Resistance to the Polyether Ionophore Antibiotic Pandavir (Nigericin) in Two Producing Strains of *Streptomyces*

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In two pandavir (nigericin) producing strains, *Streptomyces hygroscopicus* 155 and *Streptomyces albogriseolus* 444, an enzyme activity was detected leading to inactivation of the antibiotic in the presence of ATP-Na_2. Apparently, the observed inactivation is specific for the antibiotic produced by these strains. The nigericin producing strains were also found to be less permeable to pandavir than their non-producing variants.

Organisms which produce antibiotics must be protected from the lethal effects of the latter. Different resistance mechanisms have been reported in *Streptomyces*. For instance, *Streptomyces coelicolor* Müller can *O*-phosphorylate macrolides[1]. *Streptomyces fradiae*, *Streptomyces rimosus forma paromomycinus*, *Streptomyces lividus*, *Streptomyces ribosidificus*, *Streptomyces griseus*, *Streptomyces hygroscopicus* NRRL 2387, *Streptomyces vinaceus* were noted to have antibiotic modifying enzymes[2~9]. In contrast, *Streptomyces antibioticus* and *Streptomyces ambofaciens*, producers of oleandomycin and spiramycin respectively, have been shown to have a decreased permeability to oleandomycin and spiramycin, suggesting a role for the cell surface in self-resistance[10,11] and a macrolide glycosyl-transferase activity has been characterized in *S. antibioticus*[12]. However the resistance mechanisms in *Streptomyces* which produce polyether antibiotics are quite unclear. These substances have metal binding capacity forming complexes with cations. For nigericin the following order of ion selectivity was determined: $K^+ > Rb^+ > Na^+ > Cs^+ > Li^+$[13]. There is considerable evidence that the mechanism of antimicrobial action of the membrane active complexons involves a decrease of the cytoplasmic K^+ concentration and also lowering of the cytoplasmic pH. Ribosomal function depends upon K^+ ions whereas glycolysis and phosphate transport are inhibited at low pH. Microbial membranes contain the ATP synthesizing system and probably the action of these antibiotics lowers Δm_H, thereby inhibiting phosphorylation and the transport of phosphates. This is similar to the uncoupling of phosphorylation and respiration in mitochondria. In support of this model was the observed enhancement of respiration and K^+ uptake by *Mycobacterium phlei* and *Azotobacter vinelandii* in the presence of valinomycin[13].

The present study deals with resistance mechanisms to pandavir (nigericin) in the producing-organisms, *S. hygroscopicus* 155 and *S. albogriseolus* 444[14~16], respectively.

Methods

Bacterial Strains and Media

S. hygroscopicus 155 and *S. albogriseolus* 444 were from the culture collection of the Department of Microbiology, Sofia University. Stock cultures of the two strains were maintained on 2% (w/v) agar plates containing, per litre: bacto yeast extract (Difco) 10 g, bacto-dextrose (Difco) 4 g, bacto-malt extract (Difco) 10 g, pH 7.3.

Batch cultures were grown in YEME medium[17] for the preparation of S-100 fraction and in AL53 medium[18] for experiments on cellular impermeability to antibiotics. Batch cultures were grown at 28°C on orbital shakers (220 rpm).

Growth of Cells and Preparation of Cell-free Extracts (S-100)

Batch cultures of the two strains and their non-producing variants were grown in YEME liquid medium for 40 hours. Cells were collected and washed by filtration through Whatman No. 1 paper on a Buchner funnel and then twice more by resuspension in RS buffer (10 mM Tris-HCl pH 7.6 at 20°C, 10 mM $MgCl_2$, 50 mM NH_4Cl, 3 mM 2-mercaptoethanol) containing 0.5 mM Na_2 EDTA, followed by recollection as above.

Cell-free extracts were prepared by sonication, followed by treatment with deoxyribonuclease (2 μg/ml) at 4°C for 15 minutes and centrifugation at 30,000 $\times g$ for 20 minutes. The upper 4/5 of the supernatants were layered over 0.33 vol of RS buffer containing 40% (w/v) sucrose and centrifuged at 100,000 $\times g$ for 14 hours. The

middle 1/3 of the supernatants (designated S-100) was dialysed at 0°C against 500 vol of RS buffer and then rapidly frozen and stored at −70°C. Aliquots of S-100 fractions of *S. hygroscopicus* 155 and *S. albogriseolus* 444 and their non-producing variants were used as potential sources of antibiotic-modyfing enzymes.

The pandavir-inactivating enzymes (pandavir-phosphotransferase) were measured in a reaction mixtures (200 μl) consisting of 80 mM Tris-HCl (pH 7.6), 10 mM magnesium acetate, 6 mM 2-mercaptoethanol, 2 mM ATP-Na_2, 10 μg/ml pandavir and S-100 fraction from *S. hygroscopicus* 155, *S. albogriseolus* 444 and their non-producing variants. The mixtures were incubated at 28°C for 1 hour, then heated at 70°C for 10 minutes, followed by centrifugation at 14,000 rpm for 1 minute. Aliquots of the supernatants (20 μl) were placed in wells in agar plates overlayered with spores of *Bacillus subtilis* 6633. Pandavir-inactivating activity was measured by the reduced antibacterial activity of the drug after phosphorylation. The residual activity was measured as mm sterile zones.

Cell Impermeability to the Antibiotics

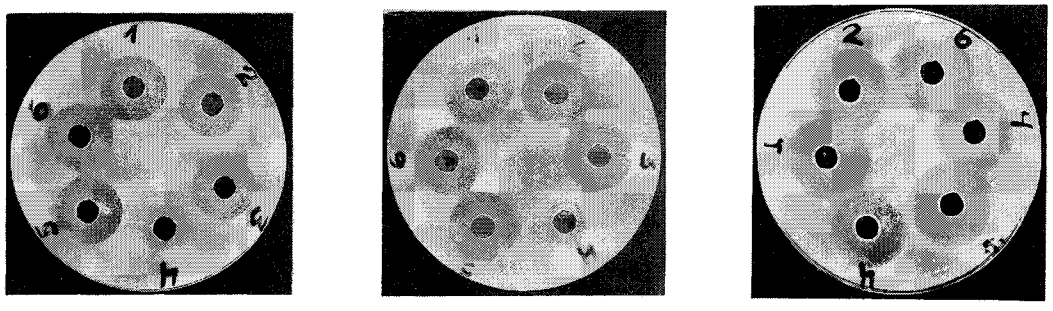
S. hygroscopicus 155, *S. albogriseolus* 444 and their non-producing variants were grown in liquid medium AL53 at 28°C on orbital shaker (220 rpm). At various time intervals (24, 48, 72 and 96 hours each) samples, containing approximately 35 mg wet weight mycelia were centrifuged, washed with the sterile AL53 medium and then resuspended in 10 ml fresh AL53 medium, containing pandavir or salinomycin (2 μg/ml in either case). After 4 hours of incubation at 28°C on orbital shaker (220 rpm), followed by centrifugation the residual antibiotic in the supernatants was determined by bioassay against *Bacillus subtilis* 6633. The residual activity was measured as mm sterile zones.

Results and Discussion

Our initial experiments indicated that *S. hygroscopicus* 155, a strain producing pandavir (nigericin) and azalomycin B was resistant to its own antibiotic up to 1,000 mg/ml when cultivated on solid medium[19]. The non-producing variant of *S. hygroscopicus* 155, deficient in aerial mycelium formation, was extremely sensitive to the antibiotic at 1 μg/ml under similar growth conditions. The resistance mechanisms in *Streptomyces* have been studied with respect to enzyme inactivation and membrane permeability. Since no resistance mechanism to polyether antibiotics had previously been detected in the respective producers we examined the possible existence of antibiotic inactivating enzymes. Postribosomal fraction (S-100) of the producing and non-producing variants of two *Streptomyces* strains (producers of pandavir) —*S. hygroscopicus* 155 and *S. albogriseolus* 444, were obtained after 40 hours growth and were tested for their ability to inactivate pandavir. The antibiotic activity decreased after incubation of pandavir with ATP and postribosomal fraction from *S. hygroscopicus* 155 (Fig. 1A) and *S. albogriseolus* 444 (data not shown). The non-producing variants also showed the same pattern of activity (Fig. 1B). Interestingly, the structurally similar polyether antibiotics monensin (Fig. 1C) and salinomycin (data not shown) were not inactivated by such S-100 preparations. Therefore we think that the enzyme inactivating pandavir was highly substrate specific and the presence of such enzymes could account for the resistance of *S. hygroscopicus* 155 and *S. albogriseolus* 444 to low concentrations of pandavir. We were surprised by the presence of such enzymes in non-producing variants of the two strains studied. This suggests that resistance is constitutively expressed and may support the view of some authors that antibiotic modifying enzymes in other strains had not only defence functions but also participated in antibiotic biosynthe-

Fig. 1. Influence of S-100 fraction from *Streptomyces hygroscopicus* 155 on activity of pandavir (A, B) and monensin (C).

A and C: producing variant; B: non-producing variant.



A B C

Reaction mixtures (200 μl) contained 80 mM Tris-HCl (pH 7.65), 10 mM magnesium acetate, 6 mM 2-mercaptoethanol and 20 μg/ml pandavir together with one of the following (1÷6).

1: Buffer I (10 mM Tris-HCl (pH 7.65), 10 mM magnesium acetate, 6 mM 2-mercaptoethanol and 30 mM NH_4Cl); 2: S-100 fraction from *S. hygroscopicus* 155 (producing and non-producing variant); 3: S-100 fraction and acetyl coenzyme A (2 mM); 4: S-100 fraction and ATP-NA_2 (2 mM); 5: buffer I and acetyl coenzyme A; 6: buffer I and ATP-Na_2.

Table 1. Pandavir uptake by the mycelia of *Streptomyces hygroscopicus* 155 and *Streptomyces albogriseolus* 444.

Age of the mycelia (hours)	Sterile zone (diameter, mm)							
	Streptomyces hygroscopicus 155				*Streptomyces albogriseolus* 444			
	Producing strain		Non-producing variant		Producing strain		Non-producing variant	
	0 hour	4 hours	0 hour	4 hours	0 hour	4 hours	0 hour	4 hours
24	23	23	23	22	23	23	23	20
48	23	23	22	19	22	22	23	19
72	22	22	22	17	22	22	22	16
96	22	22	22	17	22	22	21	14

AL53 medium (10 ml), containing 2 μg/ml pandavir was inoculated with about 35 mg washed mycelia. The residual antibiotic in the supernatants was determined immediately after inoculation (0 hour) and after 4 hours of incubation at 28°C on orbital shaker (220 rpm) by bioassay against *Bacillus subtilis* 6633. Values represent the inhibition zones (diameter, mm) and are the average of three determinations.

Table 2. Salinomycin uptake by mycelium of *Streptomyces hygroscopicus* 155.

Age of the mycelia (hours)	Sterile zone (diameter, mm)			
	Producing strain		Non-producing variant	
	0 hour	4 hours	0 hour	4 hours
24	16	16	16	13
48	16	16	16	12
72	15	15	15	12
96	15	15	15	11

AL53 medium (10 ml), containing 2 μg/ml salinomycin was inoculated with about 35 mg washed mycelia. The residual antibiotic in the supernatants was determined immediately after inoculation (0 hour) and after 4 hours of incubation at 28°C on orbital shaker (220 rpm) by bioassay against *Bacillus subtilis* 6633. Values represent the inhibition zones (diameter, mm) and are the average of three determinations.

sis[20,21].

The self defence systems of strains producing of antibiotics are in some cases complex, involving multiple defence mechanisms. For example, some strains contain enzymes that can inactivate the endogenous antibiotic *via* phosphorylation and *via* acetylation, although the latter mechanism was not detected in the present studies (Fig. 1, A, B). However, additional data suggested that the cell surfaces of *S. hygroscopicus* 155 and *S. albogriseolus* 444 were impermeable to pandavir which would also contribute to the survival strategy (Table 1).

In the two wild type strains, this impermeability functioned in the early stage of development and was kept until the end of the growth (Table 1) whereas the non-producing variants of *S. hygroscopicus* 155 and *S. albogriseolus* 444 displayed increased permeability to the exogenous antibiotics. These results were checked using another polyether antibiotic-salinomycin. It was added to the media of *S. hygroscopicus* 155 and its non-producing variant and the data obtained were similar to these obtained for pandavir (Table 2).

On the basis of these results we suggest that more than one mechanism of self defence is functional in *S. hygroscopicus* 155 and *S. albogriseolus* 444.

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