 ketoreductase, an aromatase and another oxygenase. The oviedomycin gene cluster contains all these expected biosynthetic genes for the generation of the angucyclinone polyketide, probably forming a transcriptional unit. All these deduced proteins show the highest similarities with several other angucycline antitumor gene clusters such as those for simocyclinone,[11] landomycin A,[16] jadomycin[17] and urdamycin.[18] All these compounds share as a common element a tetracyclic angular benz[*a*]anthraquinone ring system.[19]

Genes *ovmP, ovmK* and *ovmS* codify for a type II minimal PKS. The keto-acyl synthase α OvmP and the ketoacyl synthase β OvmK contain the conserved Cys174 and Gln166 residues characteristic of these enzymes, respectively. The acyl carrier protein OvmS also contains the conserved Ser144, which is involved in the 4′-phosphopantetheine attachment site. These three enzymes would be responsible for the assembly of nine malonyl-CoA extender units to the initial acetyl-CoA starter through Claisen condensations for generation of a decaketide.

The product of the *ovmT* gene contains a short-chain alcohol dehydrogenase domain and is probably involved in the reduction of the keto group at C_9 of the nascent polyketide chain to a hydroxy group, a modification required for the subsequent aromatization. As in the case of OvmPKS, its closest relatives are from other angucycline gene clusters. Aromatic polyketide biosynthetic routes in which this enzymatic activity is present produce a final aglycon in which the final hydroxy group (from malonyl-CoA) at this C_9 position is lost during the first ring aromatization (Scheme 2).

The *ovm* gene cluster contains two possible cyclase genes that are thought to carry out subsequent intramolecular aldol reactions, producing the stepwise ring closures that lead to the angular tetracyclic structure. OvmA contains two cyclase domains and it is believed to act as a cyclase/aromatase during the formation of the first two rings, a biosynthetic event which takes place between C7–C12 and C5–C14, respectively. For the formation of the final rings at positions C4–C17 and C2–C19, the activity of the other cyclase present in the gene cluster, OvmC, together with the minimal PKS, must be required.

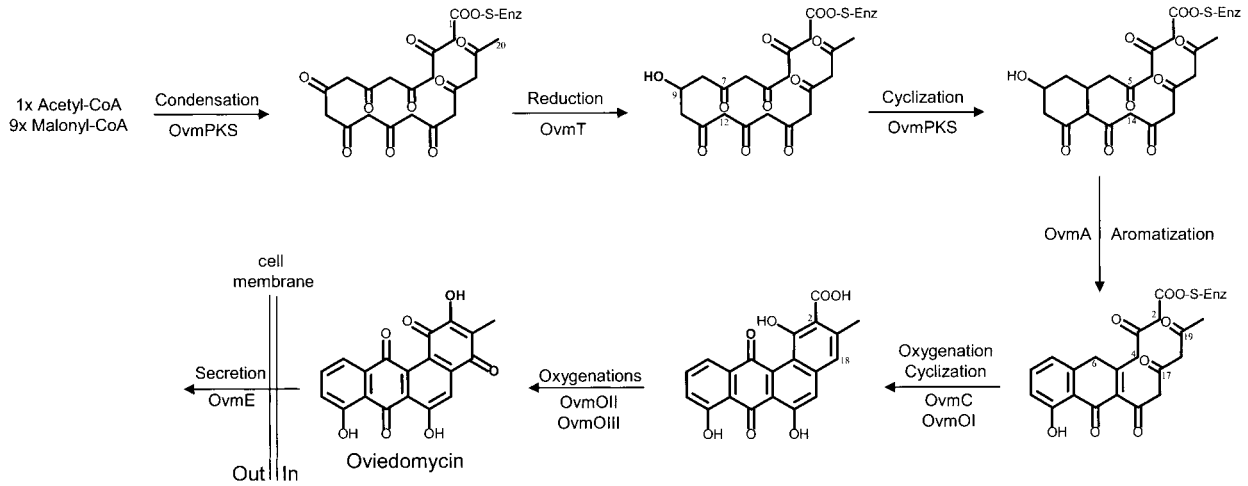
At least three tailoring oxygenations take place during oviedomycin biosynthesis (Scheme 2). The products derived from *ovmOI*, *ovmOII* and *ovmOIII* show high similarity to oxygenases from other angucycline gene clusters, especially the first two. Each of these three oxygenases only contains one domain associated with FAD-dependent oxidoreductases. The *ovmOI* and *ovmOII* genes are located in the same relative positions with respect to the PKS-cyclases region in many of these clusters, as in urdamycin,[18,20] landomycin,[16] simocyclinone[11] and auricin.[21] The oxygenase OvmOI is highly similar to UrdE, LanE and other monooxygenases responsible for the introduction of a keto group during the initial steps of angucyclinone biosynthesis.[18,20] This oxygenation step gives rise to the quinone ring C, which is present in all these angucyclines including oviedomycin. OvmOII is most similar to UrdM, SymA8 and LanM hydroxylases, which are responsible for the angular hydroxylations present at rings A–B in their corresponding aglycons.[11,16,18] However, OvmOII does not contain a fused dehydrogenase domain at the C terminus, as UrdM, SymA8 and LanM do. Moreover, oviedomycin does not contain these angular hydroxylations but has a hydroxylation and a keto group at ring A. OvmOIII presents the most different pattern in the databases, and some of its closest similarities are with hydroxylases from *Rhodococcus* species that modify 7-ethoxycoumarin[22] and rifampin.[23] According to these similarities we propose that OvmOII and OvmOIII are involved in catalysing the hydroxylation or the formation of the keto group at ring A (Scheme 2).

Figure 1. *Genetic organization of the oviedomycin biosynthetic gene cluster. B* = Bam*HI restriction sites. Minimal PKS genes are highlighted. ORFs indicated by numbers are not believed to be involved in oviedomycin biosynthesis.*

Table 1. *Deduced functions for the ORFs in the oviedomycin gene cluster.*

ORF	aa	Similar protein	Access. No., (% id./% sim.)	Proposed function
ORF1	405[a]	Glutamyl-tRNA synthetase, *Streptomyces coelicolor A3*(2)	NP 629681, 44/56	truncated Glu-tRNA-synthetase
ORF2	211	conserved hypothetical protein Atu5548, *Agrobacterium tumefaciens* plasmid AT	AD3227, 48/61	predicted flavin-nucleotide-binding protein structurally related to pyridoxine 5′-phosphate oxidase
ORF3	120	transcriptional regulator from ArsR family, *Bacillus cereus* ATCC 14579	NP 831767, 49/64	ArsR family repressor
OvmE	412	chloramphenicol resistance protein, *Streptomyces lividans*	P31141, 27/42	permease of the major facilitator superfamily
OvmX	278	putative hydroxylase, *Streptomyces nogalater*	AAF01810, 45/56	unknown
OvmY	216	putative TetR-family transcriptional regulator, *Streptomyces avermitilis* MA-4680	NP 825892, 30/47	transcriptional repressor for resistance permease
OvmZ	241	unknown, med-ORF27, *Streptomyces sp*. AM-7161	BAC79023, 34/48	unknown
OvmR	255	arginine degradation regulator, ArcR, *Halobacterium salinarum*,	CAA56903, 39/54	transcriptional regulator
OvmOI	494	C_{12} oxygenase, UrdE, *Streptomyces fradiae* Tu 2717	S54810, 67/76	ring C oxygenase
OvmC	110	jadomycin cyclase, JadI, *Streptomyces venezuelae*	AAD37852, 83/87	cyclase
OvmP	374	ketosynthase SimA1, *S. antibioticus* Tu 6040	AAK06784, 81/87	ketoacyl synthase α
OvmK	407	chain length factor, LanB, *Streptomyces cyanogenus* S136	AAD13537, 66/76	ketoacyl synthase β
OvmS	90	acyl carrier protein, *Streptomyces venezuelae*	AAB36564, 62/69	acyl carrier protein
OvmT	260	ketoreductase, *Streptomyces venezuelae*	AAB36565, 89/94	C_9-ketoreductase
OvmA	315	vyclase, UrdL, *Streptomyces fradiae* Tu 2717	AAF00205, 77/86	aromatase
OvmOII	490	oxygenase, LanM, *Streptomyces cyanogenus* S136	AAD13541, 64/72	oxygenase
OvmOIII	506	flavin-type hydroxylase, *Rhodococcus sp.* NCIMB 9784	AAM67415, 39/51	oxygenase
OvmF	293	phosphopantetheinyl transferase Med-ORF24, *Streptomyces sp.* AM-7161	BAC79025, 51/62	phosphopantetheinyl transferase, lipid-polyketide-metabolism
OvmG	514	carboxyl transferase/decarboxylase, Sim11, *Streptomyces antibioticus* Tu 6040	AAL15589, 83/90	acetyl-CoA carboxylase, β chain (carboxyl transferase subunit), lipid metabolism
OvmH	572	acyl-CoA carboxylase complex, A-subunit, *Streptomyces venezuelae*	AAD37851, 77/82	acetyl-CoA carboxylase, α chain (biotin-containing subunit),
ORF4	172	putative MarR family transcriptional regulator, *Streptomyces avermitilis* MA-4680	BAC73105, 35/53	MarR family repressor (multiantibiotic resistance)
ORF5	267	3-oxoacyl-ACP reductase, *Bacillus cereus* ATCC 14579	AAP10490, 41/61	β-ketoacyl-ACP reductase, lipid metabolism
ORF6	171	none	–	unknown
ORF7	101[a]	none	–	unknown

[a] Incomplete ORF.



Scheme 2. *Proposed biosynthetic steps during oviedomycin biosynthesis. Oxygen atoms introduced by the action of oxygenases are shown in bold.*

Other genes related to polyketide biosynthesis

The *ovm* gene cluster contains several genes related to polyketide biosynthesis that are not usually present in these gene clusters. OvmF shows high sequence similarity to 4′-phosphopantetheinyl transferases. Although this enzyme is necessary for oviedomycin biosynthesis, this function is generally present as part of the primary metabolism of the cell. A few secondary metabolite gene clusters contain an extra copy of this gene a priori, as in the case of the benzoisochromanequinone medermycin from *Streptomyces sp*. AM-7161,[24] the lipopeptide antibiotics iturin and surfactin from *Bacillus subtilis* RB14[25] and the epoxyspiroketal griseorhodin A.[26] It is more frequently found in angucycline gene clusters, such as simocyclinone from *Streptomyces antibioticus* Tü 6040,[11,27] jadomycin from *Streptomyces venezuelae*[28] and auricin from *Streptomyces aureofaciens* CCM 3239.[21]

As in the case of OvmF, the gene products of *ovmG* and *ovmH* genes are related to polyketide metabolism. They are not usually part of biosynthetic gene clusters, however, but are found elsewhere in the bacterial chromosome, where they also participate in primary metabolism functions such as fatty acids biosynthesis. OvmG and OvmH are very similar to the β- and α-chains, respectively, of the acetyl-CoA carboxylase complex. OvmH contains the conserved motif EAMKM at positions 534–538, which is the biotin attachment site for the BCCP domain (biotin-carboxylase carrier protein). This enzymatic complex catalyses the first committed step in fatty acid biosynthesis in animals, plants and bacteria: the conversion of acetyl-CoA into malonyl-CoA. Firstly, the α-chain (biotin-containing subunit) is thought to act as a biotin carboxylase (this domain is located at the OvmH N terminus), incorporating CO_2 into its biotin residue (located at the OvmH C terminus). Secondly, the β-chain (carboxyl transferase subunit, OvmG) would carry out the transcarboxylation from biotin-CO_2 to acetyl-CoA, affording malonyl-CoA. Interestingly, other angucycline antibiotic gene clusters also contain genes for one or both subunits, as in the cases of jadomycin,[28] landomycin[16] and simocyclinone.[11] Interestingly, the mutant affected in the equivalent gene to *ovmH* from the jadomycin pathway (*jadJ*) in *S. venezuelae* shows only 15% residual jadomycin production.[29] This means that, at least in this organism, the JadJ protein has been recruited specifically for polyketide biosynthesis, and that the primary metabolism acetyl-CoA carboxylase counterpart can not complement this function properly. However, *S. albus* or *S. lividans* harbouring cosAB50, an *ovmH*-less cosmid clone, still produce oviedomycin at normal rates, indicating that this gene is not absolutely essential for oviedomycin biosynthesis, although it might be necessary for a more efficient process.

Genes probably involved in oviedomycin resistance and regulation

The 5′ DNA region of the gene cluster contains several genes that could be involved in resistance and regulation of the pathway. OvmE is the only protein in the sequenced region showing high similarity to transmembrane proteins. Its closest relatives are chloramphenicol resistance proteins from *Streptomyces lividans*[30] and *Bacillus* species.[31] All these drug/H^+ antiporters belong to the major facilitator superfamily of exporters. OvmE could therefore be the secretion mechanism for oviedomycin in the producer organism, and so a self-resistance mechanism.

OvmY shows sequence similarities to several predicted TetR-family transcriptional repressors. This kind of regulator usually controls the expression of permeases of the major facilitator superfamily, as in the case of TetR repressor on the TetA resistance permease for tetracycline in several Gram-negative bacteria.[32] We therefore propose OvmY as the transcriptional regulator of the predicted oviedomycin export permease OvmE.

The deduced gene product of *ovmR* contains the conserved domain IclR, which is characteristic of many bacterial transcriptional regulators. This family of regulators includes the glycerol operon regulator from *S. coelicolor*[33] and the acetate operon repressor. These proteins have the corresponding helix-turn-helix motif at the N terminus. OvmR presents similarity with regulators of this family, two of them involved in arginine degradation in halobacteria[34] and one from the catechol degradation operon from *Rhodococcus erythropolis* (accession number CAE53840). According to this, OvmR could play a role in oviedomycin regulation.

The product of *ovmX* contains the conserved domain COG0702 and it shows similarity to nucleotide-diphosphate-sugar epimerases and to putative hydroxylases such as SnoaW from the anthracycline nogalamycin biosynthetic cluster,[35] in *Streptomyces nogalater*. A function, if any, for this protein in oviedomycin biosynthesis is unknown.

OvmZ shows no conserved domains and resembles four database proteins of unknown function. All these relatives belong to polyketide biosynthetic clusters: a silent angucycline-type gene cluster from a rubromycin B-producing *Streptomyces sp*. PGA64, Med-ORF27 from the benzoisochromanequinone medermycin,[24] Aur10 from the angucycline auricin[21] and Tyl-ORF12 from the macrolide tylosin.[36] The fact that these genes are present in polyketide gene clusters suggests that they could play a role in their corresponding pathways.

Other genes

Flanking the gene cluster are several genes that do not show any relation with polyketide biosynthesis and are probably not involved in oviedomycin biosynthesis: *orf1*, *orf2*, *orf3*, *orf4*, *orf5*, *orf6* and *orf7*. ORF1 shows similarity to glutamyl- and glutaminyl-tRNA synthetases and is incomplete. ORF2 shows sequence similarity to flavin-nucleotide-binding proteins structurally related to pyridoxine 5′-phosphate oxidases. Some of these proteins are described as possible NTP-pyrophosphohydrolases. ORF3 shows high similarity with the ArsR family (from "Arsenical Resistance") of metal-regulated transcriptional repressors.

At the other end of the sequenced region, ORF4 is probably a member of the MarR (multiple antibiotic resistance) family of repressors. The ORF5 protein shows similarities with 3-ketoacyl-acyl carrier protein reductases such as FabG, which reduces the initial keto group at carbon 3 in the ACP thioester sub-

strate to a hydroxy group during fatty acid biosynthesis in bacteria. The deduced products from ORF6 and ORF7 (incomplete) do not have conserved domains nor similar proteins in the public databases.

On the basis of the deduced functions of all these genes and of the common DNA region present in the four cosmid clones producing oviedomycin (cosAB3, cosAB4, cosAB16 and cosAB50), the boundaries defining the necessary genes for oviedomycin biosynthesis were established. The ends of cosAB16 and cosAB50 have been sequenced for this purpose. According to all these data the limits of the cluster would probably be the *ovmE* gene in the 5′ region and the *ovmH* on the 3′ region and comprise a DNA region of about 20 kb (Figure 1).

Production of oviedomycin by *S. antibioticus* and generation of a nonproducer mutant

Oviedomycin had not previously been reported as a natural product by any other organism and had never previously been detected in cultures of *S. antibioticus* ATCC 11891. We therefore carried out a more exhaustive and careful examination of cultures of this organism under different culture conditions, trying to detect either oviedomycin itself or an oviedomycin derivative. We could not detect oviedomycin production after growth of *S. antibioticus* ATCC 11891 in different liquid media. However, oviedomycin was readily detected when the organism was cultivated on solid GAE medium. Under these incubation conditions, biosynthesis of oviedomycin was a growth phase-dependent process, its biosynthesis occurring in parallel with the transition from substrate to aerial mycelium, with a further increase during spore formation (Figure 2 A), as identified by HPLC and MS analysis. This suggested that oviedomycin biosynthesis was a developmentally regulated process. The oviedomycin compound isolated from these *S. antibioticus* solid cultures was exactly the same compound as previously described from *S. albus* (cosAB3) liquid cultures, with the same HPLC mobility, absorption spectrum and mass as oviedomycin (Figure 2 B). This indicates that oviedomycin is the final product of the biosynthetic pathway.

We tried to extend these observations by looking for oviedomycin production in other *Streptomyces* species, on the assumption that oviedomycin might be a widely distributed compound in this genus. However, no oviedomycin was detected in cultures of *S. coelicolor*, *S. lividans*, *S. argillaceus*, *S. griseus* subsp. *griseus*, *S. brasiliensis*, *S. cattleya*, *S. clavuligerus* or *S. albus* on solid media at any developmental stage under the tested culture conditions (data not shown). It thus appears that oviedomycin is a product specific to *S. antibioticus*, only synthesized under specific conditions on solid cultures. There are several sequenced silent biosynthetic routes from streptomycetes for which final products still remain uncharacterized in the host. In some of these cases this is due to the fact that some secondary metabolites are produced under very specific culture conditions that might remain unknown. For example, jadomycin B, from *S. venezuelae*,[28] is produced only in the presence of high amounts of ethanol in liquid culture media.

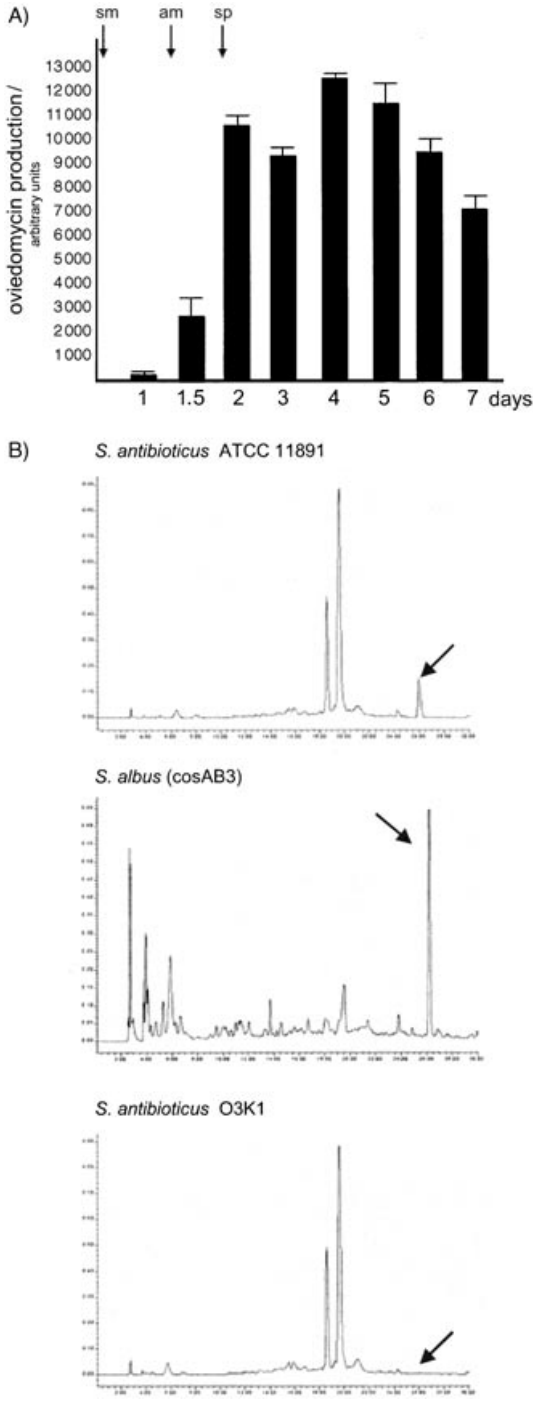


Figure 2. *A) Oviedomycin production during the* S. antibioticus *ATCC 11891 cell cycle, growing on solid GAE medium. The arrows indicate the onset of substrate mycelium (sm) formation, aerial mycelium (am) formation and sporogenesis (sp). B) HPLC chromatograms of culture extracts from wild-type* S. antibioticus *ATCC 11891,* S. antibioticus *mutant O3K1 (*ovmK*-minus mutant) and* S. albus *(cosAB3). Detection was carried out at 298 nm. Arrows show the mobility corresponding to oviedomycin.*

In other cases a biosynthetic pathway can lose the regulatory network that connects it to the host metabolism.

In order to test if growth development in *S. antibioticus* was actually linked to oviedomycin production, we decided to generate a non-producing mutant by disrupting the gene *ovmK*,

which forms part of the minimal *ovm* PKS. Plasmid pFL974, a pOJ260 suicide derivative containing an internal fragment from *ovmK* under the control of the *ermE** promoter to facilitate transcription of downstream genes (see Experimental Section), was introduced into *S. antibioticus* by conjugation from *E. coli* ET12567 (pUB307). *S. antibioticus* is an organism highly refractory to genetic manipulation: 120 conjugation experiments were necessary to provide a single mutant colony. Gene disruption in this mutant was confirmed by Southern hybridization of *Bam*HI-digested wild-type and mutant chromosomal DNAs (Figure 3), with use of a 1.3 kb *Nco*I fragment containing *ovmK* as a probe. The mutant strain O3K1 was cultivated on GAE agar plates containing apramycin, and no oviedomycin production was detected by HPLC (Figure 2B), confirming that the mutant was affected in oviedomycin production. Under these culture conditions, no differences in the growth cycle relative to the wild-type strain were observed. This mutant had normal substrate and aerial mycelium formation, and it generated spores normally, so oviedomycin does not seem to be involved in cellular differentiation, although its biosynthesis and this process probably share some kind of common regulation.

Experimental Section

Microorganisms, culture conditions and plasmids: *Streptomyces albus* J1074 (*ilv-1*, *sal-2*)[37] and *Streptomyces lividans* TK21[38] were used as hosts for expression of oviedomycin genes. *E. coli* DH10B (Invitrogene) was used as host for subcloning. *E. coli* ED8767[39] was used for gene library construction. *E. coli* ET12567 (pUB307)[40] was used as donor for intergenic conjugation. For growing of *E. coli*, TSB medium (Merck) was used. GAE medium[41] was used for sporulation of *S. antibioticus* and for detection of oviedomycin production during growth and development. R5A[42] was used for oviedomycin production in liquid cultures. "A" Medium[42] was used for sporulation of *S. albus* and *S. lividans*. When plasmid-containing clones were grown, the medium was supplemented with the appropriate antibiotics: ampicillin (100 $\mu g\,mL^{-1}$), kanamycin (25 $\mu g\,mL^{-1}$), apramycin (25 $\mu g\,mL^{-1}$) and nalidixic acid (100 $\mu g\,mL^{-1}$). The bifunctional (*E. coli–Streptomyces*) cosmid pKC505[42] was used to construct the *Streptomyces antibioticus* ATCC11891 DNA library;[12] pUC18,[44] pUK21,[45] pIJ2925,[46] pEM4,[47] pCRBlunt (Invitrogene) and pOJ260[48] were used for subcloning.

DNA manipulation: Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, DNA ligations, Southern hybridization and other DNA manipulations were performed by use of standard techniques for *Escherichia coli*[49] and *Streptomyces*.[38] Preparation of *S. albus* and *S. lividans* protoplasts, transformation and selection of transformants were carried out as described.[38] Intergenic conjugation from *E. coli* ET12567 (pUB307) to *S. antibioticus* was performed as described[38] with 48 h-old mycelium cultures in TSB. Transconjugants were plated on A medium plates containing $MgCl_2$ (10 mM).

DNA sequencing: Sequencing was performed on double-stranded templates derived from different clones in pUC18 and pUK21 and by use of the dideoxynucleotide chain termination method[50] and the Cy5 AutoCycle Sequencing Kit (Amersham Pharmacia Biotech). Both DNA strands were sequenced with primers supplied in the kits or with internal oligoprimers (17-mer) by use of an ALF-express automatic DNA sequencer (Amersham Pharmacia Biotech). Computer-aided database searching and sequence analysis were carried out with the UWGCG[51] program package and the BLAST[52] program.

Generation of mutant O3K1: For the generation of an non-oviedomycin-producing mutant, two oligoprimers were designed for amplification of an internal 0.89 kb fragment within the *ovmK* gene: FL-ovmKup 5′-GCATCGGCCGCATCACCCGCTTCG-3′ and FL-ovmKrp 5′-CATGGTCTTGGGCGCGGTGACCGGG-3′. This amplicon was cloned into pCRBlunt, generating pFL969. By use of polylinker restrictions sites from pFL969, the amplicon was subcloned as a

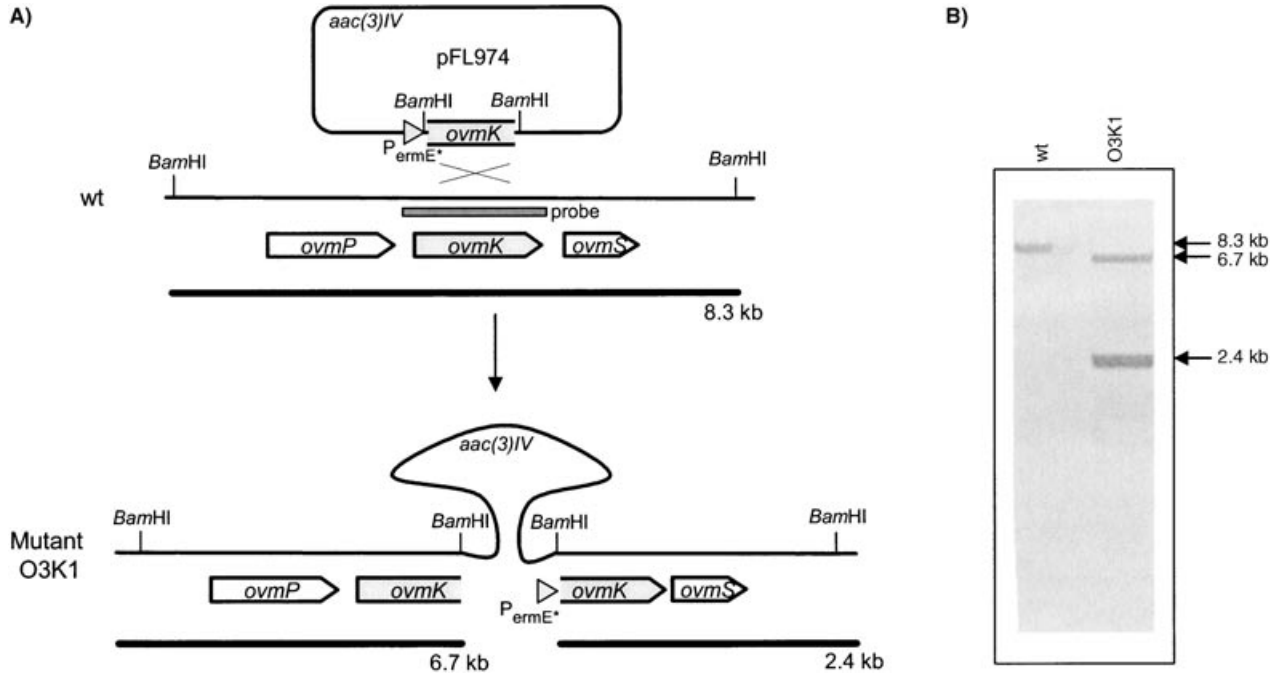


Figure 3. *Generation and analysis of mutant O3K1. A) Scheme representing gene disruption in the* ovmK *gene. Abbreviations:* aac*(3)IV, apramycin resistance gene;* PermE**,* ermE** promoter. B) Southern hybridization on* Bam*HI-digested chromosomal DNA with use of a 1.3 kb* Nco*I fragment containing* ovmK *as probe.*

*Pst*I-*Nsi*I fragment into the *Pst*I site of pEM4, giving rise to pFL973. Finally a 1.1 kb *Hind*III-*Spe*I DNA fragment from pFL973 including the *ermE** promoter was subcloned into pOJ260 previously digested with *Hind*III and *Xba*I, generating pFL974. This was the final construct for conjugation from *E. coli* ET12567 (pUB307).

HPLC analysis for oviedomycin: HPLC was performed on a Waters Alliance instrument, with a photodiode array detector model 996. Conditions used for analysis by reversed-phase HPLC were the same as previously described.[12]

Solid cultures were prepared in plates containing solid GAE medium (30 mL) that were uniformly inoculated with spores and incubated at 28 °C. Every 12 or 24 h, three plates were processed, and each culture was extracted with ethyl acetate (15 mL) containing formic acid (1 %). Liquid cultures were performed in 250 mL Erlenmeyer flasks containing GAE medium (50 mL), which was inoculated with spores and incubated at 30 °C and 250 rpm. The cultures were sampled at daily intervals by extraction of culture (3 mL) with ethyl acetate (1.5 mL) containing formic acid (1 %). In each case the organic extract was evaporated in vacuo and redissolved in methanol for subsequent HPLC analysis.

Acknowledgements

We thank Lorena Castro and Leire Peña for helpful technical assistance. This research was supported by grants from the Spanish Ministry of Science and Technology (BMC2002–03599, BIO2000–0274) and from the Plan Regional de I+D+I del Principado de Asturias (GE-MED01–05). We thank Obra Social Cajastur for financial support to F.L.

Keywords: antitumor agents · apoptosis · heterologous expression · oviedomycin · polyketides

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Received: March 15, 2004

