Resistance to spiramycin in *Streptomyces ambofaciens*, the producer organism, involves at least two different mechanisms

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During its stationary phase, *Streptomyces ambofaciens* produces the macrolide antibiotic spiramycin, and has to protect itself against this antibiotic. Young mycelia, not yet producing spiramycin, are sensitive to it, but they become fully resistant when production begins. In a sensitive mycelium, resistance could be induced by exposure to sub-inhibitory concentrations of spiramycin, and these induced mycelia, like producing mycelia were resistant not only to spiramycin but also to several other macrolide antibiotics. Ribosomes extracted from these resistant mycelia were shown *in vitro* to be more resistant to spiramycin than ribosomes extracted from sensitive mycelium, indicating that *S. ambofaciens* possesses a spiramycin-inducible ribosomal resistance to spiramycin and to macrolide antibiotics. Studies with spiramycin non-producing mutants showed that, in these mutants, resistance to spiramycin also varies during cultivation, in that an old culture was much more resistant than a young one. But with these non-producing mutants, the spectrum of resistance was narrower, and *in vitro* data showed that resistance was not due to ribosomal modification. These results suggest that *S. ambofaciens* presents at least two distinct mechanisms for spiramycin resistance; a spiramycin-inducible ribosomal resistance, and a second resistance mechanism which might be temporally regulated and which could involve decreased permeability to, or export of, the antibiotic. The two mechanisms are probably at work simultaneously in the producing mycelium, the spiramycin-inducible resistance being induced by endogenous spiramycin. In non-producing mutants, in the absence of self-induction by spiramycin, only the second mechanism is observed.

Introduction

Macrolide antibiotics are compounds consisting of a macrocyclic lactone ring of 12, 14 or 16 residues to which are attached one or more amino or deoxy sugars (Omura & Tanaka, 1984). These antibiotics inhibit protein synthesis in prokaryotes by a mechanism involving binding of the antibiotic to the 50S ribosomal subunit (Gale *et al.*, 1981). Macrolides, especially erythromycin, have been widely used in human medicine. Several mechanisms of resistance to erythromycin or to macrolides have been described. Mutations affecting the genes encoding ribosomal components can confer resistance. For instance, in *Escherichia coli*, mutants conferring erythromycin resistance have been located in the genes coding for ribosomal proteins L4 and L22 (Wittmann *et al.*, 1973) and macrolide resistance mutations have been obtained in the 23S rRNA genes (Morgan *et al.*, 1988). Resistance to erythromycin by enzymic hydrolysis of the drug has also been reported in *E. coli* (Barthélomy *et al.*, 1984). Active efflux of the drug, mediated by a protein of the ATP-binding transport family, is responsible for the resistance observed in some strains of staphylococci (Ross *et al.*, 1990). However, in pathogenic bacteria, the most widespread resistance is known as MLS resistance because it confers resistance to macrolide, lincosamide and streptogramin type B antibiotics (reviewed by Weisblum, 1983; Leclercq & Courvalin, 1991). Here resistance is due to a modification of the 50S ribosomal subunit by methylation of an adenine residue in the 23S rRNA. This MLS-resistant phenotype can be expressed constitutively or can be induced by sub-inhibitory concentrations of some MLS antibiotics, generally including erythromycin.
Several *Streptomyces* species are macrolide producers, but among *Streptomyces* species, whether producers of MLS antibiotics or not, some present the archetypal MLS resistance phenotype and others have only partial MLS resistance, suggesting that a family of macrolide and lincosamide resistance mechanisms is distributed among these species (Fujisawa & Weisblum, 1981). Genes encoding 23S rRNA methyl transferases conferring resistance have been isolated from several streptomycete strains and characterized (reviewed by Cundliffe, 1989a, b). Other resistance mechanisms have also been reported in *Streptomyces*. For instance, *Streptomyces coelicolor* Müllcr, which is not known to be a macrolide producer, can nevertheless *O*-phosphorylate macrolides (Marshall et al., 1989). *Streptomyces antibioticus*, the oleandomycin producer, has been shown to have a decreased permeability to oleandomycin, suggesting a role for cell permeability in self-resistance (Fierro et al., 1987), and a macrolide glycosyl-transferase activity has been characterized in the same strain (Vilches et al., 1992). A macrolide glycosyl-transferase is also present in *Streptomyces lividans*, and the gene encoding this enzyme has been isolated and sequenced (Jenkins & Cundliffe, 1991). *Streptomyces venezuelensis* can also inactivate erythromycin by glycosylation (Kuo et al., 1989). Macrolide producers very often have several different genes involved in self resistance. For instance, *Streptomyces fradiae*, the tylosin producer, has had four different resistance genes cloned from it (Birmingham et al., 1986; Biltz & Seno, 1988; Beckmann et al., 1989; Zalacain & Cundliffe, 1991).

*Streptomyces ambofaciens* produces spiramycin (Pinnert-Sindico, 1954), a 16-membered macrolide containing three amino sugars. In this organism, a mutation in one of the 23S ribosomal RNA genes confers a higher level of resistance to macrolide antibiotics (Pernodet et al., 1988). We report here the results of a physiological study of spiramycin resistance in *S. ambofaciens*.

**Methods**

**Bacterial strains, media and culture conditions.** The wild-type *S. ambofaciens* strain (ATCC23877), and three *S. ambofaciens* spiramycin non-producing mutants (RP17, RP167, NP3) were used. Mutants RP17 and RP167 were obtained after NTG mutagenesis and were a kind gift of Dr P. Lacroix, Rhone-Poulenc Rorer, Vitry, France. Mutant NP3 was obtained after mutagenesis by nitrous acid (Smokvina et al., 1988) and was a kind gift of Dr F.-X. Francou, Université Paris XI, Orsay, France. *S. ambofaciens* strains were maintained on plates of Hickey-Tressner (HT) medium (Pridham et al., 1957) at 30 °C. This medium contained (per litre) 1 g yeast extract (Difco), 1 g beef extract (Difco), 10 g white dextrin (Prolabo), 2 g NZ amine type A (Sheffield), 20 mg CoCl₂·6H₂O, 20 g Bacto agar (Difco). The pH was adjusted to 7.3 prior to autoclaving. Spores harvested from HT plates were separated from mycelial fragment by filtration on cotton, suspended in 20% (w/v) glycerol and stored at −20 °C until used. YEML liquid medium (Bibb et al., 1977) contained (per litre) 3 g yeast extract (Difco), 5 g Bacto-peptone (Difco), 3 g malt extract (Difco), 10 g glucose, 340 g sucrose. After autoclaving, MgCl₂ was added to a final concentration of 5 mM, and glycine to a final concentration of 0.25%. DALP liquid medium (Dr A. Sabatier, personal communication) contained (per litre) 10 g Bacto-peptone (Difco), 5 g yeast extract (Difco), 10 g white dextrin (Prolabo). Prior to autoclaving, the pH was adjusted to 7.0 and Bacto agar (Difco) was added to the medium to a final concentration of 0.2 g l⁻¹. These media were inoculated with spores and incubated at 30 °C on an orbital shaker. For spiramycin production, two different media were used. The first, SL11 (Dr A. Sabatier, personal communication), contained (per litre) 25 g white dextrin (Prolabo), 12.5 g yeast extract (Fould-Springer), 1 g MgSO₄, 1 g KH₂PO₄, 20.9 g MOPS (Sigma). The pH was adjusted to 7.1 and the medium was put into Erlenmeyer flasks. Prior to autoclaving, CaCO₃ was added to the medium to a final concentration of 5 g l⁻¹, and maize oil to a final concentration of 0.5%. SL11 medium was inoculated with spores. The second production medium, MP5, contained (per litre) 7 g yeast extract (Difco), 5 g NaCl, 1 g NaN₃, 36 ml glycerol, 20.9 g MOPS (Sigma). The pH was adjusted to 7.5 prior to autoclaving. As germination was not very good in MP5, this medium was inoculated with spores incubated for 2 h at 37 °C in pre-germination medium as described (Hopwood et al., 1985). For spiramycin production, the cultures (60 ml in a 500 ml flask) were incubated at 26 °C in an orbital shaker at 260 r.p.m. SL11 was the better medium for production, but the insoluble CaCO₃ present in this medium was an obstacle when ribosomes had to be prepared from cultures grown in production medium. In such cases, MP5 was used.

**Antibiotic production and resistance.** Antibiotic production was tested by a biological assay. *Bacillus subtilis* ATCC6633 was used as the indicator organism. A paper disk was impregnated with 80 μl *Streptomyces* culture supernatant, and was laid on the surface of an agar plate spread with a spore suspension of the indicator organism. The medium was antibiotic medium no. 8 (Difco) buffered with 100 mM-phosphate buffer, pH 8.0. After overnight incubation at 37 °C, the diameter of the inhibition zone was measured. As standards, disks impregnated with various dilutions of a solution of commercial spiramycin were also laid on the surface of the plate. The diameters of the inhibition zones for these disks were used to draw the standard curve. The threshold of detection for this method was 0.5 μg spiramycin ml⁻¹ in the production medium.

To assay mycelial spiramycin resistance, *Streptomyces* strains were grown in liquid medium, in Erlenmeyer flasks. Samples were periodically withdrawn and gently sonicated (for 20% of a period of 2 min, at 40 W with a Branson 250 sonicator) to disperse the mycelial pellets, diluted and plated on medium without spiramycin to give the total colony-forming units (c.f.u.), and on medium with spiramycin to give resistant c.f.u. Colonies were counted after 3 d incubation.

**Assay of in vivo protein synthesis.** *S. ambofaciens* was grown in the production medium. When necessary, resistance was induced by exposure to exogenous spiramycin for 2 h. The mycelium was centrifuged and resuspended in one third of the culture volume of minimal medium (Fierro et al., 1987) containing (per litre) 10 g glucose, 2 g asparagine, 2 g (NH₄)₂SO₄, 4.3 g KH₂PO₄, 0.82 g K₂HPO₄, 0.01 g FeSO₄·7H₂O, 0.5 g MgSO₄·7H₂O. The mycelium was then homogenized with a Thoma homogenizer. Samples of 300 μl were incubated at 25 °C for 15 min in the absence or presence of various concentrations of antibiotics, followed by pulse-labeling for 30 min with 2.5 μCi of [³⁵S]methionine (1123 Ci mm⁻¹, 41.5 MBq mm⁻¹).

We then added 50 μl cold methionine (250 μM) and incubated at 25 °C for 10 min more. Incorporation was stopped by the addition of 0.8 ml 20% (w/v) TCA. After 10 min on ice, the samples were heated at 90 °C for 15 min, cooled on ice and filtered through Whatman GF/C
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Preparation of ribosomes and post-ribosomal supernatant. Ribosomes and post-ribosomal supernatants (S100*) were prepared from \textit{Streptomyces ambofaciens} as described by Skeggs et al. (1985), except that the mycelium was ground with alumina in a mortar instead of being passed through a French press.

Poly-U-directed polyphenylalanine synthesis. This was carried out essentially as described by Skeggs et al. (1985). For some experiments, a post-ribosomal supernatant from \textit{Escherichia coli}, called S105, was used instead of S100\textsuperscript{*} from \textit{Streptomyces ambofaciens}. This post-ribosomal supernatant was a kind gift from J. Dondon, Institut de Biologie Physico-Chimique, Paris France.

Results

Changes in the resistance level to spiramycin during cultivation

Under our culture conditions, in the production media (SL11 and MP5), spiramycin production begins after 36 h cultivation (see below). The sensitivity of young mycelium, not yet producing after 24 h cultivation, and of mycelium producing spiramycin after 72 h of growth, were assayed. The results (Fig. 1) show clearly that old antibiotic-producing mycelium is much more resistant to spiramycin than mycelium which is not producing yet. For instance, when plated on medium containing 400 µg spiramycin ml\(^{-1}\), 100% of the c.f.u. from the producing culture grew, compared to 0.1% of the c.f.u. from the young culture. This concentration of 400 µg spiramycin ml\(^{-1}\) was used throughout this study to discriminate between sensitive and resistant mycelium. Resistance was expressed as the ratio of the c.f.u. on medium with 400 µg spiramycin ml\(^{-1}\) to the c.f.u. on medium without spiramycin. Thus, the resistance of producing mycelium was 100%. To know more precisely when the change in the resistance level of the culture occurred, mycelium grown in production medium was assayed for resistance at regular intervals throughout cultivation. The concentration of spiramycin in the supernatant was also determined, using a biological assay. The results (Fig. 2) were obtained in SL11 production medium, but identical results were obtained with MP5. The medium was inoculated with spores which are relatively resistant to spiramycin, but as they germinated they became spiramycin-sensitive, and resistance was minimal after 24 h growth, after which time no spiramycin could be detected in the medium. The resistance then increased very rapidly, and the mycelium was fully resistant after 36 h growth. Spiramycin could be detected in the medium at this time, although it could have been present in small amounts inside the mycelium before it could be detected in the supernatant. Thus the increase in the resistance level and the start of production may well be concomitant. MLS resistance can be induced by some antibiotics of the MLS group in several other bacteria. It is possible that in \textit{S. ambofaciens} sub-

![Fig. 1. Sensitivity of \textit{S. ambofaciens} to spiramycin. The mycelium was gently sonicated to disrupt the pellets and dilutions were plated on medium either without spiramycin or with various concentrations of the drug. Survival is expressed as the ratio c.f.u. on medium with spiramycin to c.f.u. on the same medium without spiramycin. ○, 24 h culture of \textit{S. ambofaciens}, before production phase; ●, 72 h culture of \textit{S. ambofaciens} during production phase.]

![Fig. 2. Spiramycin resistance and production during growth of \textit{S. ambofaciens} ATCC23877. \textit{S. ambofaciens} was grown in production medium. At regular time intervals, samples were taken of the culture. The mycelium was gently sonicated to disrupt the pellets, and spiramycin resistance (○) and production (●) were assayed as described in Methods. Resistance is expressed as the ratio c.f.u. on medium with spiramycin (400 µg ml\(^{-1}\)) to c.f.u. on the same medium without spiramycin.](image)
inhibitory concentrations of spiramycin present inside the mycelium induce a resistance mechanism of the MLS type.

Induction of spiramycin resistance by sub-inhibitory concentrations of spiramycin

In YEME medium, spiramycin production is not only reduced compared to that obtained in SL11 or MP5, but also delayed, because production starts after 3 or 4 d culture. In YEME medium, the mycelium remained very sensitive to spiramycin during the period between 18 and 72 h culture (data not shown). Induction experiments using exogenous spiramycin were therefore performed in YEME medium rather than production medium, to avoid self-induction by endogenous spiramycin. A spiramycin-sensitive mycelium, grown in YEME liquid medium for 18 h, was exposed to sub-inhibitory concentrations of spiramycin. Sub-inhibitory concentrations were defined as those antibiotic concentrations which did not affect the growth rate of the strain. The results (Fig. 3a) show that after 2 h exposure to 10 \( \mu \)g spiramycin ml\(^{-1} \), the mycelium became spiramycin-resistant, whereas the control (same mycelium but not exposed to spiramycin) remained fully sensitive (data not shown). Moreover, Fig. 3(b), shows both that the response was dependent on the spiramycin concentration, and that the spiramycin concentrations used were sub-inhibitory, as c.f.u. were not lowered on medium without spiramycin.

In the same way it was also possible to induce resistance in a mycelium grown in DALP medium or in the production media SL11 and MP5. The kinetics of induction were the same, but spiramycin concentrations as low as 1 \( \mu \)g ml\(^{-1} \) were sufficient to obtain full induction in production media (data not shown).

To investigate whether the resistance observed after induction by spiramycin was restricted to spiramycin or could be extended to other macrolide antibiotics, simple tests of alteration of the inhibition zone were performed as described by Fujisawa & Weisblum (1981). These tests showed that spiramycin could induce resistance to other antibiotics such as chalcomycin or erythromycin to which the strain is otherwise very sensitive (data not shown). These results were confirmed by the sensitivity of protein synthesis to macrolide antibiotics (see below).

Thus sub-inhibitory concentrations of spiramycin can induce resistance to spiramycin and to other macrolide antibiotics. This resistance, which is induced by exogenous spiramycin, should also be induced by endogenous spiramycin when production of the antibiotic begins. We wondered whether this resistance mechanism was the only one responsible for the resistant phenotype observed after 3 d cultivation in production medium, and if spiramycin was the only inducer. If so, this would imply that non-producing mutants should always remain sensitive to spiramycin. To test this hypothesis, spiramycin-non-producing mutants were studied.

Study of non-producing mutants

The spiramycin-non-producing mutants used in this study were blocked mutants capable of cosynthesis (P. Lacroix and F.-X. Francou, personal communication), i.e. when two different mutants were grown nearby on an
producing mutants than with the wild-type strain means that the mutants are probably affected in one or mutant, blocked at an earlier step of the pathway. This mutant RP17 grown in SL11, but identical results were followed during their cultivation in production media that they are blocked in different steps of the pathway.

a few genes of the spiramycin biosynthetic pathway, and mycelium fully resistant to induce the resistance of a sensitive mycelium, after these results, it is clear that even with non-producing strain, beginning after 24 h growth and yielding a mycelium fully resistant to 400 μg spiramycin ml⁻¹. The increase in the resistance level is less rapid with non-producing mutants than with the wild-type strain (compare Figs 2 and 4).

With all non-producing mutants used, it was possible to induce the resistance of a sensitive mycelium, after 18 h cultivation, by exposure to sub-inhibitory spiramycin concentrations. The parameters of induction were the same as for the wild-type strain, and the mycelium also became more resistant to macrolide antibiotics. This induction by exogenous spiramycin is shown in Fig. 4. The spiramycin-inducible resistance mechanism present in the wild-type strain is also functional in the non-producing mutants.

To explain the resistance observed in the non-producing mutants after 72 h cultivation, without induction by exogenous spiramycin, two hypotheses can be advanced. In the first hypothesis, the resistance observed after 72 h cultivation occurs by the same mechanism as that responsible for the spiramycin-inducible resistance. This mechanism would be active either because the so called ‘non-producing mutants’ are nevertheless producing undetected spiramycin in amounts sufficient to induce resistance, or because it could respond to an inducer other than spiramycin, perhaps a spiramycin precursor in the biosynthetic pathway, although if so, such a precursor must be the product of an early step in the pathway, prior to the steps in which the mutants are blocked, because different mutants behave in the same way. If the same mechanism is responsible for both the resistance observed after induction by exogenous spiramycin and that observed after 72 h of cultivation, then the spectrum of resistance should be the same in each. In the second hypothesis, the resistance observed after 72 h cultivation occurs by a second mechanism, different to that involved in the spiramycin-inducible resistance. This second mechanism could differ in its spectrum from that first identified. Thus the next step was to study the spectrum of resistance in the wild-type strain and in the non-producing mutants after either induction by exogenous spiramycin or 72 h cultivation.

_Susceptibility of protein synthesis in vivo_

The aim of these experiments was to determine the spectrum of resistance of the mycelia of the wild-type strain and of the non-producing mutants, after 24 h cultivation, with or without induction by exogenous spiramycin, and after 72 h cultivation without addition of exogenous spiramycin. To determine the spectrum of resistance accurately, the susceptibility of _in vitro_ protein synthesis was studied by a technique in which the mycelium was incubated in the absence or presence of various macrolide antibiotics for 10 min, followed by pulse-labelling with ³⁵S)methionine for 30 min. The mycelium was exposed to the macrolide antibiotics for too short a time to allow induction of resistance by these antibiotics. Fig. 5 shows results from one typical experiment. Similar results were reproducibly obtained with mycelia from different cultures grown in the same conditions. For the wild type strain, mycelium induced by exogenous spiramycin, and producing mycelium, are both more resistant to several macrolide antibiotics than the uninduced non-producing mycelium. In the case of the non-producing mutants, induction by exogenous spiramycin conferred the same spectrum of resistance to the non-producing mutant was grown and resistance was followed as described for the wild-type strain. No spiramycin (■) could be detected in the culture supernatant. The resistance (●) is expressed as the ratio of c.f.u. on medium with spiramycin (400 μg ml⁻¹) to c.f.u. on the same medium without spiramycin. The dotted line represents induction of resistance when exogenous spiramycin was added to a final concentration of 1 μg ml⁻¹ to a sample of the 18 h culture.

![Fig. 4. Spiramycin resistance during growth of a non-producing mutant of _S. ambofaciens_. The non-producing mutant was grown and resistance was followed as described for the wild-type strain. No spiramycin (■) could be detected in the culture supernatant. The resistance (●) is expressed as the ratio of c.f.u. on medium with spiramycin (400 μg ml⁻¹) to c.f.u. on the same medium without spiramycin. The dotted line represents induction of resistance when exogenous spiramycin was added to a final concentration of 1 μg ml⁻¹ to a sample of the 18 h culture.](image)
macrolide antibiotics as in the wild-type strain. But 3-d-old mycelium of the non-producing mutants, which is spiramycin-resistant, was less resistant to erythromycin or chalcomycin than young mycelium induced by exogenous spiramycin. Since spectrum of resistance of the 3-d-old mycelium was narrower, the mechanism responsible for spiramycin resistance in old cultures of the non-producing mutant may well be different from that in the spiramycin-inducible resistance to macrolide antibiotics.

**Susceptibility of ribosomes to spiramycin**

Study of the biochemical basis of the resistance could confirm that two different mechanisms produce the two resistance types observed. The spiramycin-inducible mechanism seems to confer resistance to macrolide antibiotics, and this type of wide-spectrum resistance could be due to a modification of the target, i.e. the ribosome. Accordingly, we studied the effect of spiramycin on poly-U-directed synthesis of polyphenylalanine. Salt-washed ribosomes and post-ribosomal supernatant (S100*) were prepared from 24 h cultures of the wild-type strain grown in DALP medium, induced or not induced by exogenous spiramycin. Ribosomes from each were supplemented with both types of S100* and assayed in cell-free protein synthesizing systems directed by poly-U, in the absence or presence of various spiramycin concentrations. The results (Fig. 6) show that ribosomes from the induced culture are more resistant to spiramycin than those from the non-induced culture, regardless of the origin of the S100* fraction. Thus ribosome modification seems to be the basis of the resistance observed after spiramycin induction. This was confirmed by poly-U-directed polyphenylalanine synthesis experiments using ribosomes from induced and non-induced cultures and S105 from *E. coli* (data not shown).

The next step was to compare the spiramycin susceptibility of ribosomes from 72 h cultures with that of ribosomes from 24 h induced and non-induced cultures, using the wild-type strain and non-producing mutants grown in MP5 medium. In all these experiments, S105 fractions from *E. coli* were used. The data shown
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Fig. 7. Effect of spiramycin on poly-U-directed polyphenylalanine synthesis in vitro. Assays contained S105 from E. coli and S. ambofaciens ribosomes. (a) Ribosomes from wild-type S. ambofaciens strain were extracted from: ○, one-day-old culture, non-induced (sensitive mycelium) (100% activity: 40176 c.p.m.); ●, one-day-old culture, induced (resistant mycelium) (100% activity: 20480 c.p.m.); ▲, three-day-old culture (resistant mycelium) (100% activity: 41165 c.p.m.). (b) Ribosomes from the spiramycin-non-producing mutant were extracted from: ○, one-day-old culture, non-induced (sensitive mycelium) (100% activity: 17893 c.p.m.); ●, one-day-old culture, induced (resistant mycelium) (100% activity: 37423 c.p.m.); ▲, three-day-old culture (resistant mycelium) (100% activity: 27840 c.p.m.).

Discussion

Antibiotic-producing organisms have developed several types of defensive strategies against their own product: modification or replacement of the target site for the antibiotic; inactivation or sequestration of the intracellular antibiotic; and exclusion of the drug from the cytoplasm by an efflux mechanism together with the erection of membrane permeability barriers. Several of these mechanisms can co-exist in the same strain (reviewed by Cundliffe, 1989a).

Analysis of the development of spiramycin resistance in antibiotic-producing S. ambofaciens led to the conclusion that at least two distinct resistance mechanisms were present. A mechanism involving ribosomal modification conferred resistance to spiramycin but also to other macrolide antibiotics. It is inducible by sub-inhibitory concentrations of spiramycin, can be induced in the sensitive mycelium by exogenous spiramycin added to the medium, and is probably induced by endogenous spiramycin when production begins. In the wild-type strain, this resistance seems to be induced before the detection of spiramycin in the medium, but the intracellular concentration of the antibiotic is probably sufficient for induction before the extracellular concentration is detectable. This intracellular concentration necessary for induction is probably lower than the extracellular concentration required for induction by exogenous spiramycin. Some spiramycin precursors might also induce this ribosomal resistance, but as no self-induction of the ribosomal resistance was observed for any non-producing mutant in this study, these mutants would have to be blocked before such inducing precursors were produced.

Another resistance mechanism can be detected using non-producing mutants. This resistance was not ribosomal, and conferred resistance to some but not all incomplete modification of the ribosomes after only 2 h induction, and the fact that the ribosome population tested is not homogeneous. In the case of the spiramycin-non-producing mutant (Fig. 7b), only ribosomes from the 24 h induced culture (resistant mycelium) display resistance to spiramycin. Ribosomes from the 72 h culture (resistant mycelium) are as sensitive to spiramycin as ribosomes from the 24 h non-induced culture (sensitive mycelium). Ribosomal modification is therefore responsible for the resistant phenotype observed after induction either by exogenous spiramycin added to 24 h culture or by endogenous spiramycin produced by the wild-type strain after 72 h cultivation. But ribosomal modification cannot account for the resistance observed in 72 h cultures of the non-producing mutants, demonstrating that another mechanism is involved.

(Fig. 7) were obtained with strain RP17, but the different non-producing mutants gave equivalent results. From Fig. 7(a), it is clear that the ribosomes from both the 72 h producing culture (resistant mycelium) and the 24 h induced culture (resistant mycelium) are more resistant to spiramycin than the one from the 24 h uninduced culture (sensitive mycelium). The difference observed between the ribosomes from the 24 h induced culture and those from the 72 h producing culture might be due to
macrolide antibiotics, possibly only to the 16-membered
macrolides. This resistance was not constitutively ex-
pressed, since it appeared specifically in 72 h cultures. It
might be regulated similarly to the production genes,
because all non-producing mutants were able to co-
synthesize spiramycin; in these mutants, the production
genes were probably turned on at the same time as in the
wild-type strain (between 36 and 48 h in our culture
conditions). The second resistance mechanism began to
be expressed at that time. Moreover, under culture
conditions retarding the onset of spiramycin production
in the wild-type strain, the appearance of resistance in
the non-producing mutants was similarly delayed (data
not shown). In some non-producing mutants, unable to
cosynthesize spiramycin, and deficient in aerial mycelium
formation, this resistance is also affected (data not
shown). These observations could indicate that the non-
ribosomal resistance is turned on at the onset of produc-
tion. Concerning the biochemical basis of this non-ribosomal resistance, Fierro et al. (1988) failed to
detect any spiramycin modification by methylation,
acetylation or adenosylation, using extract from S.
ambofaciens. These authors found that S. ambofaciens
had a decrease in permeability specific to spiramycin,
which could be the basis for resistance in the non-
producing mutants. The resistance could in fact cor-
respond to a mechanism of spiramycin secretion, and it
could be considered as the last step of the biosynthetic
pathway rather than as a resistance mechanism sensu
stricto. In the wild-type strain, during the production
phase, we have shown that the ribosomal resistance was
active, but this non-ribosomal resistance detected in the
non-producing mutants is probably also active in the
wild-type strain.

The existence of at least two different resistance
mechanisms is in agreement with the fact that several
resistance determinants, (srmA, srmB, srmC and srmD),
have been cloned from S. ambofaciens (Richardson et al.,
1987; Pernodet and others, unpublished data). More-
over, among these resistance determinants, srmA and
possibly srmD confer resistance through ribosomal
modification (Pernodet and others, unpublished data).
One or both of these determinants might be responsible
for the spiramycin-inducible ribosomal resistance to
macrolides observed in S. ambofaciens. Schoner et al.
(1992) have shown that srmB encodes a protein possess-
ing significant sequence similarity to ATP-dependent
transport proteins. This suggests that the mechanism by
which srmB confers resistance to spiramycin involves
export of the antibiotic. Thus srmB could be involved in
the second resistance mechanism that we observed.

Mechanisms involved in resistance to the produced
antibiotic have been studied for several other macrolide
producers. Ribosomal modification seems to be a
widespread resistance mechanism, as rRNA methyl
transferases conferring resistance have been characterized in
Saccharopolyspora erythraea (Skinner et al., 1983;
Uchiyama & Weisblum, 1985), Streptomyces thermo-
tolerans (Epp et al., 1987; Zalacain & Cundliffe, 1990)
and Streptomyces mycarofaciens (Hara & Hutchinson,
1990). A strain such as S. fradiae, the tylosin producer,
can possess two different genes encoding rRNA methyl
transferases: trrA, also called ermSF (Kamimiya &
Weisblum, 1988; Zalacain & Cundliffe, 1989) and trrD
(Zalacain & Cundliffe, 1991). Genes which could be
involved in an efflux mechanism are also present in
several macrolide producers: the genes trrC from S.
fradiae and carA from S. thermotolerans encode proteins
that are highly similar to that encoded by srmB in S.
ambofaciens. All these proteins possess significant se-
quence similarity to ATP-dependent transport proteins
(Roesteck et al., 1991; Schoner et al., 1992). From S.
antibioticus, the oleandomycin producer, no resistance
determinants have been cloned, but Fierro et al. (1987)
reported that this strain showed a decreased permeability
to oleandomycin, suggesting a role for cell permeability
in self resistance. In addition, Vilches et al. (1992)
reported a macrolide glycosyl transferase activity in this
strain.

To conclude, like several other macrolide produc-
ers, S. ambofaciens possesses at least two different resistance
mechanisms. The characterization of the cloned resis-
tance determinants and the study of their regulation
will be the next step in our study. The data obtained
should provide helpful information for the understand-
ing of the self-protection towards the produced antibiotic
and of the control involved in the commitment to
antibiotic production.

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