MECHANISMS OF AMINOGLYCOSIDE-RESISTANCE OF *STREPTOMYCES* HARBORING RESISTANT GENES OBTAINED FROM ANTIBIOTIC-PRODUCERS

Sir:

The antibiotic resistance genes (neomycin, thiostrepton, erythromycin and viomycin) were obtained from antibiotic-producing *Streptomyces* by THOMPSON *et al.*[1,2,3]. The mechanisms of resistance to these antibiotics were due to phosphotransferase, acetyltransferase and/or 23S rRNA methylase[4]. As reported in our previous paper[5], we cloned the resistance genes ribostamycin, kanamycin, racemomycin, destomycin and novobiocin, which were obtained from the antibiotic-producing *Streptomyces*. In the present communication, we describe the mechanisms of resistance to the aminoglycoside antibiotics involving pMS1 (coded by the ribostamycin resistance gene), pMS18 and pMS21 (coded by kanamycin resistance genes).

The resistance mechanisms of the aminoglycoside antibiotic-producing *Streptomyces* to the own producing antibiotics, were already demonstrated biochemically to be due to their aminoglycoside inactivating enzymes[6], their ribosomal resistance[7,8,9], or the both[10]. We have used DNA cloning technology, to isolate and study the genes which confer resistance.

S. ribosidificus SF-733 producing ribostamycin, *S. kanamyceticus* M1164 producing kanamycins, *S. lividans* 66 (a host strain), three strains of *S. lividans* 66 harboring pMS1, pMS18 and pMS21 were used. Strains were grown in Tryptic Soy Broth (Difco) on a reciprocal shaker at 28°C. Mycelia at the mid-logarithmic growth phase were harvested by centrifugation, washed twice and resuspended in buffer A (consisting of 10 mM Tris-HCl (pH 7.8), 60 mM ammonium chloride, 10 mM magnesium acetate and 6 mM 2-mercaptoethanol). The suspensions of mycelia were disrupted by passage through a French pressure cell at 1.4 kg/cm^2. The supernatants (designated as the S-30 fractions), were obtained after centrifugation at 30,000$\times g$, for 30 minutes. All procedures were performed at 4°C. The inactivating activities towards aminoglycoside antibiotics in each S-30 fraction were assayed according to the methods described in Table 1. The S-30 fraction prepared from *S. lividans* 66 (pMS1), showed phosphorylating activity with ribostamycin, kanamycin, kanamycin B, neamine and butirosin B, but not dibekacin, tobramycin or the gentamicin C complex. It showed no acetylating and adenylylating activities.

Table 1. The inactivating activities to the aminoglycoside antibiotic in *Streptomyces*.

Strain	Phosphorylation	Acetylation
	(Ribostamycin)	
S. ribosidificus SF-733	72,000 dpm	48,000 dpm
S. lividans 66 (pMS1)	94,000	1,000
S. lividans 66	380	400
	(Kanamycin)	
S. kanamyceticus M1164	200 dpm	4,700 dpm
S. lividans 66 (pMS18)	0	4,300
S. lividans 66 (pMS21)	0	100
S. lividans 66	150	500

The phosphorylating activities to aminoglycoside antibiotics in the S-30 fractions were assayed as follows. The reaction mixture (25 μl) consisted of 20 mM phosphate buffer (pH 6.8), 2 mM magnesium acetate, 1 mM dithiothreitol, 1 mM [γ-^{32}P]-ATP (10 mCi/mmol), 15 μl of S-30 fraction and 0.05 mM antibiotic.

After the incubation at 37°C for 60 minutes, 20 μl of the mixture was put on a phosphocellulose paper (Whatman P-81, 1.5$\times$1.5 cm) and washed in 500 ml of distilled water for 4 times, the papers were dried and placed in glass scintillation vials cotaining 10 ml of toluene-based scintillation fluid and counted. Acetylation or adenylylation of antibiotics was performed by the use of 0.2 mM [1-^{14}C]acetyl-CoA (10 mCi/mmol), or 0.3 mM [8-^{14}C]ATP (10 mCi/mmol) instead of [γ-^{32}P]ATP in the phosphorylation. Adenylylating activities were not detected in any samples.

Neamine was inactivated by the S-30 fraction in the presence of ATP, and the inactivated neamine was purified by column chromatography on the Amberlite CG-50 (NH_4^+) and CM-Sephadex C-25 (NH_4^+). The IR and NMR spectra of the inactivated neamine were coincident with those of neamine 3′-phosphate[11] (Fig. 1). The mechanism of resistance in pMS1 was confirmed to be due to an APH(3′) enzyme. Both the S-30 fractions of *S. lividans* 66 (pMS1) and *S. ribosidificus* SF-733 had a phospholylating activity similar to the APH(3′) enzyme reported

Fig. 1. The structure of inactivated neamine.

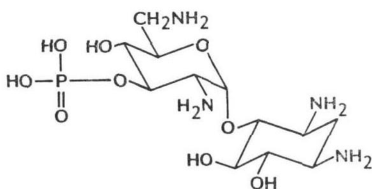
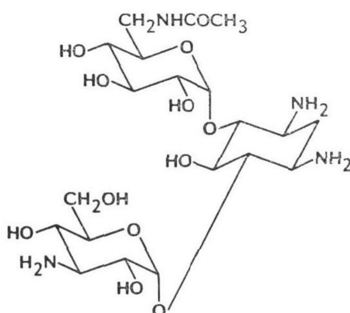


Fig. 2. The structure of inactivated kanamycin.



by DOWDING[12]. The MIC data for the various aminoglycoside antibiotics[5] were consistent with the substrate specificities of this enzyme. Cleavage maps of the APH(3′) gene in pMS1[13] by restriction enzymes were compared with that of the *aph* gene obtained from *S. fradiae*[3]. Although their cleavage maps did not resemble each other, their DNA sequences were very similar[14,15].

Kanamycin was acetylated by the S-30 fraction prepared from *S. lividans* 66 (pMS18), but not phosphorylated (Table 1). The inactivated kanamycin prepared by incubation of the S-30 fraction, ATP, acetate and CoA was purified by column chromatography on Amberlite CG-50 (NH_4^+). From the IR and NMR spectral data, the structure of the inactivated kanamycin was determined to be kanamycin 6′-acetate[16] (Fig. 2). This confirmed that pMS18 contained the AAC(6′) gene. The acetylating activities in the S-30 fractions prepared from *S. lividans* 66 (pMS18) and *S. kanamyceticus* M1164 were similar to those of the AAC(6′) enzymes reported by SATOH *et al.*[17] and HOTTA *et al.*[6]

As the S-30 fraction from *S. lividans* 66 (pMS21) showed no enzymatic inactivation of kanamycin and other aminoglycoside antibiotics, the mechanism of the resistance involving pMS21 seemed to be different from pMS1 and pMS18. Origin of the kanamycin resistance in *S. lividans* 66 (pMS21) was examined by poly(U)-directed polyphenylalanine synthesis. The S-30 fractions from *S. lividans* 66 (pMS21) or *S. lividans* 66 were centrifuged at 150,000 × *g* for 120 minutes, to obtain the supernatant (designated as the S-150 fraction) and precipitated fractions (designated as the ribosomal fraction). The respective S-150 and ribosomal fractions were dialyzed with buffer A for 18 hours at 4°C and then used for poly(U)-directed polyphenylalanine synthesis. As shown in Table 2, frac-

Table 2. Effect of kanamycin on poly(U)-directed polyphenylalanine synthesis.

	Strain	S-150 & ribosome	Incorporation of [^{14}C]phenylalanine	
			None (dpm)	Kanamycin (100 μg/ml) (dpm)
Expt 1	*S. lividans* 66	S-150	17,780	3,980
	S. lividans 66	Ribosome		
Expt 2	*S. lividans* 66 (pMS21)	S-150	20,460	16,000
	S. lividans 66 (pMS21)	Ribosome		
Expt 3	*S. lividans* 66 (pMS21)	S-150	24,400	6,0 0
	S. lividans	Ribosome		
Expt 4	*S. lividans* 66	S-150	11,280	13,680
	S. lividans 66 (pMS21)	Ribosome		

Poly(U)-directed polyphenylalanine synthesis *in vitro* were assayed as follows. The reaction mixture (50 μl) consisted with 50 mM Tris-HCl buffer (pH 7.8), 4.5 mM magnesium acetate, 1 mM dithiothreitol, 42 mM ammonium chloride, 1 mM ATP, 0.05 mM GTP, 0.5 mM phosphoenolpyruvate, 30 μg pyruvate kinase, 80 μg poly(U), 0.02 mM L-[*U*-^{14}C]phenylalanine (25 mCi/mmol), 10 μl of the S-150 fraction and ribosome (1.0 A_{260} unit). The reaction mixture was incubated at 34°C for 20 minutes, stopped by the addition of 5% TCA (0.5 ml), and then heated at 90°C for 10 minutes. The TCA insoluble fraction was collected on a membrane filter (Millipore HA) and counted in a liquid scintillation counter.

tions prepared from *S. lividans* 66 (pMS21), were inhibited slightly by the addition of kanamycin (100 μg/ml), but those from *S. lividans* 66 were inhibited about 80% by kanamycin. Therefore, the mechanism of resistance to kanamycin involving the pMS21 is probably due to resistance in poly(U)-directed polyphenylalanine synthesis. When the S-150 and ribosomal fractions were reciprocally exchanged with each other, inhibition of the polyphenylalanine synthesis with kanamycin was observed by using the S-150 fraction prepared from *S. lividans* 66 (pMS21), but not the ribosomal fraction. We conclude that resistance to kanamycin involving pMS21 is associated with the ribosome.

Recently, NAKANO *et al.*[13] shotgun-cloned the ribosomal resistance gene from *S. kanamyceticus*. We believe that this gene was very similar to the kanamycin resistance gene in pMS21. Because, the cleavage maps of the genes[13] and the resistance patterns to aminoglycoside antibiotics[5] were similar to each other.

HOTTA *et al.* reported[6], that the mechanism of self-resistance in the kanamycin producing organism, *S. kanamyceticus*, was caused by the AAC(6′) enzyme. These observations in conjunction with our data shown that the mechanism of self-resistance in *S. kanamyceticus* are can be explained in at least two ways: the AAC(6′) kanamycin inactivating enzyme and kanamycin resistant ribosomes.

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