ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1998, p. 2612–2619 Vol. 42, No. 10
0066-4804/98/$04.00+0

Characterization of a Glycosyl Transferase Inactivating Macrolides, Encoded by *gimA* from *Streptomyces ambofaciens*

ANNE GOURMELEN, MARIE-HÉLÈNE BLONDELET-ROUAULT, AND JEAN-LUC PERNODET*

Institut de Génétique et Microbiologie, UMR 2225, Université Paris-Sud XI, Orsay, France

Received 11 March 1998/Returned for modification 15 May 1998/Accepted 4 August 1998

In *Streptomyces ambofaciens*, the producer of the macrolide antibiotic spiramycin, an open reading frame (ORF) was found downstream of *srmA*, a gene conferring resistance to spiramycin. The deduced product of this ORF had high degrees of similarity to *Streptomyces lividans* glycosyl transferase, which inactivates macrolides, and this ORF was called *gimA*. The cloned *gimA* gene was expressed in a susceptible host mutant of *S. lividans* devoid of any background macrolide-inactivating glycosyl transferase activity. In the presence of UDP-glucose, cell extracts from this strain could inactivate various macrolides by glycosylation. Spiramycin was not inactivated but forocidin, a spiramycin precursor, was modified. In vivo studies showed that *gimA* could confer low levels of resistance to some macrolides. The spectrum of this resistance differs from the one conferred by a rRNA monomethylase, such as SrmA. In *S. ambofaciens*, *gimA* was inactivated by gene replacement, without any deleterious effect on the survival of the strain, even under spiramycin-producing conditions. But the overexpression of *gimA* led to a marked decrease in spiramycin production. Studies with extracts from wild-type and *gimA*-null mutant strains revealed the existence of another macrolide-inactivating glycosyl transferase activity with a different substrate specificity. This activity might compensate for the effect of *gimA* inactivation.

Macrolides inhibit protein synthesis by binding to the large ribosomal subunit (14). Several mechanisms of resistance to macrolides have been described. The most widespread consists of target modification by methylation of 23S rRNA (for a review, see reference 45). Resistance can also be the result of antibiotic export (23; reference 41 and references included) or inactivation. Various chemical modifications leading to macrolide inactivation have been described phosphorylation (24, 25), esterification of the lactone ring (2, 26), and glycosylation (11, 21, 33, 38). All these resistance mechanisms have been identified in pathogens and in *Streptomyces* spp., both those producing and those not producing macrolide antibiotics. Often, macrolide producers have several genes involved in self-resistance. For instance, in *Streptomyces fradiae*, the tylosin producer, four resistance genes have been cloned (4, 9, 35, 47).

Streptomyces ambofaciens produces the macrolide antibiotic spiramycin (31). Spiramycin is composed of a 16-atom lactone ring with two amino sugars and one neutral sugar attached. The mycelium is sensitive to spiramycin during exponential growth phase and then becomes resistant, with the concomitant production of spiramycin, during stationary phase. In *S. ambofaciens*, at least two resistance mechanisms are present (29). Several resistance determinants have been cloned (34, 39). One of them, *srmA*, encodes a monomethyltransferase acting at position A2058 of 23S rRNA (28), thereby conferring resistance to macrolides-lincosamides-streptogramin B (type I resistance phenotype [30]). Here we report the cloning, sequencing, and characterization of *gimA*, a new macrolide resistance gene located downstream of *srmA*.

MATERIALS AND METHODS

Strains and culture conditions. Strains and plasmids used in this study are listed in Table 1. *Streptomyces* strains were maintained on Hickey-Tressner (HT) medium at 30°C (32). For spiramycin production, *S. ambofaciens* strains were grown in MP5 liquid medium (29) at 27°C. *Streptomyces lividans* strains were maintained on R2YE medium (17) at 30°C. AS1 medium (3) was used for conjugation experiments, and cultures were grown at 37°C (see below). *S. lividans* strains were grown in tryptic soy broth medium (TSB; Difco) at 30°C for preparation of S30 cellular extracts. To ensure maintenance of plasmids carrying the thiostrepton resistance gene (*tsr*), nosiheptide (NO) was added to the solid medium at 40 μg/ml and to the liquid medium at 10 μg/ml for *S. lividans* cultures, and at 200 μg/ml to the solid medium for *S. ambofaciens* cultures.

Micrococcus luteus cells grown at 37°C in Luria-Bertani (LB) medium (Gibco BRL) were added to soft agar overlay on LB plates. Observation of growth inhibition zones was done after incubation of petri plates for 24 to 48 h at 37°C. The *M. luteus* congocidin (CG)-resistant mutant was grown in LB medium in the presence of CG at 5 μg/ml.

Antibiotics. The antibiotics were obtained from the following sources: hygromycin B (HM), Boehringer (Mannheim, Germany); ampicillin, Appligene (Illkirch, France); chalcomycin, tylosin, geneticin (GN), erythromycin, oleandomycin, and nalidixic acid, Sigma (St. Louis, Mo.); carbomycin and azithromycin, Pfizer (Orsay, France); josamycin, Pharmuka (Genevilliers, France); rosaramicin, Schering-Plough Corporation (Union, N.J.); neospiramycin, forocidin, spiramycin, NO, and CG, Rhône-Poulenc Rorer (Paris, France); angolamycin, lankamycin, methymycin, and pikromycin, E. Cundliffe (Leicester University, Leicester, United Kingdom).

DNA manipulations and bacterial transformation. DNA extraction and manipulation and transformations of *Escherichia coli* and *Streptomyces* were performed according to standard protocols (17, 36).

DNA sequencing. The DNA sequencing protocol was as described by Sanger et al. (37), with modifications as described by Biggin et al. (8). A Deaza T7 sequencing kit was obtained from Pharmacia, and α-^{35}S-dATP was obtained from Amersham. Sequences were determined for both strands.

Computer-assisted sequence analysis. Sequence comparisons with databases were performed with the FASTA (27) and BLAST programs (1).

Preparation of crude extracts. This preparation was performed essentially as described by Cundliffe (11), except that cell breakage was obtained by ultrasonic treatment. Spores were used to inoculate TSB. Cultures were grown with vigorous shaking for 24 h. Mycelia were harvested by filtration, washed twice with HS buffer (10 mM HEPES-KOH [pH 7.5], 10 mM $MgCl_2$, 1 M NH_4Cl, 5 mM β-mercaptoethanol) and once with HRS buffer (10 mM HEPES-KOH [pH 7.5], 10 mM $MgCl_2$, 50 mM NH_4Cl, 5 mM β-mercaptoethanol). Mycelia were resuspended in HRS buffer prior to cell breakage by ultrasonication, followed by centrifugation at 30,000 × *g* for 30 min. The supernatant was then dialyzed against HM buffer (10 mM HEPES-KOH [pH 7.5], 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10% glycerol), resulting in S30 extracts, and stored as aliquots at −70°C. For *S. ambofaciens*, cultures were grown in TSB containing spiramycin at 10 μg/ml for 20 h, and 10 μg of spiramycin per ml was again added 90 min before harvesting. Spiramycin was added because the cultivation time was too short to allow production and appearance of the resistance. As the expression of some resistance genes (29), including *srmA* and probably *gimA* (15), could be induced by spiramycin, this treatment ensured their early expression.

* Corresponding author. Mailing address: Laboratoire de Biologie et Génétique Moléculaire, Institut de Génétique et Microbiologie, Bât. 400, Université Paris-Sud XI, 91405 Orsay Cedex, France. Phone: 33 (0) 1 69 15 69 13. Fax: 33 (0) 1 69 15 72 96. E-mail: pernodet@igmors.u-psud.fr.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
S. ambofaciens ATCC 23877	Wild-type strain	31
S. ambofaciens OS81	Non-spiramycin-producing mutant of ATCC 23877	29
S. ambofaciens OS41.99	Hm^r; derivative of ATCC 23877, with *gimA* inactivated by gene replacement	This study
S. ambofaciens OS41.99NP	Hm^r; derivative of OS81, with *gimA* inactivated by gene replacement	
S. lividans OS456	Hm^r; derivative of TK21 (18), with *lrm* and *mgt* inactivated by gene replacement; highly sensitive to macrolides	30
M. luteus DSM1790	Indicator strain, highly sensitive to macrolides	
M. luteus Cg^r	Mutant strains of *M. luteus* DSM1790, resistant to CG	This study
E. coli DH5α	Strain used for DNA cloning	Clontech
E. coli S17.1	Donor strain used for *E. coli*/*Streptomyces* conjugation	40
Plasmids		
pHP45 Ω*hyg*	Ap^r Hm^r; source of the Ω*hyg* cassette	10
pWED1	Ap^r; cosmid vector derived from pWE15 (44) by deletion of the 4.1-kb *Hpa*I-*Hpa*I fragment, removing the mammalian expression module	This study
pIJ4070	Ap^r; pIJ2925 (19) containing the *ermE*-up promoter mutant *ermEp** (6)	5
pIJ903	Ap^r No^r; low-copy-number *Streptomyces*/*E. coli* vector	22
pOJ260	Gn^r; replicative vector in *E. coli*, nonreplicative in *Streptomyces*; used for conjugation experiments	7
pOS41.78	Ap^r; cosmid from the *S. ambofaciens* ATCC 23877 gene library in pWED1 hybridizing with *srmA*	This study
pOS41.80	Ap^r; derived from pOS41.78; 3.6-kb *Bam*HI fragment hybridizing with the end of *srmA* cloned into pUC19 (Fig. 1)	This study
pOS41.85	Ap^r; derived from pOS41.80; *Sgr*AI-*Bam*HI fragment cloned into pIJ4070 *Bam*HI (Fig. 1)	This study
pOS41.90	Ap^r No^r; derived from pOS41.85; fragment containing *ermEp** and *gimA* cloned into pIJ903 (Fig. 1)	This study
pOS41.105	Ap^r No^r; derived from pOS41.90; in-frame deletion in *gimA* from *Nru*I to *Sma*I (Fig. 1)	This study
pOS41.98	Ap^r Hm^r; derived from pOS41.80; *Nco*I-*Bam*HI fragment with the internal *Spl*I-*Spl*I fragment replaced by Ω*hyg* (Fig. 1)	This study
pOS41.99	Gn^r Hm^r; insert from pOS41.98 cloned into pOJ260 (Fig. 1)	This study

In vitro inactivation of macrolides. Complete reaction mixtures (total volume, 100 μl) contained dialyzed S30 extract (90 μl), UDP-glucose (1 mM), and macrolide (100 μg/ml) and were incubated at 30°C. At time zero and after various times of incubation, samples were removed and frozen at −20°C to stop the reaction. They were then applied to Whatman AA paper discs (6 mm in diameter), and residual antibiotic activity was examined by bioassay with *M. luteus*.

Glycosyl transferase assays. The experiments were performed according to the method developed by Cundliffe (11) except that S30 extracts were used instead of S100*. S30 extract was incubated (total volume, 110 μl) with 0.125 μCi of UDP-[^{14}C]glucose (specific activity, 335 mCi/mmol or 12.4 GBq/mmol) and 50 μg of macrolide antibiotic. The extent of ^{14}C-glycosylation of the drug was calculated by the subtraction of background values obtained from drug-free control assays. For *S. lividans* harboring the cloned gene, 10 μl of S30 extracts was used, while for *S. ambofaciens*, 100 μl was needed for the assays.

Resistance conferred by *gimA*. Various dilutions of spore suspensions of *S. lividans* OS456(pOS41.90) or -(pIJ903) (control) were plated on HT medium supplemented with increasing concentrations of various macrolides or without antibiotics. The cultures were then incubated for 48 h before counting was performed.

Spiramycin production assays. For spiramycin production, MP5 was inoculated with spores at a concentration of 2.5×10^6 spores/ml. No pregermination treatment was done before inoculation. The cultures (70 ml in 500-ml baffled flasks) were incubated at 27°C in an orbital shaker at 250 rpm. In order to take in account the variability of individual cultures, six identical cultures of each strain were included in the study. At 0, 32, 45, 54, 72, and 94 h, samples were withdrawn, and supernatants were independently frozen. The presence of spiramycin in the supernatant of each sample was detected and quantified by bioassay against *M. luteus* and by high-pressure liquid chromatography (HPLC).

For bioassays, 70 μl of supernatant was applied to Whatman AA paper discs (12 mm in diameter) in parallel with known quantities of spiramycin. Discs were laid on plates containing *M. luteus*. A CG-resistant mutant (Table 1) obtained for this purpose was used because *S. ambofaciens* produces CG in addition to spiramycin. Plates were incubated at 4°C for 2 h to allow antibiotic diffusion and then were incubated at 37°C. Diameters of growth inhibition zones were measured after 48 h. Analytic HPLC was carried out as described by Dary et al. (12). Mixtures of spiramycin I, II, and III were used as standards.

Conjugal transfer from *E. coli* to *S. ambofaciens*. Conjugal transfer was performed essentially as described by Bierman et al. (7). Spores of *S. ambofaciens* ATCC 23877 or OS81 (Table 1) pregerminated as described by Hopwood et al. (17) were used. The optimal conditions were found to be 10^6 *S. ambofaciens* CFU and 6×10^7 *E. coli* CFU per plate. Plates were incubated at 37°C. After 18 h, they were washed with LB and gently scraped to remove the *E. coli* layer. Then they were covered with an overlay containing HM (at a final concentration of 50 μg/ml) to select transconjugants and nalidixic acid (at a final concentration of 50 μg/ml) to inhibit *E. coli* growth. Incubation was continued for 5 days.

Nucleotide sequence accession number. The nucleotide sequence obtained in this study has been deposited in the GenBank/EMBL database under accession no. AJ223970.

RESULTS

Cloning and sequencing of *gimA*. Downstream of macrolide resistance gene *srmA* (28), sequence analysis revealed a truncated open reading frame (ORF). The putative GTG start codon of that ORF overlapped the *srmA* stop codon (GTGA). To investigate a possible role of this second ORF in resistance, the cloning of the complete ORF was undertaken. An *S. ambofaciens* gene library constructed in cosmid pWED1 (Table 1) was probed by colony hybridization with a DNA fragment containing *srmA* and the truncated ORF. This led to the isolation of cosmid pOS41.78, from which a 3.6-kb *Bam*HI fragment containing *srmA* and 1.7 kb of downstream DNA sequence was subcloned into pUC19 to yield pOS41.80. The sequence of the region downstream of *srmA* was determined. It revealed the presence of a 1,254-bp ORF which could encode a 45-kDa protein (data not shown). Its GTG start codon was preceded, 8 bp upstream, by the sequence GAGGAG, which could constitute a good Shine-Dalgarno sequence. This ORF showed a typical *Streptomyces* codon usage (46). The analysis of the deduced protein sequence revealed high degrees of similarity to proteins deduced from *mgt* of *S. lividans* (82.5% identity) (20) and *oleD* of *Streptomyces antibioticus* (80.9% identity) (16). Mgt from *S. lividans* is a glycosyl transferase inactivating macrolides by the addition of a glucose residue (11, 20). The gene *mgt* is also cited as *mgtA* in the literature. *S. lividans* is not known to produce any macrolide. In *S. antibioticus*, producer of the macrolide oleandomycin, OleD is a glycosyl transferase inactivating several macrolides including

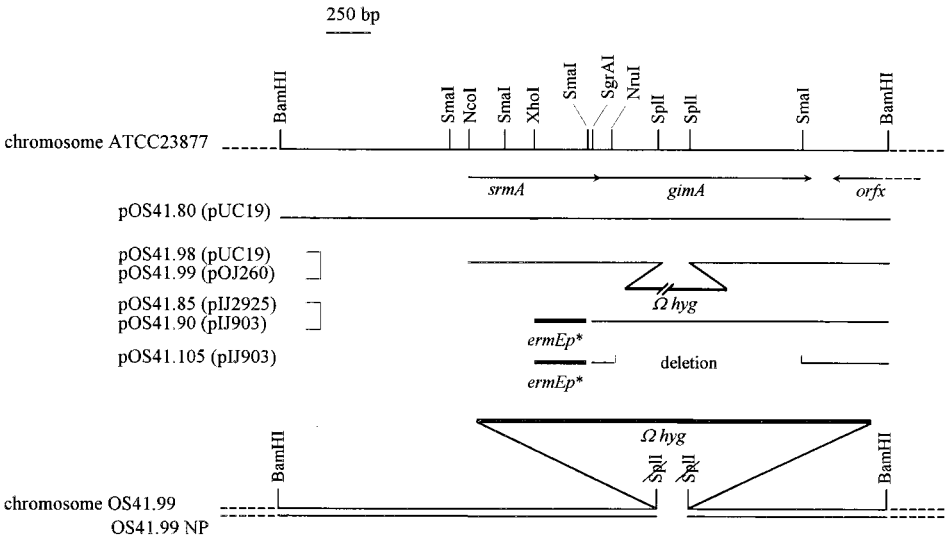

FIG. 1. Map of the chromosome of *S. ambofaciens* in the region of *srmA* and *gimA*. The inserts found in the plasmid constructions used for this study are indicated. The names of the corresponding vectors are in parentheses. The *srmA-gimA* chromosomal regions of *S. ambofaciens* strains, in which *gimA* has been inactivated by gene replacement, are also presented.

oleandomycin (33). Because of the strong similarity of the product of the ORF downstream of *srmA* to Mgt from *S. lividans*, we hypothesized that this product might also be a glycosyl transferase inactivating macrolides. Therefore, the ORF was called *gimA*.

The *gimA* structural gene was cloned downstream from the strong, constitutive *ermE*-up promoter mutant *ermEp** (6), yielding plasmid pOS41.90 (Table 1; Fig. 1). This plasmid was used for most of the experiments described below.

Activity of the *gimA* product. In a first approach, experiments were performed to test if the *gimA* product could inactivate macrolides. As *S. ambofaciens* possesses several other resistance genes, this was done with the cloned *gimA* gene. pOS41.90 was introduced into *S. lividans* OS456 (Table 1), a mutant strain of *S. lividans* in which the resistance genes *lrm* and *mgt* have been inactivated, providing a host strain highly sensitive to macrolides and with no background Mgt activity (30). Crude S30 extracts were prepared from OS456(pOS41.90) and from OS456(pIJ903) (control strain containing the vector without insert) and used for various tests: inactivation of macrolides followed by biological assay of residual antibiotic activity with *M. luteus* as the indicator strain and glycosylation tests using UDP-[^{14}C]glucose.

Extracts from OS456(pOS41.90) were able to inactivate some macrolides in the presence of UDP-glucose. In particular, chalcomycin and rosaramicin were completely inactivated after 1 h at 30°C, while no spiramycin inactivation could be detected. In the absence of UDP-glucose, no inactivation was observed (Fig. 2). This UDP-glucose-dependent inactivation was not detected with extracts from OS456(pIJ903). Inactivation was also observed with UDP-galactose as a cofactor (data not shown).

Extracts from OS456(pOS41.90) were used, with UDP-[^{14}C]glucose, to test and quantify the transfer of glucose to macrolides. Chalcomycin, which was rapidly inactivated, was used in this assay. [^{14}C]glucose was transferred to chalcomycin, and almost all the [^{14}C]glucose available in the reaction mixture was transferred within 6 min (see below and Fig. 3).

These results demonstrated that the *gimA* product could inactivate macrolide antibiotics by transfer of a glucose residue. UDP-galactose could replace UDP-glucose, but as discussed by Cundliffe (11) it is unknown if UDP-galactose is used per se or if it must be converted to UDP-glucose (or vice versa). Therefore, GimA activity was called glycosyl transferase rather than glucosyl transferase.

Substrate specificity of GimA. The radiotransfer assay allows quantification of the GimA activity. This assay was used to study the substrate specificity of GimA, in order to compare it with those of other known macrolide-inactivating glycosyl transferases from *S. lividans* (11), *S. antibioticus* (33), and

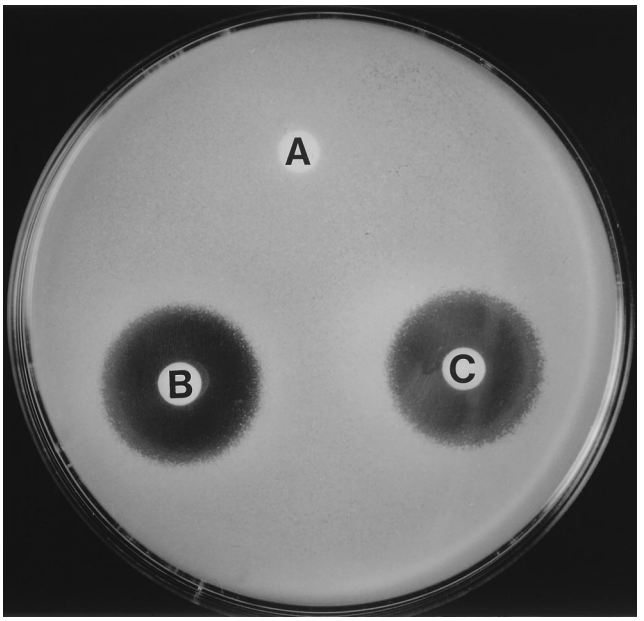

FIG. 2. In vitro inactivation of chalcomycin by S30 extracts from *S. lividans* OS456(pOS41.90). Paper disks impregnated with samples from inactivation assays were used to test residual antibiotic activity on *M. luteus*. Samples came from complete reaction mixture: S30 extract, chalcomycin, and UDP-glucose (A); reaction mixture without UDP-glucose (B); and reaction mixture without S30 (C).

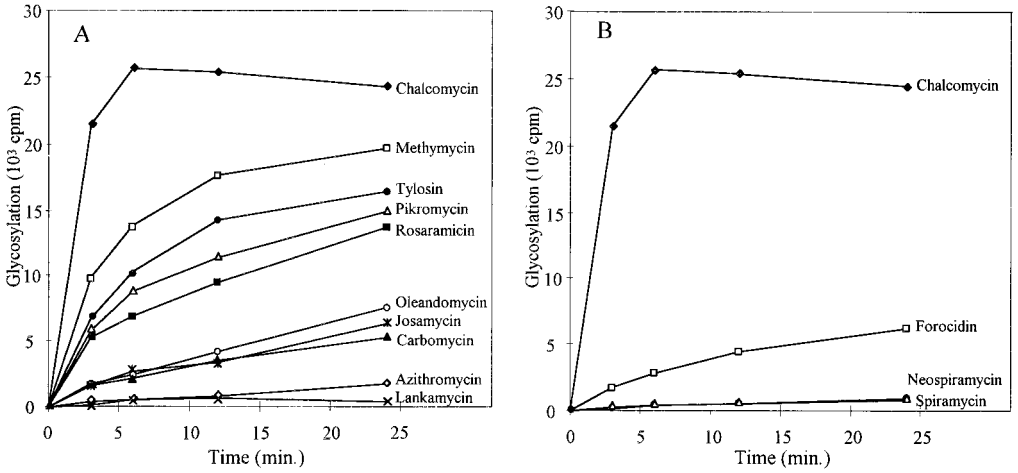

FIG. 3. Glycosylation of macrolide antibiotics with extracts from *S. lividans* OS456(pOS41.90). Reaction mixtures contained crude GimA together with 5 μl of UDP-[^{14}C]glucose and 5 μl of macrolide solution in dimethyl sulfoxide (at 10 mg/ml) as described in Materials and Methods. (A) Glycosylation of various macrolides. The same results were obtained with erythromycin, angolamycin, and lankamycin; only those for lankamycin are shown. (B) Glycosylation of spiramycin and its precursors compared with glycosylation of chalcomycin.

Streptomyces hygroscopicus (38). Moreover, the radiotransfer assay should indicate if GimA could glycosylate spiramycin precursors in the biosynthetic pathway. This last point could not be studied by inactivation, because some of the precursors of spiramycin lacked antibiotic activity.

With UDP-[^{14}C]glucose as the cofactor, crude S30 extracts from OS456(pOS41.90) were tested on various macrolides (Fig. 3A). Among those, chalcomycin was the most active substrate. Methymycin, tylosin, pikromycin, and rosaramicin were four of the best substrates. Oleandomycin, josamycin, and carbomycin were glycosylated to a lesser extent. Macrolides that were found to be as poor substrates of GimA as lankamycin were erythromycin and angolamycin. Spiramycin was also a very poor substrate (Fig. 3B). As *gimA* originates from a spiramycin producer, it was interesting to study the glycosylation of spiramycin precursors. Forocidin consists of the 16-atom lactone ring with only mycaminose attached. Neospiramycin possesses two amino sugars, mycaminose and forosamine. The biosynthetic pathway ends with spiramycin with three sugars attached, the mycarose being attached to mycaminose. The results presented in Fig. 3B demonstrate that neospiramycin was as poorly modified as spiramycin but, interestingly, that forocidin could be glycosylated.

Resistance profile conferred by *gimA*. It was unknown whether a macrolide-inactivating glycosyl transferase could confer a resistant phenotype or not. To answer this question, the resistances of *S. lividans* OS456(pOS41.90) to various macrolides were compared to those of *S. lividans* OS456(pIJ903) (control strain). This study could only be performed with the cloned *gimA* gene and not with *S. ambofaciens*, which possesses several resistance mechanisms.

The results are presented in Fig. 4. Some of the macrolides tested in the radiotransfer assay were not available in sufficient amounts for this experiment. Among the ones tested, those for which the difference in survival between OS456(pOS41.90) and the control strain was the greatest were chalcomycin, rosaramicin, tylosin, and oleandomycin. In these cases, the presence of *gimA* increased the survival by at least a factor of 100 at several concentrations of the antibiotics. These four antibiotics were among the best substrates for GimA in vitro. However, for other macrolides tested, no simple correlation between the level of resistance in vivo and the in vitro activity could be established. For instance, with spiramycin, a clear difference in survival between the strain harboring *gimA* and the control strain was observed, even though spiramycin was one of the poorest substrates. These results might indicate that different macrolides are taken up and/or accumulated by *S. lividans* with different efficiencies.

Gene replacement inactivating *gimA* in *S. ambofaciens*. In order to determine the role played by *gimA* in the self-protection of *S. ambofaciens* from spiramycin, its inactivation was attempted both in the ATCC 23877 wild-type strain and in the non-spiramycin-producing mutant OS81 (Table 1). As shown in Fig. 1, an *Spl*I fragment internal to *gimA* was deleted and replaced by the Ω*hyg* cassette, generating pOS41.98 (in pUC19) and then pOS41.99 (in conjugative vector pOJ260; conferring GN resistance) (Table 1). Then, pOS41.99 was introduced into *S. ambofaciens* via conjugal transfer from *E. coli* S17.1 (40) and HM selection was applied. Five days after conjugation, Hm^r transconjugants were picked and examined for their GN resistance. Hm^r Gn^s clones were obtained from both strains. Such clones had presumably lost the pOJ260 part by a double-crossover recombination event. The replacement of *gimA* by its disrupted counterpart from pOS41.99 was confirmed by Southern blot analysis (data not shown). For instance, the *gimA* probe revealed that the hybridizing *Bam*HI fragment had increased from 3.6 kb in the wild type to 6.1 kb in the mutant strains. Hybridization with Ω*hyg* confirmed its presence in the mutants and its absence in the wild-type and OS81 strains. One mutant strain with *gimA* interrupted derived from the wild-type strain was designated OS41.99. One equivalent mutant derived from OS81 was designated OS41.99NP. These two strains were not affected in their growth or sporulation.

Effect of *gimA* on spiramycin production. The issue investigated was the ability of strain OS41.99 to produce spiramycin, and the possible effect of *gimA* disruption or overexpression on the level of spiramycin production. OS41.99 was first grown in MP5 production medium, and the supernatant activity was tested by a bioassay using *M. luteus* Cg^r (Table 1) and further characterized by HPLC. It was found that OS41.99 could still produce spiramycin, indicating that *gimA* is not a resistance gene essential for the survival of the strain, even under antibiotic production conditions.

Therefore, as GimA could glycosylate some spiramycin pre-

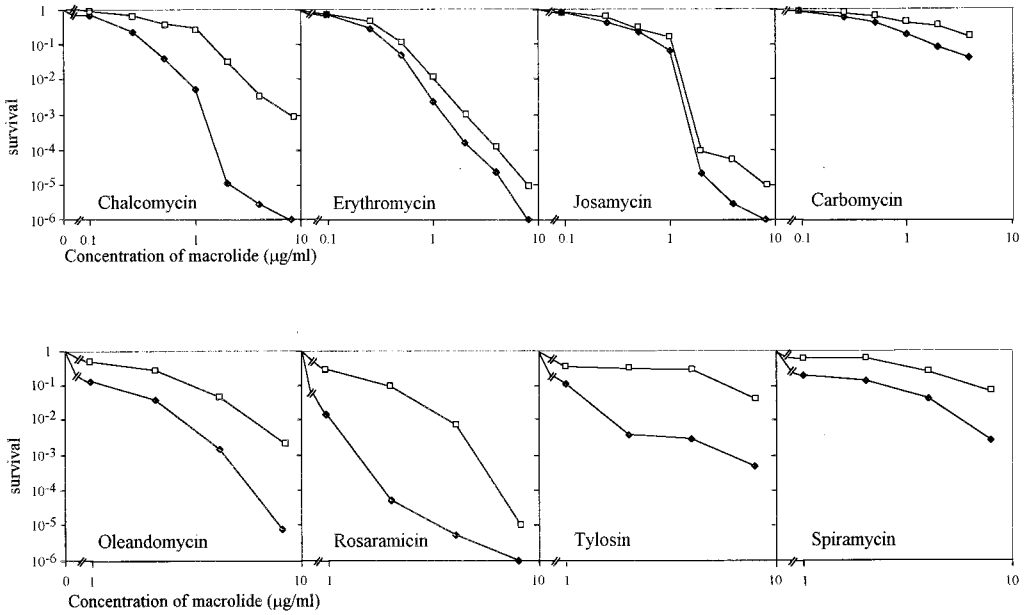

FIG. 4. Resistance conferred by *gimA*. Spores from *S. lividans* OS456(pOS41.90) and OS456(pIJ903) (control) were plated on HT medium without or with different macrolides at various concentrations. The survival rates at various macrolide concentrations, each calculated as the ratio of the number of CFU on the medium with the macrolide to the number of CFU on the same medium without the antibiotic (log scale), are presented for OS456(pOS41.90) (white squares) and OS456(pIJ903) (black squares).

cursors, and as GimA could confer resistance towards spiramycin in vivo, it was of interest to investigate whether or not the inactivation or the constitutive expression of *gimA* had an effect on the spiramycin production level. The production of spiramycin by the following four *S. ambofaciens* strains was studied: wild-type strain ATCC 23877, OS41.99 where *gimA* is inactivated, OS41.99(pOS41.90) (where *gimA* expression is governed by the strong and constitutive *ermE*-up promoter mutant *ermEp**), and, as a control, OS41.99(pOS41.105). The last plasmid was derived from pOS41.90 by an in-frame deletion removing most of the *gimA* gene (Fig. 1). This control was done to ensure that a possible effect observed with pOS41.90 was due to the presence of *gimA*, and not to the presence of the vector carrying *ermEp**. As has been reported, introduction of plasmid vectors could affect antibiotic production in some strains (42).

All strains were grown under the same conditions, with no selective pressure. The presence of the low-copy-number replicative plasmids pOS41.90 and pOS41.105 was checked at different times by plating the dispersed mycelia on medium without antibiotics, followed by replica plating on medium with NO. After 94 h of growth in MP5, the plasmids were still present in 100% of the CFU. The growth rates of the different strains in MP5 were similar (data not shown). The levels of spiramycin production were determined by HPLC.

The mean values for spiramycin production are presented in

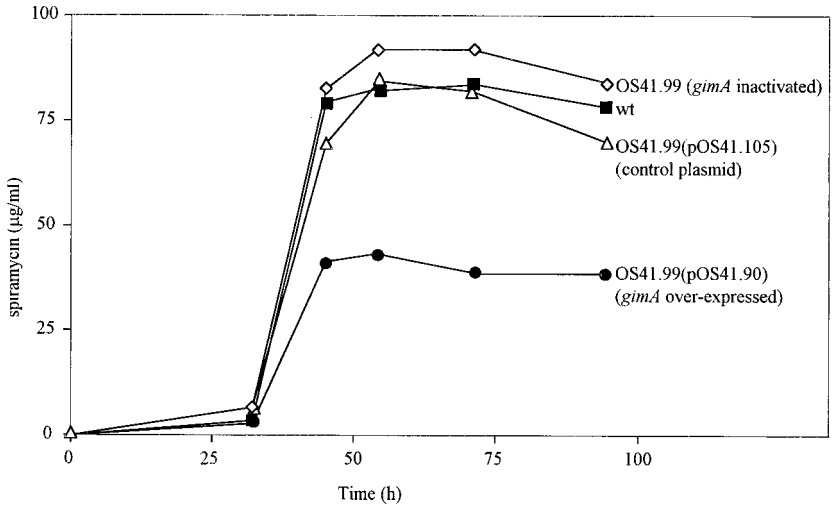

FIG. 5. Effect of *gimA* expression on spiramycin production in *S. ambofaciens*. Six identical independent cultures of each strain were grown in MP5 medium. Samples were withdrawn at 0, 32, 45, 54, 71, and 94 h. Spiramycin was detected and quantified by HPLC. For each strain, the curve was drawn from the averages of the six independent values obtained for each time. wt, wild type.

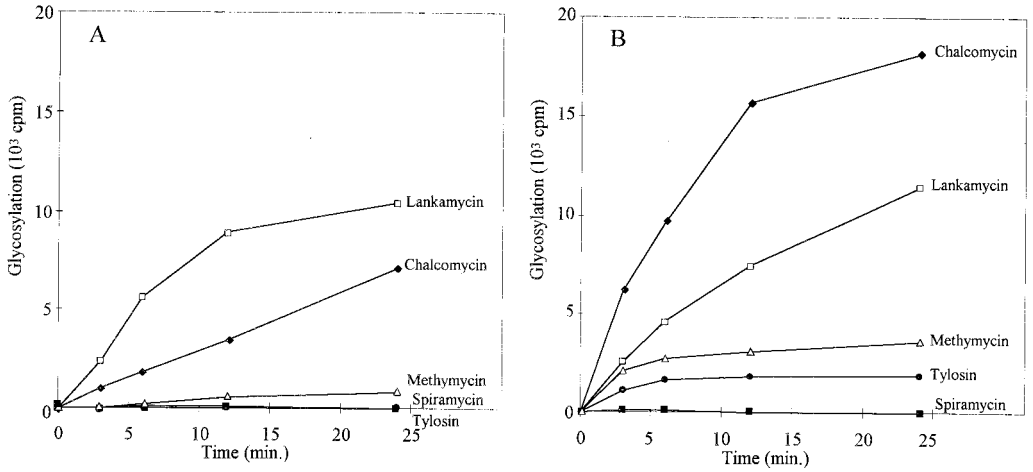

FIG. 6. Glycosylation of macrolides with S30 extracts from *S. ambofaciens*. Reaction mixtures contained crude extract together with 5 μl of UDP-[^{14}C]glucose and 5 μl of macrolide solution in dimethyl sulfoxide (at 10 mg/ml) as described in Materials and Methods. (A) Extracts from OS41.99 (*gimA*-null mutant); (B) extract from the wild-type strain.

Fig. 5. The observed slight increase in spiramycin production between the *gimA* mutant OS41.99 and the wild-type strain is not significant (data not shown). However, for strain OS41.99 (pOS41.90), where *gimA* is constitutively expressed, the level of spiramycin production was significantly decreased, by a factor of 2, in comparison with that of OS41.99 and wild-type strains. This effect could be attributed to the expression of *gimA*, carried by pOS41.90, as in the same strain the presence of control plasmid pOS41.105 caused a nonsignificant decrease.

Evidence for the existence of another glycosyl transferase inactivating macrolides in *S. ambofaciens*. S30 extracts were prepared from the *S. ambofaciens* wild-type strain and the OS41.99 mutant. Surprisingly, extracts from both strains inactivated chalcomycin in the presence of UDP-glucose (data not shown). Moreover, these extracts were used in radiotransfer assays, and a comparison of the results presented in Fig. 3A and 6A shows that the substrate specificity of GimA was clearly different from that of the activity detected in OS41.99. For instance, lankamycin, which was a very poor substrate for GimA (Fig. 3A), was found to be a very active substrate for the activity detected in both *S. ambofaciens* strains (Fig. 6). The activity observed in the wild-type strain (Fig. 6B) could result from the addition of both activities. A comparison of results presented in Fig. 6A and 6B shows that lankamycin was glycosylated to comparable levels with extracts from both strains but that *gimA* inactivation led to a decrease in the glycosylation levels of chalcomycin, tylosin, and methymycin, which were good substrates for GimA. These observations indicated the existence of another macrolide-inactivating glycosyl transferase activity, with a substrate specificity different from that of GimA.

DISCUSSION

The mechanism of macrolide inactivation by glycosylation at the C-2′ position has been most often described for *Streptomyces*: this glycosyl transferase activity was first described for *Streptomyces vendargensis* acting on erythromycin A (21) and seems widespread among *Streptomyces* strains (38). It was studied for strains not producing macrolides such as *S. lividans* (11), and *S. hygroscopicus* (38). In *S. antibioticus*, producer of the macrolide oleandomycin, two glycosyl transferases (OleD and OleI) and a glycosidase (OleR) are involved in oleandomycin modification during its biosynthesis (33).

In *S. ambofaciens*, the *gimA* gene was found immediately downstream of *srmA* (28), a gene encoding a methyltransferase conferring macrolide resistance by target modification. As shown in Fig. 7, macrolide-inactivating glycosyl transferase genes are

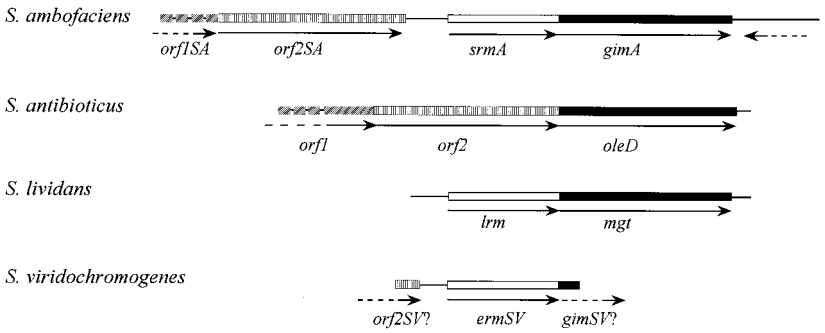

FIG. 7. Comparison of the genetic environments around macrolide resistance genes in various *Streptomyces* species. *gimA*, *oleD*, and *mgt* gene products have about 80% identity. *srmA*, *lrm*, and *ermSV* encode monomethyltransferases with about 80% identity. The deduced products of *orf1SA* and *orf2SA* from *S. ambofaciens* and of *orf1* and *orf2* from *S. antibioticus* have, respectively, 75 and 76% identity. The deduced product of the end of an ORF upstream of *ermSV* has 69% identity with the deduced products of *orf2* and *orf2SA*. The deduced product of the short sequence downstream of *ermSV* is similar to glycosyl transferases inactivating macrolides. The GenBank/EMBL accession numbers are AJ223970 for *gimA*, Z22577 for *oleD*, M74717 for *mgt*, and U59450 for *ermSV*.

associated with methyltransferase genes in *S. ambofaciens*, in *S. lividans*, and probably in *Streptomyces viridochromogenes*. The last two strains are not known to produce any macrolide antibiotics. For *S. antibioticus*, no methyltransferase gene is found upstream of *oleD* or *oleI*. This is in agreement with the observation that ribosomes from *S. antibioticus* are sensitive to oleandomycin, even during production (13). Interestingly, the two ORFs upstream of *oleD* have high degrees of similarity to those upstream of *srmA*. This could indicate that some deletion or insertion events occurred at the corresponding loci in *S. antibioticus* or in *S. ambofaciens*. In *S. lividans*, the region located upstream of *lrm* is completely different from those found in the three other strains, indicating transfer may have occurred in this region.

The substrate specificities of macrolide-inactivating glycosyl transferases from *S. lividans*, *S. antibioticus*, and *S. hygroscopicus* and that of GimA from *S. ambofaciens* are different. The size of the lactone ring does not seem to be a factor discriminating the poor and good substrates as discussed by Cundliffe (11). As in other cases, the sugar glycosylated by GimA is probably the one attached to the C-5 position of the 16- and 14-member lactone rings (21) (corresponding to C-3 in the 12-member lactone ring of methymycin). On this sugar, the position modified is probably that of the 2′-OH group. As expected in this case, angolamycin, which lacks this hydroxyl group, was revealed to be a very poor substrate of GimA. Our results, with the exception of those for tylosin, are in agreement with the suggestion that macrolides monosubstituted at position C-5 are better substrates than those disubstituted (43).

The ability of glycosyl transferases inactivating macrolides to confer resistance in vivo has never been investigated until now. This was done with the cloned gene *gimA*, introduced into a strain susceptible to macrolides, *S. lividans* OS456. Resistance was indeed observed, but the levels of resistance were very low, in particular, much lower than those conferred by target modifying enzymes: those encoded by either *tlrD*, *ermE* (30), or *srmA* (28). The low level of resistance was probably not due to a low level of expression of *gimA*. Indeed, cellular extracts prepared from OS456(pOS41.90), the strain used for the whole-cell resistance study, showed greater glycosyl transferase activity than the ones prepared from the *S. ambofaciens* wild-type strain. Although the level of resistance conferred by GimA was low, GimA nevertheless conferred resistance to chalcomycin and tylosin, while SrmA was unable to confer any resistance to these two macrolides.

In a strain producing a macrolide antibiotic, a gene encoding a glycosyl transferase inactivating macrolides might play a role in the self-protection of the strain, as *oleI* does in *S. antibioticus*. Nevertheless, gene *gimA* could be inactivated in *S. ambofaciens* without any deleterious effect, even during production of spiramycin. But *S. ambofaciens* possesses additional macrolide resistance mechanisms (including a second macrolide-inactivating glycosyl transferase), which might provide protection against the produced antibiotic.

In *S. antibioticus*, *oleI* is located among oleandomycin biosynthetic genes and its product is supposed to be the enzyme responsible for oleandomycin glycosylation during biosynthesis. *oleD* is not located in the oleandomycin cluster and might play a more general role, one not directly involved in oleandomycin biosynthesis (33). In *S. ambofaciens*, *gimA* is dispensable; it is probably not located in the spiramycin cluster, and its product is not very active on spiramycin. A comparison of genetic organization and protein sequences indicated that *gimA* is more related to *mgt* and *oleD* than to *oleI*. Therefore, *gimA* could be involved in the resistance to exogenous macrolides rather than in self-protection against spiramycin during biosynthesis. However, the constitutive overexpression of *gimA* led to a decrease in spiramycin production. This effect could be due to the action of GimA on spiramycin itself, or more probably on spiramycin precursors such as forocidin. These glycosylated precursors might not be substrates for later steps of the biosynthetic pathway, or the glycosylated spiramycin produced might not be reactivated.

ACKNOWLEDGMENTS

We are very grateful to E. Cundliffe for introducing us to the use of the radiotransfer assay for the measurement of macrolide-inactivating glycosyl transferase activity, for the kind gift of various macrolides, and for helpful discussions. We thank P. Lacroix for the kind gift of spiramycin precursors and CG, J. Hugueville for HPLC analysis, P. Mazodier for providing *E. coli* S17-1, and M. Bibb for providing pIJ4070. We thank M. Guérineau for support and critical reading of the manuscript.

This work was supported by CNRS and the Alliance Programme. A.G. received a PhD fellowship from the Ministère de l'Education Nationale, de la Recherche et de la Technologie.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Arthur, M., D. Autissier, and P. Courvalin.** 1986. Analysis of the nucleotide sequence of the *ereB* gene encoding the erythromycin esterase type II. Nucleic Acids Res. **14:**4987–4999.
3. **Baltz, R. H.** 1980. Genetic recombination by protoplast fusion in *Streptomyces*. Dev. Ind. Microbiol. **21:**43–54.
4. **Baltz, R. H., and E. T. Seno.** 1988. Genetics of *Streptomyces fradiae* and tylosin biosynthesis. Annu. Rev. Microbiol. **42:**547–574.
5. **Bibb, M. J.** Personal communication.
6. **Bibb, M. J., J. White, J. M. Ward, and G. R. Janssen.** 1994. The mRNA for the 23S rRNA methylase encoded by the *ermE* gene of *Saccharopolyspora erythraea* is translated in the absence of a conventional ribosome-binding site. Mol. Microbiol. **14:**533–545.
7. **Bierman, M., R. Logan, K. O'Brien, E. T. Seno, R. N. Rao, and B. E. Schoner.** 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene **116:**43–49.
8. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80:**3963–3965.
9. **Birmingham, V. A., K. L. Cox, J. L. Larson, S. E. Fishman, C. L. Hershberger, and E. T. Seno.** 1986. Cloning and expression of a tylosin resistance gene from a tylosin-producing strain of *Streptomyces fradiae*. Mol. Gen. Genet. **204:**532–539.
10. **Blondelet-Rouault, M. H., J. Weiser, A. Lebrihi, P. Branny, and J. L. Pernodet.** 1997. Antibiotic resistance gene cassettes derived from the omega interposon for use in *E. coli* and *Streptomyces*. Gene **190:**315–317.
11. **Cundliffe, E.** 1992. Glycosylation of macrolide antibiotics in extracts of *Streptomyces lividans*. Antimicrob. Agents Chemother. **36:**348–352.
12. **Dary, A., N. Bourget, N. Girard, J. M. Simonet, and B. Decaris.** 1992. Amplification of a particular DNA sequence in *Streptomyces ambofaciens* RP181110 reversibly prevents spiramycin production. Res. Microbiol. **143:**99–112.
13. **Fierro, J. F., C. Hardisson, and J. A. Salas.** 1987. Resistance to oleandomycin in *Streptomyces antibioticus*, the producer organism. J. Gen. Microbiol. **133:**1931–1939.
14. **Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. J. Waring.** 1981. The molecular basis of antibiotic action. Wiley, London, United Kingdom.
15. **Gourmelen, A.** Unpublished data.
16. **Hernandez, C., C. Olano, C. Mendez, and J. A. Salas.** 1993. Characterization of a *Streptomyces antibioticus* gene cluster encoding a glycosyltransferase involved in oleandomycin inactivation. Gene **134:**139–140.
17. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*. A laboratory manual. The John Innes Foundation, Norwich, United Kingdom.
18. **Hopwood, D. A., T. Kieser, H. M. Wright, and M. J. Bibb.** 1983. Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. J. Gen. Microbiol. **129:**2257–2269.
19. **Janssen, G. R., and M. J. Bibb.** 1993. Derivatives of pUC18 that have BglII sites flanking a modified multiple cloning site and that retain the ability to identify recombinant clones by visual screening of *Escherichia coli* colonies. Gene **124:**133–134.
20. **Jenkins, G., and E. Cundliffe.** 1991. Cloning and characterization of two

genes from *Streptomyces lividans* that confer inducible resistance to lincomycin and macrolide antibiotics. Gene **108:**55–62.
21. **Kuo, M. S., D. G. Chirby, A. D. Argoudelis, J. I. Cialdella, J. H. Coats, and V. P. Marshall.** 1989. Microbial glycosylation of erythromycin A. Antimicrob. Agents Chemother. **33:**2089–2091.
22. **Lydiate, D. J., F. Malpartida, and D. A. Hopwood.** 1985. The *Streptomyces* plasmid SCP2*: its functional analysis and development into useful cloning vectors. Gene **35:**223–235.
23. **Mendez, C., and J. A. Salas.** 1998. ABC transporters in antibiotic-producing actinomycetes. FEMS Microbiol. Lett. **158:**1–8.
24. **Noguchi, N., A. Emura, H. Matsuyama, K. O'Hara, M. Sasatsu, and M. Kono.** 1995. Nucleotide sequence and characterization of erythromycin resistance determinant that encodes macrolide 2′-phosphotransferase I in *Escherichia coli*. Antimicrob. Agents Chemother. **39:**2359–2363.
25. **Noguchi, N., J. Katayama, and K. O'Hara.** 1996. Cloning and nucleotide sequence of the *mphB* gene for macrolide 2′-phosphotransferase II in *Escherichia coli*. FEMS Microbiol Lett. **144:**197–202.
26. **Ounissi, H., and P. Courvalin.** 1985. Nucleotide sequence of the gene *ereA* encoding the erythromycin esterase in *Escherichia coli*. Gene **35:**271–278.
27. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
28. **Pernodet, J. L.** Unpublished data.
29. **Pernodet, J. L., M. T. Alegre, M. H. Blondelet-Rouault, and M. Guerineau.** 1993. Resistance to spiramycin in *Streptomyces ambofaciens*, the producer organism, involves at least two different mechanisms. J. Gen. Microbiol. **139:** 1003–1011.
30. **Pernodet, J. L., S. Fish, M. H. Blondelet-Rouault, and E. Cundliffe.** 1996. The macrolide-lincosamide-streptogramin B resistance phenotypes characterized by using a specifically deleted, antibiotic-sensitive strain of *Streptomyces lividans*. Antimicrob. Agents Chemother. **40:**581–585.
31. **Pinnert-Sindico, S.** 1954. Une nouvelle espèce de *Streptomyces* productrice d'antibiotiques: *Streptomyces ambofaciens* n. sp. caractères culturaux. Ann. Inst. Pasteur (Paris) **87:**702–707.
32. **Pridham, T. G., P. Anderson, C. Foley, L. A. Lindenfelser, C. W. Hesseltine, and R. C. Benetdict.** 1957. A selection of media for maintenance and taxonomic study of *Streptomyces*. Antibiot. Annu. 1956–1957:947–953.
33. **Quiros, L. M., I. Aguirrezabalaga, C. Olano, C. Mendez, and J. A. Salas.** 1998. Two glycosyltransferases and a glycosidase are involved in oleandomycin modification during its biosynthesis by *Streptomyces antibioticus*. Mol. Microbiol. **28:**1177–1185.
34. **Richardson, M. A., S. Kuhstoss, P. Solenberg, N. A. Schaus, and R. N. Rao.** 1987. A new shuttle cosmid vector, pKC505, for streptomycetes: its use in the cloning of three different spiramycin-resistance genes from a *Streptomyces ambofaciens* library. Gene **61:**231–241.
35. **Rosteck, P. R., Jr., P. A. Reynolds, and C. L. Hershberger.** 1991. Homology between proteins controlling *Streptomyces fradiae* tylosin resistance and ATP-binding transport. Gene **102:**27–32.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
38. **Sasaki, J., K. Mizoue, S. Morimoto, and S. Omura.** 1996. Microbial glycosylation of macrolide antibiotics by *Streptomyces hygroscopicus* ATCC 31080 and distribution of a macrolide glycosyl transferase in several *Streptomyces* strains. J. Antibiot. (Tokyo) **49:**1110–1118.
39. **Schoner, B., M. Geistlich, P. Rosteck, Jr., R. N. Rao, E. Seno, P. Reynolds, K. Cox, S. Burgett, and C. Hershberger.** 1992. Sequence similarity between macrolide-resistance determinants and ATP-binding transport proteins. Gene **115:**93–96.
40. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilisation system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
41. **Sutcliffe, J., A. Tait-Kamradt, and L. Wondrack.** 1996. *Streptococcus pneumoniae* and *Streptococcus pyogenes* resistant to macrolides but sensitive to clindamycin: a common resistance pattern mediated by an efflux system. Antimicrob. Agents Chemother. **40:**1817–24.
42. **Thomas, D. I., J. H. Cove, S. Baumberg, C. A. Jones, and B. A. Rudd.** 1991. Plasmid effects on secondary metabolite production by a streptomycete synthesizing an anthelminthic macrolide. J. Gen. Microbiol. **137:**2331–2337.
43. **Vilches, C., C. Hernandez, C. Mendez, and J. A. Salas.** 1992. Role of glycosylation and deglycosylation in biosynthesis of and resistance to oleandomycin in the producer organism, *Streptomyces antibioticus*. J. Bacteriol. **174:** 161–165.
44. **Wahl, G. M., K. A. Lewis, J. C. Ruiz, B. Rothenberg, J. Zhao, and G. A. Evans.** 1987. Cosmid vectors for rapid genomic walking, restriction mapping, and gene transfer. Proc. Natl. Acad. Sci. USA **84:**2160–2164.
45. **Weisblum, B.** 1995. Erythromycin resistance by ribosome modification. Antimicrob. Agents Chemother. **39:**577–585.
46. **Wright, F., and M. J. Bibb.** 1992. Codon usage in the G+C-rich *Streptomyces* genome. Gene **113:**55–65.
47. **Zalacain, M., and E. Cundliffe.** 1991. Cloning of *tlrD*, a fourth resistance gene, from the tylosin producer, *Streptomyces fradiae*. Gene **97:**137–142.