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Functional Angucycline-Like Antibiotic Gene Cluster in the Terminal Inverted Repeats of the *Streptomyces ambofaciens* Linear Chromosome

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Streptomyces ambofaciens has an 8-Mb linear chromosome ending in 200-kb terminal inverted repeats. Analysis of the F6 cosmid overlapping the terminal inverted repeats revealed a locus similar to type II polyketide synthase (PKS) gene clusters. Sequence analysis identified 26 open reading frames, including genes encoding the β -ketoacyl synthase (KS), chain length factor (CLF), and acyl carrier protein (ACP) that make up the minimal PKS. These KS, CLF, and ACP subunits are highly homologous to minimal PKS subunits involved in the biosynthesis of angucycline antibiotics. The genes encoding the KS and ACP subunits are transcribed constitutively but show a remarkable increase in expression after entering transition phase. Five genes, including those encoding the minimal PKS, were replaced by resistance markers to generate single and double mutants (replacement in one and both terminal inverted repeats). Double mutants were unable to produce either diffusible orange pigment or antibacterial activity against *Bacillus subtilis*. Single mutants showed an intermediate phenotype, suggesting that each copy of the cluster was functional. Transformation of double mutants with a conjugative and integrative form of F6 partially restored both phenotypes. The pigmented and antibacterial compounds were shown to be two distinct molecules produced from the same biosynthetic pathway. High-pressure liquid chromatography analysis of culture extracts from wild-type and double mutants revealed a peak with an associated bioactivity that was absent from the mutants. Two additional genes encoding KS and CLF were present in the cluster. However, disruption of the second KS gene had no effect on either pigment or antibiotic production.

Streptomyces spp. are gram-positive soil-inhabiting filamentous bacteria which undergo a complex process of morphological differentiation. One of the most striking traits of these microorganisms is their ability to produce a vast array of secondary metabolites, many of which possess antibiotic or other pharmacologically useful activities (9).

Polyketide synthases (PKSs) are involved in the production of a large number of these antibiotics and are usually classified into three types. Modular (type I) and aromatic (type II) PKSs are the most common in streptomycetes, although type III PKSs, members of the chalcone synthase superfamily of condensing enzymes previously characterized in plants, have also been described (36). In type I PKSs, the catalytic sites for the various biosynthetic steps are present as domains along the length of large multifunctional proteins. In contrast, type II PKSs comprise several generally monofunctional proteins that possess one enzymatic activity which is used reiteratively.

The core component of type II PKSs is called the minimal PKS. This is responsible for assembling the polyketide chain and is composed of a β -ketoacyl synthase (KS), a chain length

factor, and an acyl carrier protein (22). Additional PKS subunits, usually cyclase/dehydrase and ketoreductase enzymes, are responsible for modification of the nascent chain into a specific cyclic polyketide compound, while open reading frames (ORF) encoding enzymes such as oxygenases or methylases tailor the polyketide backbone into the final product.

With the advent of whole-genome sequencing programs, previously uncharacterized gene clusters deduced to be responsible for secondary metabolite biosynthesis have been found in *Streptomyces*. In the avermectin-producing species *Streptomyces avermitilis*, 30 secondary metabolite gene clusters, including 12 type I and II PKS clusters, were identified by searching for homologues to polypeptides with a defined role in secondary metabolism (24, 38). *Streptomyces coelicolor* A3(2), the model *Streptomyces* strain, has long been known to produce four chemically distinct antibiotics (23), but on completion of its genome sequence, new type I and type II PKS gene clusters were revealed among a total of 18 additional clusters that code for enzymes characteristic of secondary metabolism (6). The *Streptomyces* linear chromosome is composed of a central core comprising about half the chromosome and a pair of chromosome arms, and it appears that the distribution of these clusters shows some preponderance in the arms (6, 38).

Following our studies on genetic instability in *Streptomyces ambofaciens*, a phenomenon that affects morphological and

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties ^a	Reference
<i>S. ambofaciens</i>		
ATCC 23877	Wild type	41
SM1, SM2	<i>alpIABCD</i> locus on left chromosomal arm replaced by the <i>aac(3)IV/oriT</i> cassette	This study
DM1, DM4	<i>alpIABCD</i> loci on two chromosomal arms replaced by the <i>aac(3)IV/oriT</i> cassette	This study
DM2, DM3	<i>alpIABCD</i> loci on two chromosomal arms replaced by the <i>aadA/oriT</i> cassette	This study
DM5	One copy of the <i>alpIABCD</i> locus replaced by the <i>aac(3)IV/oriT</i> cassette, the second copy replaced by the <i>aadA/oriT</i> cassette	This study
H3, H4, H5	DM2(mF6)	This study
J1, J2, J3	DM2(pNSA100)	This study
KS1, KS3	<i>alpR</i> loci on two chromosomal arms replaced by <i>aac(3)IV/oriT</i> cassette	This study
<i>E. coli</i>		
DH5 α	General cloning strain	21
ET12567	Strain used for conjugation between <i>E. coli</i> and <i>Streptomyces</i> spp.	32
BW25113	Strain used for PCR targeted mutagenesis	11
<i>B. subtilis</i> ATCC6633	Strain used as indicator in the bioassays	
Plasmids/cosmids		
F6	Cosmid from the genomic library of <i>S. ambofaciens</i> ATCC 23877	29
pUZ8002	Used to mobilize in <i>trans</i> cosmid containing the <i>oriT</i> locus	39
pIJ773	<i>oriT</i> , <i>aac(3)IV</i>	19
pIJ778	<i>oriT</i> , <i>aadA</i>	19
pIJ790	<i>gam</i> , <i>bet</i> , <i>exo</i> , <i>cat</i>	19
pSET152	<i>oriT</i> , <i>attP</i> , <i>int</i> , <i>aac(3)IV</i>	7
Supercos1	<i>bla</i> , <i>neo</i>	15
pNSA100	<i>bla</i> , <i>oriT</i> , <i>attP</i> , <i>int</i> , <i>aac(3)IV</i>	This study
mF6	<i>bla</i> , <i>oriT</i> , <i>attP</i> , <i>int</i> , <i>aac(3)IV</i>	This study

^a *cat*, chloramphenicol resistance gene; *gam*, inhibits host exonuclease V; *bet*, single-stranded DNA-binding protein; *exo*, exonuclease promoting recombination together with *bet*; *bla*, ampicillin resistance gene; *aac(3)IV*, apramycin resistance gene; *aadA*, spectinomycin and streptomycin resistance gene; *neo*, kanamycin resistance gene; *attP*, attachment site of ϕ C31; *int*, integrase gene of ϕ C31; *oriT*, origin of transfer.

biochemical differentiation and is associated with large genomic rearrangements in the terminal regions of the chromosome (including deletion, amplification, translocation, and chromosomal fusion) (28), we initiated a sequencing program of the terminal 2.6 Mb (1.3 Mb on each arm) of the 8-Mb *S. ambofaciens* ATCC 23877 chromosome (29). *S. ambofaciens* ATCC 23877 is known to produce two antibiotics: spiramycin, a polyketide derivative, and congocidin (or netropsin), a basic oligopeptide. Spiramycin is a macrolide antibiotic that is synthesized by a type I PKS (42) encoded by a cluster located in the central part of the chromosome (unpublished data). Sequence analysis of an amplifiable locus from the unstable region of the chromosome revealed the possible presence of a second type I PKS gene cluster in *S. ambofaciens* (2) located approximately 650 kb from the right chromosomal end. The only evidence for activity of this putative cluster was associated with its amplification in spontaneous mutants isolated from the wild-type strain. Thus, amplification is correlated with the loss of spiramycin production and with the production of a yellowish pigment detectable by thin-layer chromatography analysis (2, 46).

Here we report the characterization of a new type II PKS gene cluster present in the terminal inverted repeats (TIRs) of the *S. ambofaciens* ATCC 23877 chromosome, a structure characteristic of the linear replicon ends in *Streptomyces*, and demonstrate that it is responsible for the biosynthesis of an orange pigment and an antibacterial compound. We named this cluster *alp*, for angucycline-like polyketide, based on sequence comparisons with known polyketide clusters.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. All strains and plasmids used in this study are described in Table 1. For production of spores, *Streptomyces* strains were grown at 30°C on HT agar (25). Cultures grown in YEME medium (25) were used for pulsed-field gel electrophoresis and DNA extraction, and pigment and antibiotic production was assessed on solid or in liquid R2 medium (25). R2 liquid cultures were also used for RNA isolation. Phenotypes were analyzed as necessary by growing on MM glucose, MM mannitol, SMMS, HT, R2, and R2YE agar plates (25). Luria-Bertani (LB) and SOB liquid media (25) were used for culturing *Escherichia coli*, and LB was used for *Bacillus subtilis*. Antibiotics (Sigma) were added to growth media when required. For PCR-targeted mutagenesis (see below), L-arabinose (10 mM final concentration; Sigma) was added to SOB medium from a 1 M sterile filtered stock solution to induce λ red genes under the control of the pBAD promoter (11).

Nucleic acid manipulation, reagents, and enzymes. Genomic DNA from *S. ambofaciens* was prepared as described previously (29). Cosmid and plasmid DNA was extracted from *E. coli* by the alkaline lysis method (45). Restriction enzymes and molecular biology reagents were purchased from New England Biolabs and Roche Diagnostics. Preparation of chromosomal DNA for digestion and analysis by pulsed-field gel electrophoresis were performed essentially as described by Leblond et al. (29).

PCR products were purified with the High Pure PCR product purification kit (Roche), while restriction fragments were purified from agarose gels with the GeneClean procedure (Bio 101). Southern blots were performed with Hybond-N nylon membranes (Amersham-Pharmacia) and a vacuum transfer system (Bio-Rad). Probe DNA was labeled with digoxigenin-dUTP (Roche), and hybridization conditions, washing, and detection were performed according to the manufacturer's instructions. Light emission was detected with a Fluor-S MultiImager (Bio-Rad).

DNA sequencing of the F6 cosmid was done by Genoscope (Centre National de Séquençage, Evry, France) as part of a sequencing project of the terminal regions of *S. ambofaciens* ATCC 23877. Computer-aided sequence analysis was performed with Artemis (44). All sequence similarity searches were performed with the Blast programs and the nr database at the National Center for Biotechnology Information (3).

TABLE 2. Oligonucleotide primers used in this study^a

Primer	Sequence (5'-3')
ACP-F	GACCTGGACGGCGACATC
ACP-R	CTGGGCGTTGACGACCTC
KSI-F	CCACGACGAACCCGAGTG
KSI-R	GTAGGCGTTGGAGCGGGT
hrdB-F	CGCGCATGCTCTTCT
hrdB-R	AGGTGGCGTACGTGGAGAAC
ID-F	AGAACCCTTCCGTGCAACCGCCGAGGAGCCAACCCT <u>ATGTGTAGGCTGGAGCTGCTTC</u>
ID-R	GTCGGGGTCGGGTGCCCGGAAAAATCAGGGGCGGGGTCA <u>ATTCCGGGGATCCGTCGACC</u>
R-F	CGACCACGTGAACGCCAGCCCGCGGGAGGCCGACGGT <u>GATTCCGGGGATCCGTCGACC</u>
R-R	CCGGGTGGGGCTGCGGGCGGGCGGGGCGGGTGGCT <u>CA</u> TGTAGGCTGGAGCTGCTTC
CK1	TGGTGGGCGGGAAGATGC
CK2	CGAAGGGCGTGCGGCTCC
CK3	GGCCTGGCCCAACACGTC
CK4	ACCAGGGCCTGGTGTGTGTG
Modify-1	GCTCTAGAGGTT <u>CATGTGCAGCTCCA</u>
Modify-2	CGGGATCCAGGCTT <u>CCCGGGTGTCTC</u>

^a For primers ID-F and R-F, the 39-nt sequence in bold corresponds to the sequence immediately upstream of *alpI* (ID-F) and *alpR* (R-F), respectively, and includes the start codon (underlined). The 19 nt (ID-F) and 20 nt (R-F) at the 3' end match a different end of the disruption cassette. For primers ID-R and R-R, the 39-nt sequence in bold corresponds to the sequence immediately downstream from *alpD* (ID-R) and *alpR* (R-R), respectively, and includes the stop codon (underlined). The 20 nt (ID-R) and 19 nt (R-R) at the 3' end match a different end of the disruption cassette. For the primers Modify-1 and -2, the sequence in bold corresponds to the upstream sequence of the *aac(3)IV* gene and to the downstream sequence of the *int* gene from pSET152, respectively. The *XbaI* (Modify-1) and *BamHI* (Modify-2) sites are underlined.

Total RNA was isolated from R2 liquid-grown cultures of *S. ambofaciens* ATCC 23877 as previously described (25).

Reverse transcription-PCR. Five micrograms of total RNA was annealed to 2 µg of random hexamer primers (pdN6, Amersham Pharmacia Biotech Inc.) at 65°C for 5 min, then mixed with reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol), 20 µM desoxynucleoside triphosphates (Roche Diagnostics GmbH), 31.25 units of Guard RNase inhibitor (Amersham Pharmacia Biotech) and 10 units of DNase I (Amersham Pharmacia Biotech), producing a final volume of 25 µl. Reactions were carried out at 37°C for 20 min and stopped by heating at 95°C for 5 min. For each reaction, 2-µl aliquots were then taken out and used as the template in the control PCR analysis detailed below, while the remaining 23 µl received 200 units of Moloney murine leukemia virus reverse transcriptase (USB Corporation). The reverse transcriptase reactions were incubated at 37°C for 70 min and then stopped by heating at 95°C for 5 min.

Primer pairs KSI-F/KSI-R, ACP-F/ACP-R, and hrdB-F/hrdB-R (Table 2) were then used to analyze the cDNAs for *alpA*, *alpC*, and *hrdB*-like gene expression, respectively [*hrdB* encodes the major sigma factor in *S. coelicolor* A3(2) (8)]. PCR conditions were as follows: 4 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, followed by a final extension of 7 min at 72°C. PCR products of 175 bp, 177 bp, and 109 bp were expected for *alpA*, *alpC*, and *hrdB*, respectively. In parallel, control PCRs were similarly performed with the 2 µl of RNA taken out before the reverse transcription step to confirm that amplified products were not derived from chromosomal DNA.

Real-time PCR. Real-time PCR was performed with 5 µl of cDNA, 12.5 µl of Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 3.3 µl of SYBR Green I (1:10,000 dilution, Sigma), together with 10 pmol of each primer in a final volume of 25 µl. Thermal cycle conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 60 repeats of 30 s at 95°C and 1 min at 60°C (annealing and extension) and then 80 steps of 10 s with a temperature gradient increase of 0.5°C per step from 55°C to 94°C. This last step allowed the melting curve of the PCR products, and consequently their specificity, to be determined. For each run, a standard dilution of the cDNA was used to check the relative efficiency and quality of primers. A negative control (distilled water) was included in all real-time PCR assays, and each experiment was performed in duplicate. *hrdB* was used as an internal control to quantify the relative expression of target genes. Real-time PCR was carried out with the iCycler iQ real-time PCR detection system (Bio-Rad), and the data were analyzed with the software provided by the supplier.

PCR-targeted mutagenesis. The strategy used for gene disruption was based on the efficient PCR targeting system developed in *S. coelicolor* A3(2) by Gust et al. (19). The 3,945-bp sequence from *alpI* to *alpD* and the 1,257-bp *alpR* sequence (Fig. 1) were replaced by the *aac(3)IV/oriT* or *aadA/oriT* cassette. The primer sets ID-F/ID-R (*alpABCD* locus replacement) and R-R/R-F (*alpR* replacement) used to amplify the cassettes *aac(3)IV/oriT* (from pIJ773) (19) and

aadA/oriT (from pIJ778) (19) are described in Table 2. Both cassettes have the same ends and therefore could be amplified with the same set of primers.

PCR conditions for amplification of the resistance cassettes, the method for gene replacement in the cosmid, and the method for allelic exchange in *S. ambofaciens* were as previously described for *S. coelicolor* (19). Allelic exchanges were confirmed by Southern blot and PCR analysis. For the *alpABCD* locus, control PCRs were performed with primers CK1 (162 nucleotides downstream from the stop codon of *alpD*) and CK2 (165 nucleotides upstream from the start codon of *alpI*) (Table 2) giving a product of 1,711 bp or 1,751 bp after replacement, respectively, with the *aac(3)IV/oriT* or *aadA/oriT* cassette. In order to check replacement of *alpR*, PCRs were performed with primers CK-4 (87 nucleotides upstream from the start codon) and CK-3 (68 nucleotides downstream from the stop codon) (Table 2). The size expected of a PCR product with CK3/4 as primers was 1,412 bp for the wild-type allele and 1,539 bp after gene replacement with the *aac(3)IV/oriT* cassette.

Construction of pNSA100, a modified form of Supercos1. The primers Modify-1 and Modify-2 (Table 2) were used to amplify the 3.8-kb region of pSET152 (7) containing *aac(3)IV*, *oriTRK2*, *attP*, and *int* (*attP* and *int* correspond, respectively, to the attachment site and integrase gene of the bacteriophage φC31). PCR was performed with DyNAzyme DNA polymerase (Finnzyme). The reaction mixture was prepared according to the manufacturer's recommendations. The reaction program consisted of 5 min of initial heating at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C, then a final extension of 7 min at 72°C. The PCR product was digested by *BamHI* and *XbaI* and gel purified. It was then ligated with the 5.4-kb *XbaI*-*BglII* fragment of Supercos1 (15). Transformants of *E. coli* DH5α were selected on LB agar containing apramycin (50 µg/ml) and ampicillin (100 µg/ml) and characterized by restriction analysis.

Construction of mF6, a conjugative and integrative form of cosmid F6. Gel-purified linear DNA of pNSA100 cut by *BamHI* was introduced by electroporation into competent cells of *E. coli* BW25113(pIJ790) (19) containing F6. Competent cells were prepared from an SOB culture containing 10 mM L-arabinose to induce the λ *red* genes (λ-Red was used to promote homologous recombination between the linear form of pNSA100 and F6). Transformants were selected at 37°C on LB agar containing apramycin (50 µg/ml) and ampicillin (100 µg/ml) and characterized by restriction analysis.

Conjugation and screening of *S. ambofaciens* exconjugants. Conjugation between *S. ambofaciens* and *E. coli* ET12567(pUZ8002) containing the cosmid of interest were performed according to Kieser et al. (25), except HT agar medium supplemented with MgCl₂ (10 mM) was used instead of SFM MgCl₂ (10 mM). Exconjugants were selected by overlaying the plates with the appropriate antibiotic, apramycin or spectinomycin, at a final concentration of 50 µg/ml, plus nalidixic acid (final concentration 25 µg/ml) to eliminate *E. coli* cells. When necessary, clones were replica-plated onto HT containing nalidixic acid or apra-

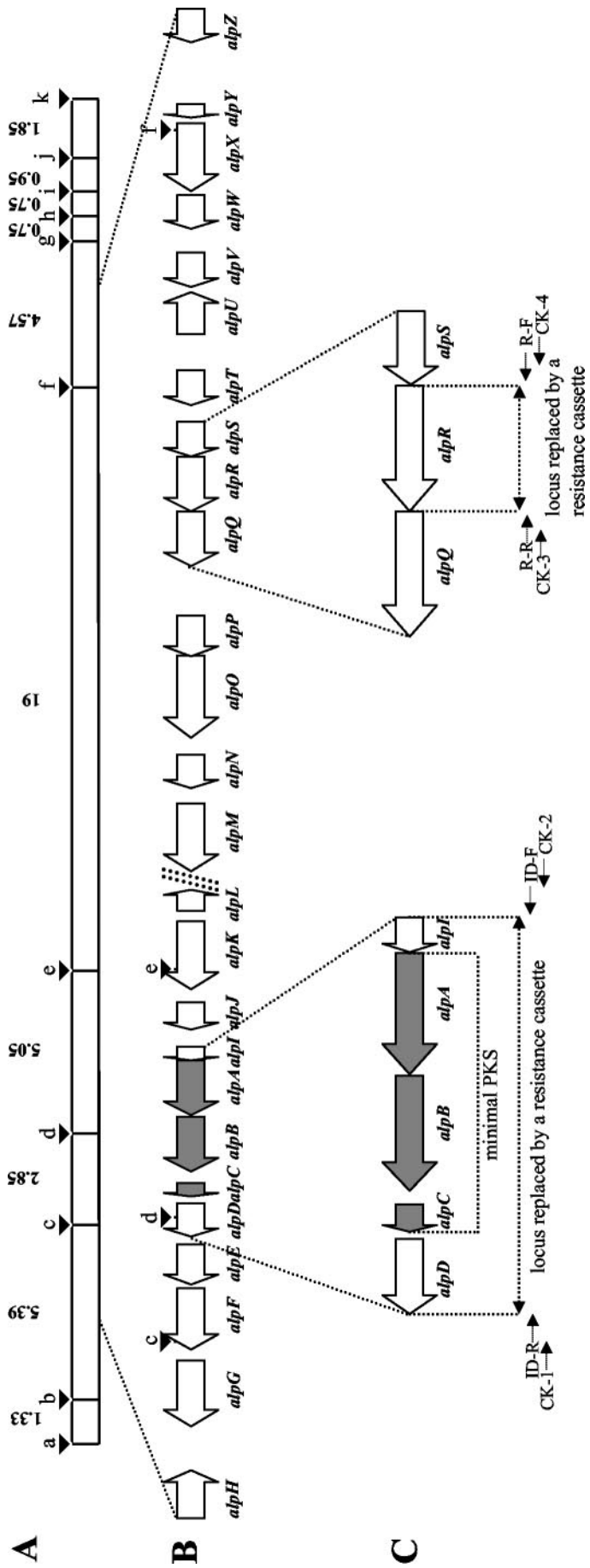


FIG. 1. (A) *Bam*HI restriction map of the F6 cosmid. The *Bam*HI sites are indicated by black triangles. The size of the fragments is given in kilobases. (B) Genetic organization of the *alp* gene cluster deduced from the sequence of F6. The genes encoding the minimal PKS are shaded. The double parallel dotted lines indicate a gap in the sequence. (C) Replacement of loci by resistance cassettes. The positions of primers used for PCR-targeted mutagenesis and for the analysis of disruptants (see Materials and Methods) are indicated as dotted arrows. The loci replaced by resistance markers are indicated by the dotted line with double arrows.

mycin (or spectinomycin) with or without kanamycin (50 µg/ml) to select for double-crossover exconjugants (Kan^r, Apra^r, and/or Spec^r).

Bioassay. Bioactivity from *S. ambofaciens* ATCC 23877 strains was tested by transferring a plug of clones grown on R2 agar onto LB agar seeded with indicator bacteria. Plates were then incubated overnight at 30°C, and activity assessed by measuring zones of inhibition. Similar assays were also performed on culture supernatants, extracts of culture supernatants, and high-pressure liquid chromatography (HPLC) fractions with paper disks inoculated with an aliquot of the sample to be tested.

Reversed-phase high-performance liquid chromatography (RP-HPLC). R2 liquid cultures (20 ml) of *S. ambofaciens* ATCC 23877 and its derivative strains were centrifuged for 5 min at 4,500 rpm at room temperature. Supernatants were collected and extracted twice with half the volume of ethyl acetate. The combined organic extracts were pooled, evaporated to dryness, then dissolved in HPLC-grade methanol (250 µl). Aliquots (40 µl) were analyzed by HPLC (Alliance model 2690, Waters, Milford, Mass.) with a reverse phase LichroCart C₁₈ column (250 by 4 mm inner diameter, 5-µm particle size, 10-nm porosity; Merck, Darmstadt, Germany). For the elution of spiramycin and congocidin, a linear gradient from 5% to 85% acetonitrile was applied in the presence of 0.1% trifluoroacetic acid for 80 min with a flow rate of 1 ml/min. The absorbance was monitored with a diode array detector (Waters model 996) scanning wavelengths from 200 to 350 nm. To elute the compound synthesized by the Alp PKS, a linear gradient from 40% to 70% acetonitrile in the presence of 0.1% trifluoroacetic acid was applied for 60 min with a flow rate of 1 ml/min, and absorption was monitored at 250 nm. Spiramycin and congocidin standards were purchased from Sigma.

To test for antibacterial activity, HPLC fractions of interest were taken to dryness by freeze-drying, resuspended in methanol, and analyzed with the paper disk bioassay method.

Pigment extraction. To extract the diffusible orange pigment produced in R2 medium, supernatants were first treated with an equal volume of ethyl acetate to remove the antibacterial compound (see Results). The aqueous phase was then acidified by addition of 1.5 ml of HCl (1 M) per 50 ml, before being reextracted with an equal volume of ethyl acetate. After evaporation of the solvent, the pigment was tested for its bioactivity.

Nucleotide sequence accession numbers. The two nucleotide sequences from cosmid F6 were deposited in GenBank under accession numbers AY338477 and AY338478.

RESULTS

Evidence of a type II PKS gene cluster in the TIRs of *S. ambofaciens* ATCC 23877. As a part of our sequencing project, the TIRs (200 kb) of the *S. ambofaciens* ATCC 23877 chromosome were sequenced with an ordered cosmid library (29). Based on restriction and hybridization analysis, the terminal inverted repeats are considered identical (or nearly identical). Therefore, we were unable to determine the origin (from the left or right chromosomal end) of the cosmids overlapping the terminal inverted repeats. Sequence analysis of two contigs (13.6 and 19.2 kb) from the F6 cosmid revealed 26 ORFs (Fig. 1) which exhibited a codon usage typical for *Streptomyces* genes (52). The gap between the two contigs is estimated to be about 1-kb long and has been resistant to sequencing so far. Predicted products encoded by the ORFs exhibit high end-to-end similarities to proteins from type II PKS gene clusters from various *Streptomyces* species, enabling putative functions to be assigned to many (Table 3). Based on homology, we named the genes of the cluster *alp*, standing for angucycline-like polyketide.

Polyketide core genes. Three of the genes, *alpA*, *alpB*, and *alpC*, encode proteins comprising the minimal PKS from this new type II PKS gene cluster. Thus, AlpA, AlpB, and AlpC exhibit high sequence similarity to KS, chain length factor, and acyl carrier protein subunits, respectively, involved in angucycline antibiotic biosynthesis, such as jadomycin produced by *Streptomyces venezuelae* (20). The *alpN* gene encodes a phos-

phopantetheinyl transferase homologue that presumably performs the posttranslational modification of the acyl carrier protein subunit AlpC, a prerequisite for polyketide biosynthesis (27).

Surprisingly, two other genes, *alpR* and *alpQ*, also encode KS and chain length factor subunits, respectively, but show highest similarity to proteins involved in mithramycin biosynthesis (31) (Table 3). However, no gene coding for an acyl carrier protein subunit could be detected next to the *alpRQ* locus, indicating that these genes are not organized as a minimal PKS locus.

Several genes might encode enzymes involved in the modification of the nascent polyketide chain. Thus, the *alpD* gene encodes a product similar to the ketoreductase subunits, while *alpE* and *alpP* encode proteins related to bifunctional cyclase/dehydrase enzymes involved in the formation of the first aromatic ring of the aromatic polyketides (22). The *alp* cluster contained another proposed cyclase gene, *alpI*, that encodes a protein very similar to JadI from the angucycline gene cluster of *S. venezuelae* (26).

It is noticeable that the genetic organization of the *alpI*, *alpA*, *alpB*, *alpC*, *alpD*, and *alpE* genes (Fig. 1) is identical to that seen for their homologues involved in the biosynthesis of the angucycline antibiotics jadomycin (*jadIABCE*) (20, 26), urdamycin (*urdFABCDL*) (12, 16), landomycin (*lanFABCDL*) (50), and kinamycin (the proposed *kinIABCE* locus; accession number AY228175).

An interesting feature of the *alp* cluster is the presence of *alpS*, immediately upstream of *alpR*. The predicted product of *alpS* has high end-to-end similarity with thioesterases. To our knowledge, only four thioesterase genes have so far been found in type II PKS systems (30, 35, 48) (accession number AJ416377). In common with its homologues, AlpS could be involved in polyketide chain release, or alternatively may function as an esterase responsible for the hydrolysis of ester intermediates, as proposed by Li and Piel (30).

Genes encoding post-PKS tailoring steps. The *S. ambofaciens alp* cluster contains three genes, *alpF*, *alpG*, and *alpK* (Fig. 1), the predicted products of which resemble oxygenases from angucycline biosynthetic clusters. We also identified a gene, *alpH*, encoding a protein similar to the *O*-methyltransferase enzymes such as CalO6 from the calicheamicin gene cluster of *Micromonospora echinospora* (1). The deduced product of the *alpJ* gene exhibits similarity to anthrone oxidases proposed to be responsible for an oxidation leading to the formation of an angucyclinone intermediate (17).

The product of *alpL* is similar to KinX from the proposed kinamycin cluster (accession number AY228175) and to JadX from the jadomycin cluster (49), but the functions of these proteins remain unclear. Downstream from *alpL* there is a gap in the sequence of about 1 kb (Fig. 1) that has so far proved impossible to close. At the end of the next sequenced contig is a gene, *alpM*, that codes for a protein (507 amino acids) possessing similarity to the condensation domain (about 450 amino acids in length) of nonribosomal peptide synthases. This is surprising because peptide synthases are usually large multifunctional proteins comprising from one to several modules, each containing several catalytic domains (33). Further genes putatively involved in modifying steps are *alpO*, encoding an

TABLE 3. Deduced functions of *alp* gene products^a

Gene	No. of amino acid residues	Putative function	Sequence similarity and antibiotic cluster	% Identity/% similarity	Reference(s) or source
<i>alpH</i>	361	O-Methyltransferase	CalO6, calicheamicin, <i>M. echinospora</i>	39/54	1
<i>alpG</i>	508	Oxygenase	SimA7, simocyclinone, <i>S. antibioticus</i>	58/70	48
<i>alpF</i>	491	Oxygenase	LanM, landomycin, <i>S. cyanogenus</i>	61/71	50
<i>alpE</i>	314	Cyclase/dehydrase	Aur1H, putative auricin, <i>S. aureofaciens</i>	75/84	37
<i>alpD</i>	262	Ketoreductase	Aur1G, putative auricin, <i>S. aureofaciens</i>	89/94	37
<i>alpC</i>	89	Acyl carrier protein	JadC, jadomycin, <i>S. venezuelae</i>	66/78	20
<i>alpB</i>	403	Chain length factor	JadB, jadomycin, <i>S. venezuelae</i>	77/86	20
<i>alpA</i>	423	Ketoacyl synthase	JadA, jadomycin, <i>S. venezuelae</i>	84/91	20
<i>alpI</i>	109	Cyclase	JadI, jadomycin, <i>S. venezuelae</i>	88/92	20, 26
<i>alpJ</i>	232	Anthrone oxidase	JadG (ORF7), jadomycin, <i>S. venezuelae</i>	54/80	20, 53
<i>alpK</i>	491	Oxygenase	SimA7, simocyclinone, <i>S. antibioticus</i>	54/69	48
<i>alpL</i>	169	Unknown	JadX, jadomycin, <i>S. venezuelae</i>	34/69	49
<i>alpM</i>	507	Peptide synthetase	SepsA (condensation domain), nonribosomal peptide, <i>Saccharothrix mutabilis</i> subsp. <i>capreolus</i>	34/47	AF512431
<i>alpN</i>	269	Phosphopantetheinyl transferase	GrhF, griseorhodin, <i>Streptomyces</i> sp. JP95	48/60	30
<i>alpO</i>	607	Acyl-coenzyme A dehydrogenase	<i>pks8</i> cluster, <i>S. avermitilis</i>	41/53	38
<i>alpP</i>	306	Cyclase/dehydrase	GrhE, griseorhodin, <i>Streptomyces</i> sp. JP95	41/54	30
<i>alpQ</i>	422	Chain length factor	MtmK, mithramycin, <i>S. argillaceus</i>	62/72	31
<i>alpR</i>	423	Ketoacyl synthase	MtmP, mithramycin, <i>S. argillaceus</i>	67/80	31
<i>alpS</i>	259	Thioesterase	MtmZ, mithramycin, <i>S. argillaceus</i>	48/61	AJ416377
<i>alpT</i>	273	Regulator	GrhR2, griseorhodin, <i>Streptomyces</i> sp. JP95	74/85	30
<i>alpU</i>	342	Regulator	TylT, tylosin, <i>S. fradiae</i>	50/58	5
<i>alpV</i>	279	Regulator	TylS, tylosin, <i>S. fradiae</i>	70/78	5
<i>alpW</i>	230	Regulator	TylQ, tylosin, <i>S. fradiae</i>	45/53	5
<i>alpX</i>	530	Decarboxylase	SimA12, simocyclinone, <i>S. antibioticus</i>	79/88	48
<i>alpY</i>	76	Unknown	SimX2, simocyclinone, <i>S. antibioticus</i>	40/50	48
<i>alpZ</i>	237	Butyrolactone receptor protein	TarA, nikkomycin, <i>S. tendae</i>	50/68	14

^a Additional comments: most of the genes from *alpG* to *alpL* encode products exhibiting the highest sequence similarity to the products of the *kin* genes from the proposed kinamycin cluster of *S. murayamaensis*, but no experimental evidence of the functionality of this cluster has been published so far. Most of the products from the *alpM* to *alpZ* genes exhibit the highest similarity to the products of genes carried by pSLA2-L, a linear plasmid from *S. rochei* (35), but no role for these genes in antibiotic production has been described.

acyl-coenzyme A dehydrogenase, and *alpX*, encoding a carboxyl transferase (or decarboxylase).

Genes encoding regulators. Type II PKS clusters usually contain regulatory genes that ensure proper expression of the cluster. The *alp* cluster contains up to five genes, *alpT*, *alpU*, *alpV*, *alpW*, and *alpZ*, which may be involved in regulation. *AlpT*, *AlpU*, and *AlpV* belong to the SARP (*Streptomyces* antibiotic regulatory protein) family of regulators (51) and may therefore be responsible for turning on the expression of biosynthetic genes in the *alp* cluster. *AlpT* is closely related to the product of *orf71* from the mithramycin-like gene cluster of pSLA2-L (35) and to GrhR2 from the griseorhodin A cluster (30), whereas the *alpU* and *alpV* products appear to be orthologues of TylT and TylS, respectively, from the *S. fradiae* type I cluster, responsible for the production of the macrolide antibiotic tylosin (5).

The *AlpW* regulatory protein exhibits sequence similarity to proteins known to act as transcriptional repressors, such as TylQ of *S. fradiae* (47). The deduced product of *alpZ* shares homology with γ -butyrolactone receptor proteins that usually negatively regulate antibiotic production in streptomycetes.

Interestingly, the *alpU*, *alpV*, *alpW*, and *alpZ* genes are congregated in a regulatory subcluster, showing an organization similar to that of the *tylT*, *tylS*, *tylQ*, and *tylP* loci of the tylosin producer *S. fradiae* (5). An ORF encoding a cytochrome P450

separates *tylP* and *tylQ* in the latter cluster, while two genes, *alpY* and *alpX* (see above), are present between *alpZ* and *alpW*. The product of *alpY* is a small protein of 76 amino acids homologous to SimX2, a protein of unknown function from the simocyclinone biosynthetic gene cluster of *S. antibioticus* Tü6040 (48).

Gene expression analysis of the *S. ambofaciens alp* cluster. With the exception of *alpH*, *alpL*, and *alpU*, all the *alp* genes are transcribed in the same direction (Fig. 1), and some, such as *alpLAB*, *alpPO*, and *alpSRQ*, are terminally overlapping. This arrangement is suggestive of translational coupling, although previous reports indicate that this is not necessarily the case in this situation (18). In addition, short intergenic regions (less than 50 bp) are occasionally present between adjacent genes, and it is therefore possible that the cluster might be partially transcribed as polycistronic mRNAs.

The *alpA* (KS) and *alpC* (acyl carrier protein) genes were selected as representative of the biosynthetic gene cluster, and their expression profiles were analyzed by reverse transcription-PCR. Total RNA was extracted at different points corresponding to exponential, transition, and stationary phases (Fig. 2A). As shown in Fig. 2B, *alpA* and *alpC* were expressed constitutively, but the expression seemed to be significantly induced following entry into transition phase after 14 h. In contrast, *hrdB* was expressed fairly constantly throughout

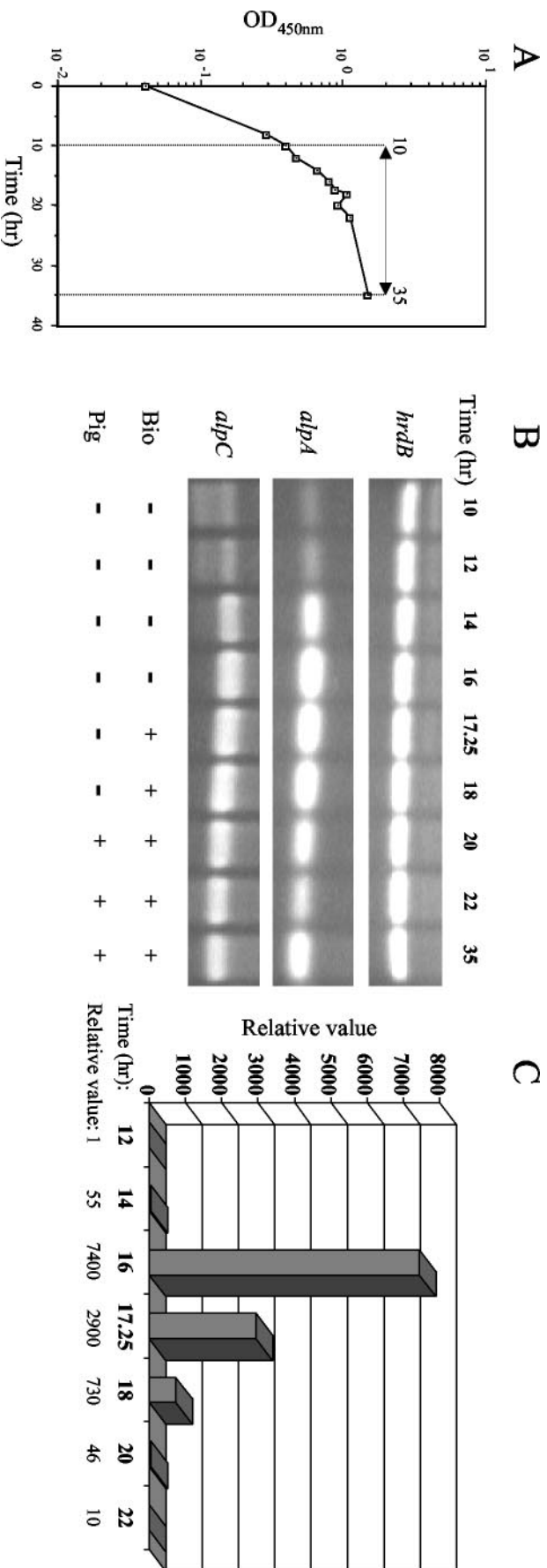


FIG. 2. Transcriptional analysis of the *alp* cluster. (A) Growth curve of the wild-type strain in R2 liquid medium. The points corresponding to culture times ranging from 10 to 35 h and from which total RNA samples were prepared are indicated by a double arrow. (B) Transcriptional analysis by reverse transcription-PCR of *hrdB*, *alpA*, and *alpC* at different times during growth. Assessment of the antibacterial activity against *B. subtilis* 6633 (Bio) and the production of pigment (Pig) for each sample is indicated under the panels (+, detected; -, not detected). (C) Quantification of *alpA* expression from 12 to 22 h of growth by real-time PCR analysis. Values are in comparison to expression of *hrdB*, which was used as an internal control (see Materials and Methods). The relative value for the 12-h sample was arbitrarily assigned as 1. The 10- and 35-h samples were excluded from the analysis because of the discrepancy of *hrdB* expression at these two points.

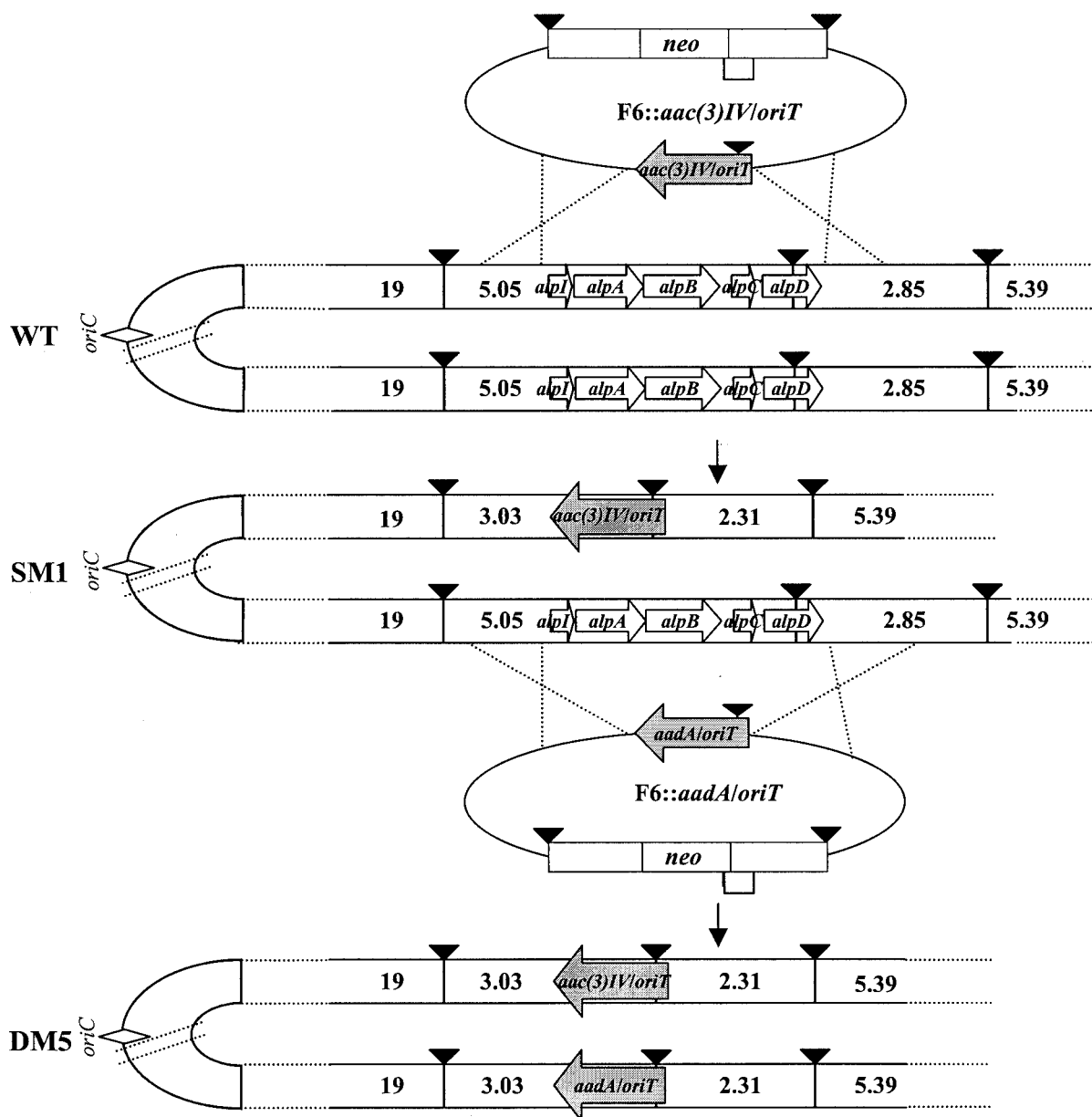


FIG. 3. Strategy used for replacement of the two *alpIABCD* loci in the chromosome. The *alpIABCD* locus was first replaced by an apramycin resistance cassette, *aac(3)IV/oriT*, in the F6 cosmid, and the mutated cosmid, F6::*aac(3)IV/oriT*, was introduced into the wild-type *S. ambifaciens* strain through intergeneric conjugation. Double-crossover integration at the *alpIABCD* locus on one chromosomal arm produces single mutants (e.g., SM1). Cosmid F6 in which the *alpIABCD* locus had been replaced by a spectinomycin resistance cassette (*aadA/oriT*) was then similarly introduced into mutant SM1. Double crossover disrupts the remaining copy of the *alpIABCD* locus on the second arm, generating double mutants (e.g., DM5). The white rectangle including the *neo* gene (conferring resistance to kanamycin) represents the vector part of the mutated F6 cosmid. The black triangles show relevant *Bam*HI sites.

growth. In order to quantify the observed increase in *alpA* expression, real-time PCR analysis was performed with the same RNA samples (Fig. 2C). The relative expression of *alpA* was determined in comparison to that of *hrdB*, with the value determined for the 12-h sample as the reference (i.e., this value was fixed arbitrarily at 1). While a 55-fold increase in transcription was detected between 12 h (late exponential phase) and 14 h (early transition phase) of growth, a considerably more dramatic increase of 7,000-fold was observed over the subse-

quent 2 h. After this strong induction, the expression level of *alpA* decreased slowly to a relative value of 10 in the 22-h sample (stationary phase).

Disruption of the *S. ambifaciens alp* gene cluster. Gene disruption of the *alp* cluster was carried out in order to investigate its role. This analysis was complicated by the fact that the chromosome contains two identical copies of the cluster, one on each arm. A strategy was therefore designed to first disrupt one cluster with an apramycin resistance marker and then the

other copy with a spectinomycin cassette (or vice versa; Fig. 3). The region selected for replacement was a 3.9-kb chromosomal sequence corresponding to the second codon of *alpI* to the penultimate codon of *alpD* (Fig. 1), removing a total of five genes, including the minimal PKS. The strategy is described in Materials and Methods and in the legend to Fig. 3. Exconjugants resistant to apramycin (or spectinomycin) but sensitive to kanamycin were selected directly following the conjugation step.

Southern and pulsed-field gel electrophoresis analysis confirmed that these clones corresponded to single mutants in which only one copy of the *alp* cluster was replaced. Pulsed-field gel electrophoresis analysis also showed that no large rearrangement associated with the disruption event had occurred. In order to generate double mutants, clones in which the two *alp* cluster copies were disrupted, we repeated the same procedure with an apramycin-resistant single mutant in combination with a mutated form of cosmid F6 in which the five genes (from *alpI* to *alpD*, see above) had been replaced by a spectinomycin cassette. By Southern and pulsed-field gel electrophoresis analysis, we confirmed that the isolated Kan^s Apra^r Spec^r clones were disrupted in both copies of the *alp* cluster, the one on the left chromosomal arm by the apramycin cassette and the one on the right arm by the spectinomycin cassette. One of these clones, the DM5 strain was used for further analysis.

We also took advantage of the high frequency of “conversion” between the *S. ambofaciens* TIRs (data not shown) and of the inability of the double mutants to produce an orange pigment to screen for double replacement events giving rise to mutants resistant to spectinomycin only and useful for the complementation experiments (see below). After two rounds of sporulation of single Spec^r mutants without selection pressure, clones were screened for Spec^r and the loss of pigment production. Southern analysis of pulsed-field gel electrophoresis profiles with the spectinomycin cassette as the probe confirmed the gene conversion event, i.e., both copies of the PKS genes were replaced by the resistance cassette.

Phenotypic analysis of the single and double *alp* mutants.

Comparison of the single and double mutants with the wild-type strain grown on HT, MM, R2YE, SMMS, and R2 solid medium showed no difference related to growth rate, aerial mycelium formation, sporulation, or spore color. This indicates that replacement of the PKS genes did not affect the growth and differentiation of *S. ambofaciens* and that the putative polyketide compound produced by the *alp* cluster was not a spore pigment. However, while the wild-type strain produced a diffusible orange pigment on R2 solid medium, the double mutant clones were completely defective in production of this pigment (Fig. 4A, DM1), and single mutant clones showed a significant reduction (Fig. 4A, SM1). Similar phenotypes were observed on SMMS plates but not on HT or R2YE, on which wild-type and mutant strains did not produce pigment.

Some angucyclines are known to be pigmented, e.g., jadomycin and urdamycin. Therefore, antibiotic activity assays were carried out. After 3 days of growth on R2 medium, an agar plug of each strain was transferred onto an LB plate seeded with *B. subtilis* spores. After overnight incubation, a large zone of growth inhibition was observed with the wild-type strain plug (Fig. 4A), but this inhibitory zone was decreased with the

single mutants and completely absent with the double mutants. The same results were observed when similarly analyzing supernatants of DM1 and wild-type cultures grown in R2 medium for activity (Fig. 4B). Note that the cultures tested grew very similarly. Together, these results suggest that the orange pigment and the antibacterial agent are both related to the *alp* PKS genes (no effect on spiramycin biosynthesis, a macrolide antibiotic produced by *S. ambofaciens*, was observed; see below).

The two copies of the *alp* gene cluster are both functional in vivo, as they showed a gene dosage effect in both pigment production and bioactivity. In R2 liquid cultures of the wild-type strain, bioactivity was detected immediately following a strong increase in transcription of the *alpA* gene encoding the KS subunit, and the pigment appeared about 2 h later (Fig. 2B, 2C, and 4B). Therefore the antibacterial compound and the orange pigment could be two distinct molecules derived from the same pathway, as was later confirmed (see below).

Complementation of the double PKS mutants. Complementation experiments were carried out with a modified form of the F6 cosmid (named mF6, see Materials and Methods) which can be introduced into *S. ambofaciens* by conjugation and can also integrate into the chromosomal ϕ C31 *attB* site. Since mF6 carries the apramycin resistance gene *aac(3)IV*, DM2, a double mutant resistant only to spectinomycin (see above), was used as a host strain for the complementation experiment. As a control, DM2 was transformed with pNSA100 (Fig. 5). Pulsed-field gel electrophoresis and Southern blot analysis of three independent exconjugants containing mF6 (H3, H4, and H5) and three containing pNSA100 (J1, J2, and J3) confirmed site-specific integration into the chromosomal *attB* site (data not shown). The exconjugants did not show any apparent differences in growth and morphological differentiation compared to the wild-type strain on different media (HT, MM, R2, and R2YE). However H3, H4, and H5 but not J1, J2, and J3 were partially restored in production of the diffusible orange pigment on R2 medium (Fig. 4A). They also recovered bioactivity against *B. subtilis* (Fig. 4A), although, as for pigment production, it was not completely restored to the wild-type level. This is consistent with the reintroduction of a single copy of the *alpIABCD* locus into the exconjugants, the wild-type *S. ambofaciens* ATCC 23877 normally possessing two active copies. This indicates that the *alp* cluster is responsible for the synthesis of both the orange pigment and the antibacterial compound.

Disruption of *alpR*, the gene encoding a second KS subunit.

As described above, a second gene encoding a KS subunit, *alpR*, was present in the cluster. As shown by reverse transcription-PCR, *alpR* presented an expression profile similar to that of *alpA* (data not shown). The whole *alpR* ORF (with the exception of the start and stop codons) was replaced by an apramycin cassette (see Materials and Methods). The *alpR* double mutants (i.e., replacement in both arms) were not affected in growth and differentiation on HT, MM, R2, and R2YE media. In addition, they were still able to produce the orange pigment and antibacterial agent on R2 medium. Therefore, *alpR* does not seem to be involved in the biosynthesis of these compounds.

HPLC analysis of *S. ambofaciens* ATCC 23877 metabolites.

In order to isolate the antibacterial compound produced by the

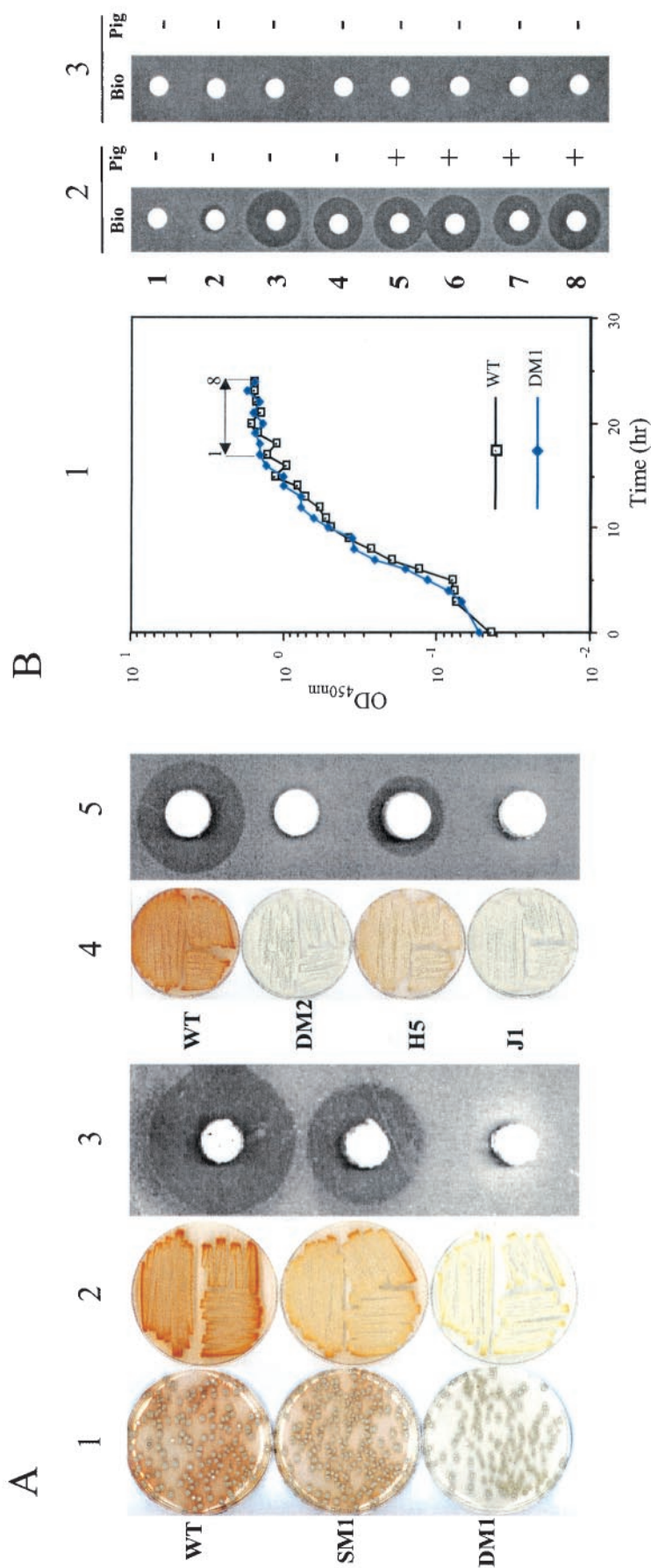


FIG. 4. (A) Pigment production and antibacterial activity on agar medium. Pigment production on R2 agar by isolated colonies (column 1) and confluent lawns of mycelia (column 2) of the wild-type, SM1, and DM1 strains and analysis of antibacterial activity against *B. subtilis* 6633 in wild-type, SM1, and DM1 strains (column 3). Columns 4 and 5 show pigment production and antibacterial activity against *B. subtilis* 6633 of the wild-type, DM2, H5 (DM2 transformed with mF6), and J1 (DM2 transformed with pNSA100) strains, respectively. For columns 3 and 5, bacteria were first grown on R2 agar, and a plug of this mycelium was then placed on an LB plate seeded with *B. subtilis* 6633 (see Materials and Methods for details). The plates in columns 2 and 4 were photographed from below. (B) Pigment production and antibacterial activity of the wild-type and DM1 strains grown in R2 liquid medium. Growth curves are given in panel 1, and the results of bioassays (Bio) and assessment of pigment production (Pig) from time points 1 to 8 in these curves are presented in panels 2 (wild type) and 3 (DM1).

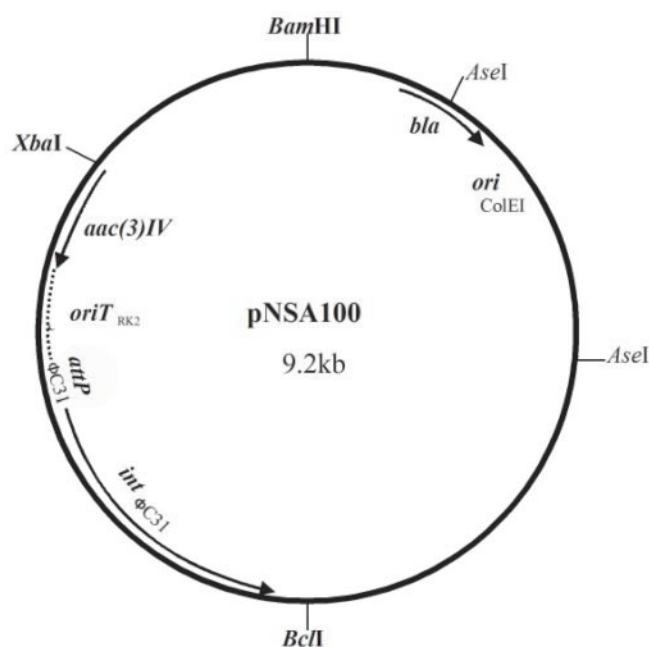


FIG. 5. Physical map of pNSA100, a modified form of SupercoS1. *bla* represents a gene conferring resistance to ampicillin, while *aac(3)IV* confers resistance to apramycin. *int* and *attP* represent the genes encoding the integrase of ϕ C31 and the ϕ C31 attachment site, respectively. The origin of transfer and the origin of replication are marked as *oriT*_{RK2} and *ori* ColE1, respectively.

alp gene cluster, ethyl acetate extracts from supernatants of R2 liquid-grown wild-type and DM1 strains were analyzed by HPLC. Supernatants were collected from stationary-phase cultures. Only the supernatant from the wild-type strain was pigmented and showed antibacterial activity against *B. subtilis* (data not shown). Extracts were separated on HPLC with an acetonitrile gradient (from 40 to 70%, trifluoroacetic acid, 0.1%; flow rate, 1 ml/min). In these conditions, a peak was eluted in the wild-type sample at an acetonitrile concentration of 46.8% which was not present in the DM1 extract (Fig. 6A). The fraction corresponding to the peak exhibited antibacterial activity against *B. subtilis* (Fig. 6B), while the corresponding fraction from DM1 did not (Fig. 6B). The peak and the associated bioactivity were restored in H5 (a double mutant complemented with mF6) but not in J1 (a double mutant containing pNSA100; Fig. 6). Under the same conditions, standards of spiramycin and congocidin, the two antibiotics known to be produced by *S. ambofaciens* ATCC 23877, were not retained on the HPLC column but were eluted with the flowthrough. The antibacterial compound of the bioactive peak does not therefore correspond to either spiramycin or congocidin. Together, these results strongly suggest that the peak detected only in the wild-type and H5 extracts is directly related to the presence of an active *alp* cluster.

HPLC analysis with a 5 to 85% acetonitrile gradient revealed that the double mutant produced levels of spiramycin similar to those of the wild-type strain in R2 medium (data not shown), indicating that the *alpIABCD* gene replacement did not affect the production of this antibiotic. Since bioassay with the culture supernatant of the double mutant strain showed no

antibacterial effect in the experimental conditions used (Fig. 4), the observation that spiramycin is synthesized by the double mutant strain grown in R2 suggests that the levels produced are below the limit of detection of the assay. The detection of a weak bioactivity from DM1 supernatant after significant concentration following ethyl acetate extraction and evaporation of the organic solvent (data not shown) is in agreement with this hypothesis. Congocidin is not soluble in organic solvents, and its production could therefore not be checked.

Treatment of the supernatant of the R2-grown wild-type strain with ethyl acetate extracted only the antibacterial compound into the organic phase, confirming that the orange pigment and antibacterial compound related to the *alp* cluster are two different molecules. Pigment could be extracted into the organic phase by acidifying the supernatant, and it was possible to separate it from the antibacterial compound (see Materials and Methods). The extracted pigment did not exhibit any effect on *B. subtilis* growth (data not shown), indicating that it does not possess antibiotic activity under these experimental conditions.

DISCUSSION

Sequencing of the terminal inverted repeats of the *S. ambofaciens* ATCC 23877 linear chromosome led us to identify a new type II PKS gene cluster, which is therefore present in two copies on the genome. Several genes, including the genes encoding the minimal PKS (Fig. 1), exhibited high sequence similarities with the biosynthetic gene clusters of angucycline antibiotics. Phylogenetic analysis of KS gene fragments from 99 actinomycetes also showed that the KS gene from this cluster, *alpA*, belongs to the branch of the angucycline antibiotic genes (34; Mikko Metsä-Ketelä, personal communication). This strongly implies that the identified DNA sequence is involved in the synthesis of an angucycline-like antibiotic. In addition, the *alpIABCDE* genes exhibit the same genetic organization and are highly homologous to the six genes of the jadomycin cluster (*jadIABCE*) described as essential for the synthesis of UWM6, the first isolable intermediate of the jadomycin biosynthetic pathway with angucyclic characteristics (26). However, as no genes involved in glycosylation of the polyketide were present among the 26 *alp* genes, we cannot rule out the possibility that the *alp* cluster is responsible for the production of an angucyclinone antibiotic, a sugarless angucyclic compound (43).

Gene replacement of the two copies (one in each TIR) of the *alpIABCD* locus revealed that the *alp* cluster is responsible for the production of an antibacterial compound on R2 medium. This was confirmed by complementing the mutants with the wild-type *alpIABCD* alleles. The fact that a single-copy replacement was accompanied only by partial loss of antibiotic production indicates that both copies of the cluster encode a functional PKS. The timing of production of this antibacterial compound is in good agreement with the transcription pattern of *alpA* (KS), a pattern characteristic of genes involved in antibiotic biosynthesis.

The antibacterial compound produced by the Alp proteins was isolated by HPLC. HPLC analysis also showed that it did not correspond to spiramycin, a macrolide antibiotic previously reported in *S. ambofaciens* that is the product of a type I PKS

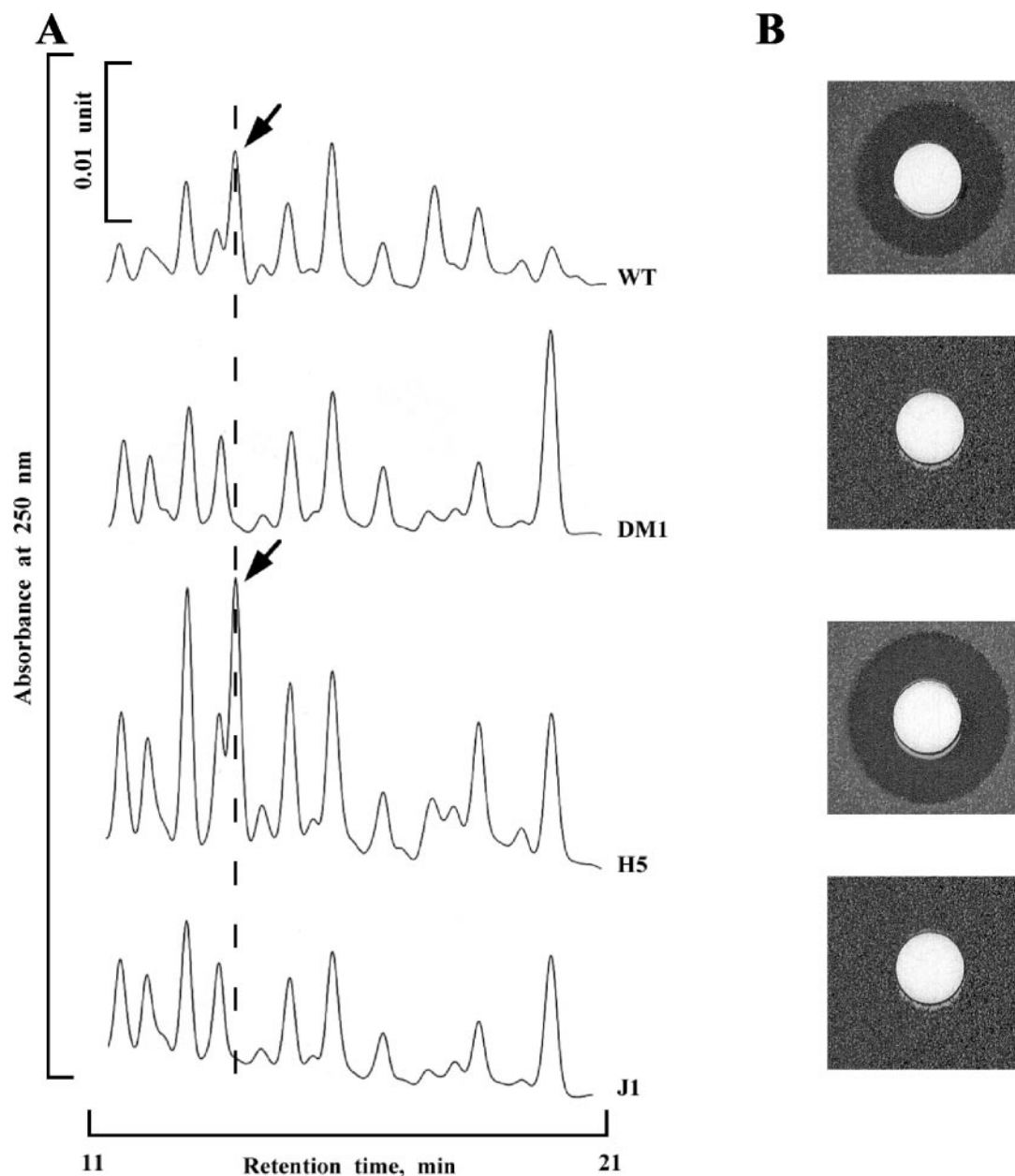


FIG. 6. (A) HPLC analysis of ethyl acetate extracts of the wild-type, DM1, H5, and J1 strains. The arrows indicate a peak present in the wild-type and H5 extracts but absent from the DM1 and J1 extracts. Extracts were separated with a linear gradient from 40% to 70% acetonitrile applied in the presence of 0.1% trifluoroacetic acid (TFA) with a flow rate of 1 ml/min. The absorbance was measured at 250 nm. For clarity, only portions of the HPLC profiles are shown. (B) Bioassay of the HPLC fractions corresponding to the dotted line for antibacterial activity against *B. subtilis* ATCC 6633.

cluster (42), or to congocidin (or netropsin), an oligopeptide compound. Therefore, we conclude that we have identified and purified a third antibiotic produced by *S. ambofaciens* ATCC 23877 and that this is likely to be a member of the angucyclinone or angucycline family.

In addition to the antibacterial activity, another phenotype, production of an orange pigment, was related to the *alp* cluster. Some angucyclic antibiotics are colored molecules, including urdamycins A to F (13) and jadomycin (4). However, we showed that the antibiotic produced by the *alp* cluster and the

pigment were two distinct molecules. Furthermore, we demonstrated that the pigment itself had no antibacterial activity. Comparison of the timing of their appearance in cultures implies that the bioactive compound may be an intermediate in the biosynthesis of the pigment. Several tailoring proteins encoded by the *alp* genes could modify the bioactive compound into a pigmented molecule.

Surprisingly, we identified two additional genes in the cluster, *alpR* and *alpQ*, encoding the KS and chain length factor subunits, respectively that make up two-thirds of a minimal

PKS. AlpR and AlpQ exhibit high similarities to KS and chain length factor proteins involved in mithramycin biosynthesis in *S. argillaceus*, an antibiotic from the aureolic acid group (31), and to the products of *orf69* and *orf68* from the mithramycin-like PKS gene cluster of pSLA2-L (35). However, no gene encoding an acyl carrier protein, the third subunit of a minimal PKS, was identified near the *alpRQ* locus. This subunit could be encoded by a gene located outside the cluster, or possibly AlpC (acyl carrier protein) is shared. Gene replacement of *alpR* (KS) had no obvious effect on either pigment production or antibacterial activity. Therefore *alpR*, and presumably also the overlapping *alpQ* gene (chain length factor), do not appear to be involved in the synthesis of the newly identified polyketide compound.

Although some minor differences could be detected, the *alpTSRQ* locus appears to correspond to a cluster of four ORFs (ORFs 1 to 4) previously described in *S. ambofaciens* ATCC 15154 (10). The authors reported that a mutant of ORF3, the *alpR* homologue, exhibited reduced antibacterial activity against *Micrococcus luteus* in SY medium. Paradoxically, the *orf3* mutant grown on R2YE medium behaved in the opposite way (10). In our hands, the *alpR* double mutant exhibited a slightly higher inhibitory effect on *M. luteus* than the wild-type strain on both SY and R2YE media (data not shown). Therefore, the compound active against *M. luteus* does not correspond to the antibacterial molecule derived from the *alp* cluster.

We propose that AlpR and AlpQ, in combination with AlpA, AlpB, and AlpC (and with the other Alp proteins), might be involved in the biosynthesis of a hybrid antibiotic resulting from the condensation of two different polyketide molecules. The condensation of the two molecules could be catalyzed by AlpM, which shows similarity to the condensation domains of peptide synthases. A natural hybrid antibiotic, the simocyclinone D8, containing an angucycline moiety and partly produced by two sets of PKS has already been described (48).

Interestingly, the *alpTSRQ* locus shows the same genetic and transcriptional organization as the locus from *orf71* to *orf68* identified in the large linear plasmid pSLA2-L of *Streptomyces rochei* (35). The proteins encoded by these loci exhibit high similarity. Therefore, this part of the *alp* cluster could have arisen from horizontal gene transfer involving plasmid-chromosome interactions, as already reported (40). Other parts may also have been acquired by horizontal transfer. The 11 genes from *alpL* to *alpG* indeed exhibit the same order as the genes from *kinX* to *kinO2* from the proposed kinamycin cluster (accession number AY228175). In both cases, the gene products are also highly similar. In addition, the location of the *alp* cluster in the subtelomeric regions is also in favor of acquisition by horizontal transfer. Comparison of the complete genomes of *S. coelicolor* and *S. avermitilis* revealed a 6.5-Mb highly conserved central core that may have evolved from an ancestor possessing a circular chromosome common to all actinomycetes. In contrast, the chromosomal arms, in which more than half of the secondary metabolite clusters are located, are not conserved and may have been acquired subsequently and accumulated separately (6, 24).

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