INVOLVEMENT OF CELL IMPERMEABILITY IN RESISTANCE TO MACROLIDES IN SOME PRODUCER STREPTOMYCETES

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The self-resistance mechanism in macrolide antibiotic-producing organisms is well known only in the case of *Streptomyces erythraeus* (erythromycin producer). This organism possesses a RNA methylase which generates N^6-dimethyladenine in 23S rRNA[1,2]; the gene responsible has been cloned and expressed in *Streptomyces lividans*[3]. This methylase confers cross-resistance to macrolides, lincosamides and streptogramins B (MLS-resistance). Although constitutive MLS-resistance has been found only in *S. erythraeus*, inducible MLS-resistance has been described in several producers of macrolides, lincosamides and streptogramins B, being associated with the appearance of mono- and/or dimethylated adenine in 23S rRNA[4]. However, in the latter cases, no causal connection between methylation and resistance has been demonstrated. Resistance to two other macrolides in their respective producers has been studied in some detail. *Streptomyces antibioticus*, oleandomycin producer (an erythromycin closely related antibiotic) has been reported to have oleandomycin-sensitive ribosomes and it has been suggested that this organism synthesizes the antibiotic as an inactive intracellular compound, the organism being perhaps impermeable to the drug which would prevent re-entry of the antibiotic into cells once excreted[5]. A tylosin-resistance gene from *Streptomyces fradiae* has been cloned which confers resistance to tylosin and also to carbomycin, niddamycin and vernamycin B[6]. However, the biochemical basis for this resistance is not known. On the other hand, phosphorylation of macrolide antibiotics in a non producer strain, *Streptomyces coelicolor*, has also been reported[7]. Here we report a study on several macrolide producing strains, suggesting a role of cell impermeability in the survival strategy.

Determinations of MICs revealed that various antibiotic producers were resistant to the produced antibiotic (MIC>200 μg/ml) but were sensitive to other macrolides (Table 1). *Streptomyces albus* G, a strain which produces no macrolides and was susceptible to all the macrolides tested was used as control.

To examine the possible existence of antibiotic-inactivating enzymes, cell-free extracts of the producing strains were obtained at different times of the cell cycle and tested for their ability to inactivate the antibiotic produced. The extracts, in 50 mM Tris-HCl pH 7.5 at 20°C, were incubated at 35°C for 3 hours with 10 μg/ml of spiramycin, carbomycin or tylosin in the presence of *S*-adenosylmethionine, acetyl coenzyme A and ATP (1 mM). The residual antibiotic activity was tested by bioassay against *Micrococcus luteus*. Under these incubation conditions, the producers all failed to inactivate

Table 1. MICs of macrolide producers.

Organism	Antibiotic produced	MIC (μg/ml)					
		SPI	CAR	TYL	LIN	ERY	OLE
Streptomyces ambofaciens ATCC 15154	Spiramycin	>200	1	10	10	1	20
S. halstedii NRRL 2331	Carbomycin	50	>200	20	40	1	10
S. tendae FD 22242	Carbomycin	20	>200	10	5	5	20
S. fradiae ATCC 21096	Tylosin	10	5	>200	10	5	10
S. albus G	Salgiomycin[a]	1	30	1	20	1	1

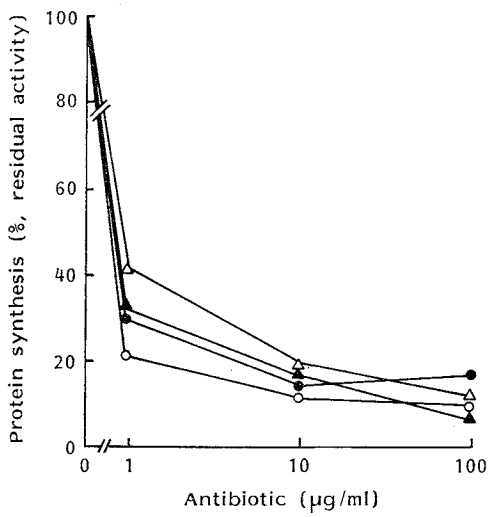
Abbreviations: SPI; Spiramycin, CAR; carbomycin, TYL; tylosin, LIN; lincomycin, ERY; erythromycin, OLE; oleandomycin. ATCC; American Type Culture Collection. NRRL; Northern Regional Research Center. FD; Pfizer International Inc.

[a] Non macrolide antibiotic.

the antibiotic they produce and thus did not appear to contain macrolide-inactivating enzymes.

Since macrolide antibiotics act on the ribosome, we tested target site modification as a mechanism of self-resistance. High-salt washed ribosomes of the four producers and of the control strain were obtained as described[5] and their antibiotic susceptibility was assayed using a coupled transcription-translation system[8] directed by plasmid DNA and sensitive to macrolide antibiotics. The ribosomes of the four producers (Fig. 1) and of *S. albus* G (data not shown) were equally sensitive to the macrolides, even when isolated at different stages of the growth curve. As a positive control, *S. erythraeus* (erythromycin producer) ribosomes were also tested and found, as expected, to be highly resistant to erythromycin. Therefore, self-resistance in these macrolide-producers does not appear to be due to modification of the target site.

Fig. 1. Effect of macrolides on "*in vitro*" protein synthesis by ribosomes of the macrolide producers.
○ Ribosomes of *Streptomyces halstedii* against carbomycin, ● ribosomes of *Streptomyces tendae* against carbomycin, △ ribosomes of *Streptomyces ambofaciens* against spiramycin, ▲ ribosomes of *Streptomyces fradiae* against tylosin.



Ribosomes (30 pmol per 30 μl assays) were incubated for 5 minutes at 4°C with different concentrations of antibiotic. Then, the assay of coupled transcription-translation was initiated by adding the components of the reaction mixture[7]. After 45 minutes at 30°C, duplicate samples (10 μl) were removed and processed as described[5]. The values are the average of two determinations.

A third resistance mechanism is impermeability of the producing cells to the antibiotic. The mycelia of each producer and of *S. albus* G were incubated, for 4 hours at 35°C with 5 μg/ml of the different antibiotics, centrifuged and the residual antibiotic in the supernatant determined by bioassay against *M. luteus*. As shown in Table 2, *S. albus* G incorporated the carbomycin, tylosin or spiramycin present in the medium and the antibiotics could be recovered active (about 90%) by boiling the cells for 5 minutes. In contrast, in each case, none of the four macrolide-producing organisms incorporated detectable amounts of the antibiotic they produced. Therefore, it can be concluded that the four producing organisms have decreased permeability to the antibiotic they synthesize. The three macrolides used each contain a 16-membered lactone ring and differ only in their aminosugar substituents but nonetheless the alteration in

Table 2. Macrolides uptake by mycelium of *Streptomyces*.

Antibiotic	Halo (diameter, mm)			
	Streptomyces albus G		Respective producer	
	0 hour	4 hours	0 hour	4 hours
Spiramycin	17	0	16	16[a]
Carbomycin	14	0	13	13[b]
Carbomycin	14	0	15	14[c]
Tylosin	15	0	14	13[d]

[a] *Streptomyces ambofaciens.* [b] *Streptomyces halstedii.* [c] *Streptomyces tendae.* [d] *Streptomyces fradiae.*
Each organism was incubated at 35°C with 5 μg/ml of antibiotic and, after 0 and 4 hours of incubation, the antibiotic in the supernatant was determined by bioassay against *Micrococcus luteus*. Values represent the inhibition halo after subtracting the Oxford tower diameter (8 mm) and are the average of three determinations.

permeability must be selective for the antibiotic produced since the four producing strains were sensitive to, and able to concentrate other macrolides (Table 1). It should be mentioned that we could not detect any antibiotic activity in cell-free extracts of the producers, even after extraction and concentration of the extracts with chloroform.

As a conclusion we suggest that, as has been recently suggested for the oleandomycin producing *S. antibioticus*[5], certain macrolide producers possessing macrolide-sensitive ribosomes could defend themselves from their own toxic product by synthesizing the antibiotic as an inactive compound intracellularly and expressing a specific reduction in permeability to the antibiotic produced following secretion.

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