RESISTANCE MECHANISMS OF KANAMYCIN-, NEOMYCIN-, AND STREPTOMYCIN-PRODUCING STREPTOMYCETES TO AMINOGLYCOSIDE ANTIBIOTICS

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Streptomyces kanamyceticus ISP5500, *S. fradiae* ISP5063 and *S. griseus* ISP5236, which produce kanamycin, neomycin or streptomycin respectively, were highly resistant to the antibiotics they produced. Polyphenylalanine synthesis in cell free systems was also resistant to the action of the antibiotics. Reciprocal exchange between ribosomes and S150 fractions from the three strains revealed that the S150 fraction of each strain had an enzyme activity that inactivated the appropriate antibiotic whereas the ribosomes were susceptible to the antibiotics. It was concluded that the resistance of the *in vitro* polyphenylalanine synthesizing systems of these antibiotics was due to the presence of inactivating enzymes.

Furthermore, *S. fradiae* and *S. kanamyceticus* were highly resistant to aminocyclitol-containing aminoglycoside antibiotics other than those produced by the two strains. In these cases, the inactivating enzymes were found to have a major role in the resistance mechanism. However, the resistance of *S. kanamyceticus* ISP5500 to streptomycin seems to be due to resistance at the ribosomal level.

The resistance mechanisms in streptomycetes which produce antibiotic inhibitors of protein synthesis such as streptomycin[1~6], erythromycin[6,7], viomycin[8], chloramphenicol[9] and thiostrepton[10~12] have been studied with respect to membrane permeability[3,9], enzymatic inactivation[3,4,8] and sensitivity of ribosomes[3,6,7,9~12]. Among aminoglycoside antibiotic producing strains, the streptomycin-producing *Streptomyces griseus* has been studied in detail and CELLA and VINING[2,3] concluded that membrane permeability to streptomycin plays a major role in resistance and that streptomycin kinase activity was only partly responsible. On the basis of studies of the inhibition of protein synthesis *in vivo* and the formation of "stuck" ribosomes by streptomycin, they inferred that the ribosomes of *S. griseus* should be sensitive to streptomycin, although they failed to confirm it by *in vitro* protein synthesis studies. Subsequently, VALU and SZABO[13] and SUGIYAMA *et al.*[6] reported that the addition of a protease inhibitor to cell homogenates made it possible to prepare an active *in vitro* protein synthesis system from *S. griseus*. SUGIYAMA *et al.*[6] reported that streptomycin-producing *S. griseus* HUT6037 had ribosomes susceptible to streptomycin. In their report, however, they did not consider the streptomycin-inactivating enzyme which should be present in the S150 fraction and interfere with the action of streptomycin on protein synthesis. TERAOKA and TANAKA[5], on the other hand, reported that the ribosomes from a streptomycin-producing strain of *S. griseus* had a much lower affinity for dihydrostreptomycin, as compared with the ribosomes of an erythromycin-producing strain of *S. erythreus*. In this case, they pointed out the possibility that this was due to the presence of a streptomycin-inactivating enzyme in the *S. griseus* ribosomal fraction or to a low streptomycin affinity of the ribosomes. It has also been reported that, for a neomycin-producing strain of *S. fradiae*, protein syn-

thesis in cell free extracts was strongly inhibited by neomycin[14], although the extract should have contained neomycin-inactivating enzyme(s)[15~17]. Thus, the roles of ribosomes and inactivating enzymes in the resistance of streptomycete strains which produce streptomycin or neomycin to these antibiotics remain unclear.

In order to characterize the resistance mechanisms, it is necessary to examine the effect of antibiotics on *in vitro* protein synthesizing system reconstituted by reciprocal exchange between ribosomes and S150 fractions from the producing organism and another organism which is susceptible to the antibiotic, as has reported in the case of an organism producing a peptide antibiotic, viomycin[8]. By this approach, we revealed that an aminoglycoside antibiotic (istamycin)-producing *S. tenjimariensis* SS-939 has a unique resistance mechanism at ribosomal level to several aminoglycoside antibiotics as well as to the one it produced[18,19]. The extract of the strain SS-939 showed no enzyme activity inactivating the aminoglycoside antibiotics.

In this paper, we investigated the effect of kanamycin, neomycin and streptomycin on polypeptide synthesis *in vitro* in extracts of the strains producing these antibiotics and have confirmed that the ribosomes of all the strains were sensitive to their "own" antibiotics and that the inhibitory action of antibiotics on the protein synthesis *in vitro* was reduced in the presence of the appropriate inactivating enzymes.

Materials and Methods

Organisms

Streptomyces kanamyceticus ISP5500, *S. fradiae* ISP5063 and *S. griseus* ISP5236 were obtained from IFO (Institute of Fermentation, Osaka, Japan).

Antibiotics

Sulfates of kanamycin A, gentamicin C complex, neomycin B, lividomycin A and streptomycin were used. Other antibiotics were used as free bases. They were all obtained from the collection of antibiotics of the Institute of Microbial Chemistry.

Antibiotic Resistance

Cultures of each strain in logarithmic growth phase in Tryptic Soy Broth (Difco) at 27°C on a rotary shaker were transferred at a dilution of 1: 400 into fresh medium containing antibiotics listed in Table 1 at varied concentrations. Growth was measured after incubation for 3 days at 27°C on a rotary shaker.

Preparation of S150 Fractions and Ribosomes

Mycelia from the logarithmic growth phase of the strains ISP5500, ISP5063 and ISP5236 grown in Tryptic Soy Broth at 27°C on a rotary shaker were disrupted and ribosomes and S150 fractions were prepared according to the method previously described[18].

Polyuridylate-Directed Polypeptide Synthesis

PolyU-directed polyphenylalanine synthesis was carried out according to the method previously described[18]. Ribosomes and S150 fractions from the neomycin-, kanamycin- and streptomycin-producing strains were reciprocally combined to reconstitute the *in vitro* synthesis system. Ribosomes, S150 fraction and [U-^{14}C]phenylalanine (25 mCi/mole, The Radiochemical Centre, Amersham) were added to the reaction mixture (100 μl) at the concentration of 0.33 μM, 4.5 mg/ml and 20 μM respectively. The reaction mixture was incubated at 37°C.

Inactivating Enzyme Activity

The S150 fraction from each strain was incubated with each antibiotic (100 μg/ml) for 60 minutes at 37°C under conditions for polypeptide synthesis without addition of ribosomes. The inactivating-

enzyme activity was also measured under the condition suitable for the acetylation of kanamycin[20]; the reaction mixture (100 μl) consisted of antibiotic (100 μg/ml), ATP disodium salt (2.4 μmoles), coenzyme A (9.8 nmoles), magnesium acetate (760 nmoles), tris-HCl (150 mM) and S150 (28 μl), pH 7.8. Incubation was for 60 minutes at 37°C. The antibiotic activity in the reaction mixture was assayed by the paper disc method using *Bacillus subtilis* PCI219 as the test organism.

Results

Antibiotic Resistance *In Vivo*

Table 1 shows the maximum concentrations of antibiotics permitting the growth (MCG) of kanamycin-, neomycin- and streptomycin-producing strains. Kanamycin-producing *S. kanamyceticus* ISP5500 grew in the presence of high concentrations of kanamycin A, neamine, dibekacin, butirosin A and streptomycin. Neomycin-producing *S. fradiae* ISP5063 was highly resistant to neomycin B, ribostamycin, neamine and paromomycin. This strain was also resistant to kanamycin A, but it was variable: Occasionally strain ISP5500 did not grow in the presence of 50 μg/ml of kanamycin A. Streptomycin producing *S. griseus* ISP5236 was resistant to its own antibiotic only.

Resistance to Antibiotics *In Vitro*

Since the kanamycin, neomycin and streptomycin producers used here were known to possess enzymes inactivating their own antibiotics, four *in vitro* polyphenylalanine synthesizing systems were reconstituted by reciprocal exchange between their ribosomes and S150 fractions.

Table 1. Resistance of aminoglycoside antibiotic producers to aminoglycoside antibiotics *in vivo*.

Antibiotic	*S.kanamyceticus* ISP5500	*S. fradiae* ISP5063	*S. griseus* ISP5236
Neamine	1000 μg/ml	500 μg/ml	10 μg/ml
Kanamycin A	1000	100	<2
Dibekacin	200	50	<2
Gentamicin C	5	25	<2
Ribostamycin	500	2000	2
Butirosin A	200	25	<2
Neomycin B	25	500	<2
Paromomycin	10	500	nt*
Lividomycin	25	50	<2
Streptomycin	100	50	400
Istamycin A	25	10	<2

nt: not tested

Fig. 1. Effect of neomycin B on polyphenylalanine synthesis using ribosomes and S150 fractions from *S. fradiae* and *S. kanamyceticus*.

(a) Both ribosomes and S150 fractions from *S. fradiae* ISP5063.

(b) Ribosomes from *S. fradiae* ISP5063 and S150 fraction from *S. kanamyceticus* ISP5500.

(c) Ribosomes from *S. kanamyceticus* ISP5500 and S150 fraction from *S. fradiae* ISP5063.

(d) Both ribosomes and S150 fraction from *S. kanamyceticus* ISP5500.

○: without neomycin B; ●: with neomycin B (100 μg/ml).

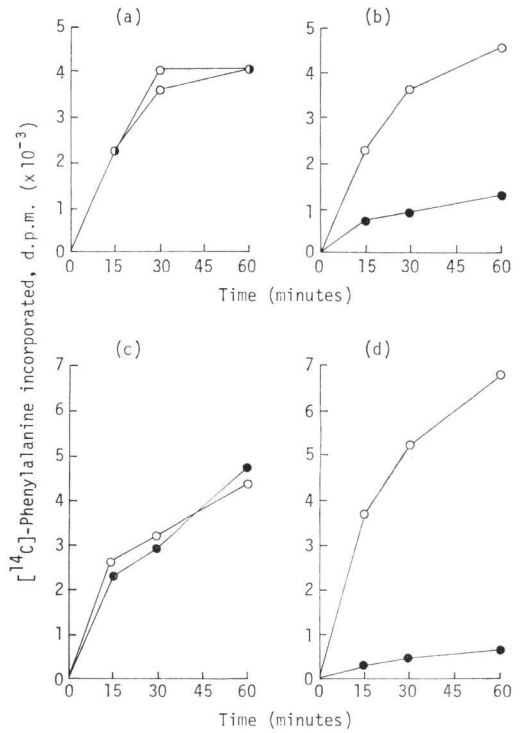
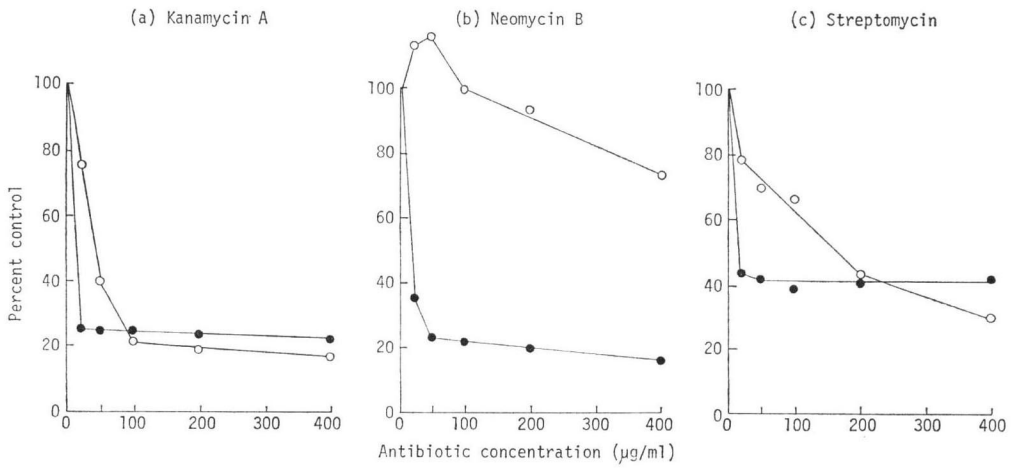


Fig. 1 shows the effect of neomycin B on polypeptide synthesis *in vitro* using ribosomes and S150 fractions from *S. fradiae* ISP5063 and *S. kanamyceticus* ISP5500. The latter was significantly sensitive to neomycin B as shown in Table 1. Polyphenylalanine synthesis was not inhibited by 100 μg/ml of neomycin B when both ribosomes and S150 fraction were derived from *S. fradiae* ISP5063 (Fig. 1-a). A marked inhibition was, however, observed when the S150 fraction of *S. fradiae* was substituted with that of *S. kanamyceticus* ISP5500 (Fig. 1-b) while no inhibition was observed when the *S. fradiae* S150 fraction was combined with *S. kanamyceticus* ribosomes (Fig. 1-c). Thus we concluded that *S. fradiae* ribosomes are sensitive and the S150 fraction determines resistance to neomycin B. The S150 fraction of *S. fradiae* ISP5063 inactivated neomycin B completely under conditions for polypeptide synthesis *in vitro* as shown in Table 4. Polyphenylalanine synthesis using ribosomal and S150 fractions from *S. kanamyceticus* ISP5500 was markedly inhibited by neomycin B (Fig. 1-d). Although this S150 fraction contained a neomycin-inactivating activity (Table 4), it was significantly weaker than that of *S. fradiae* ISP5063.

Similar results were obtained with ribosomes and S150 fractions of *S. griseus* ISP5236 and *S. kanamyceticus* ISP5500 in response to antibiotics produced by these strains.

Polyphenylalanine synthesis *in vitro* was examined in the presence of various concentrations of each antibiotic in order to determine the effect of the inactivating enzyme activity of each S150 fraction (Fig. 2). Synthesis using the S150 fraction and ribosomes of *S. kanamyceticus* ISP5500 was not inhibited by a low concentration (25 μg/ml) of kanamycin but was markedly inhibited at higher concentrations (Fig. 2-a). Ribosomes of this strain were found to be fully sensitive to kanamycin A since polyphenylalanine synthesis was strongly inhibited when the S150 fraction from *S. griseus* ISP5236

Fig. 2. Sensitivity of ribosomes and effect of S150 fractions on polyphenylalanine synthesis.
(a) Ribosomes from *S. kanamyceticus* ISP5500 and S150 fraction from *S. kanamyceticus* ISP5500 (○) or *S. griseus* ISP5236 (●). Incorporation of ^{14}C-Phe of controls (without antibiotic) were 15,262 dpm (○) and 13,756 dpm (●).
(b) Ribosomes from *S. fradiae* ISP5063 and S150 fraction from *S. fradiae* ISP5063 (○) or *S. kanamyceticus* ISP5500 (●). Incorporation of ^{14}C-Phe of controls were 4,248 dpm (○) and 4,563 dpm (●).
(c) Ribosomes from *S. griseus* ISP5236 and S150 fraction from *S. griseus* ISP5236 (○) or *S. fradiae* ISP5063 (●). Incorporation of ^{14}C-Phe of controls were 10,814 dpm (○) and 10,640 dpm (●).
Reaction mixtures were incubated for 60 minutes at 37°C.



was combined with the ribosomes of the kanamycin-producing strain. Polyphenylalanine synthesis using the S150 fraction and ribosomes of *S. fradiae* ISP5063 was highly resistant to its own antibiotic as shown in Fig. 2-b. Only about 30% inhibition was observed when as much as 400 μg/ml (4/5 of MCG) of neomycin B was added. However, a marked inhibition was observed at 25 μg/ml of neomycin B when the S150 fraction from *S. kanamyceticus* was used. In the case of *S. griseus* ISP5236, the result obtained was intermediate between that of *S. kanamyceticus* and that of *S. fradiae*. Polypeptide synthesis using ribosomes and S150 fraction from strain ISP5236 was gradually inhibited with increasing concentrations of streptomycin (Fig. 2-c). The inhibition reached the maximum when 200 μg/ml (1/2 of MCG) of streptomycin was added. The ribosomes of *S. griseus* appeared to be susceptible to streptomycin since complete inhibition of polypeptide synthesis was observed with the addition of 25 μg/ml of streptomycin to a system containing *S. griseus* ribosomes and the S150 of *S. fradiae* ISP5063.

Resistance to Aminoglycoside Antibiotics Other than Those Produced by *S. fradiae*, *S. kanamyceticus* and *S. griseus*

The effect of aminoglycoside antibiotics other than those produced was examined in *in vitro* polyphenylalanine synthesizing systems containing the ribosomes and S150 fraction of *S. fradiae* ISP5063 or *S. kanamyceticus* ISP5500 since these strains were resistant not only to the antibiotics they produced but also to other aminoglycoside antibiotics as shown in Table 1.

Table 2 shows that the S150 fraction from *S. fradiae* ISP5063 was important in determining this multiple resistance. With ribosomes and S150 fraction of *S. fradiae*, the protein synthesizing system was not inhibited by 100 μg/ml of ribostamycin, paromomycin, neamine and neomycin B, but was markedly inhibited by kanamycin A and butirosin A to which strain ISP5063 showed a much lower resistance. When the S150 fraction of *S. fradiae* was substituted with that of *S. griseus* ISP5236, polyphenylalanine synthesis was markedly inhibited by all the antibiotics tested. On the other hand, the S150 fraction of *S. fradiae* ISP5063 protected polyphenylalanine synthesis using ribosomes of *S. griseus* from the inhibitory action of the antibiotics. These results indicate that the ribosomes of *S.*

Table 2. Effect of aminoglycoside antibiotics on *in vitro* polypeptide synthesis by *Streptomyces fradiae* ISP5063.

Antibiotic*	Resistance *in vivo*		PolyU-directed protein synthesis *in vitro**			
			f-Ribosome		g-Ribosome	
	f**	g**	f-S150 (%)	g-S150 (%)	f-S150 (%)	g-S150 (%)
None (Control)	—	—	100.0 (8,248 dpm)	100.0 (6,217 dpm)	100.0 (6,609 dpm)	100.0 (5,620 dpm)
Neomycin B	R	S	64.8	15.2	57.2	11.7
Ribostamycin	R	S	86.8	31.3	98.3	16.7
Paromomycin	R	S	109.5	29.6	107.5	11.4
Neamine	R	S	86.3	23.4	90.0	16.1
Kanamycin A	S	S	22.3	22.2	19.5	11.9
Butirosin A	S	S	24.0	28.9	15.9	10.9

* Antibiotic concentration was 100 μg/ml. Incorporation of ^{14}C-phenylalanine into TCA insoluble fraction was determined after 60 minutes incubation.

** f and g refer to *S. fradiae* ISP5063 and *S. griseus* ISP5236. R refers to resistant to 50 μg/ml of antibiotic and S refers to sensitive.

Table 3. Effect of aminoglycoside antibiotics on *in vitro* polypeptide synthesis by *Streptomyces kanamyceticus* ISP5500.

Antibiotic*	Resistance *in vivo*		PolyU-directed protein synthesis *in vitro**			
			k-Ribosome		g-Ribosome	
	k**	g**	k-S150 (%)	g-S150 (%)	k-S150 (%)	g-S150 (%)
None (Control)	—	—	100.0 (5,742 dpm)	100.0 (15,478 dpm)	100.0 (3,658 dpm)	100.0 (11,299 dpm)
Kanamycin A	R	S	30.0	20.7	14.8	11.9
Neamine	R	S	32.4	15.0	16.5	12.7
Ribostamycin	R	S	28.6	16.6	15.8	14.5
Dibekacin	R	S	41.6	16.3	14.8	11.2
Butirosin A	R	S	29.6	19.4	13.0	10.4
Streptomycin	R	R	63.7	115.1	38.5	81.4
Neomycin B	S	S	12.5	8.1	11.1	9.5

* Antibiotic concentration was 100 μg/ml. Incorporation of ^{14}C-phenylalanine into TCA insoluble fraction was determined after incubating 60 minutes.

** k and g refer to *S. kanamyceticus* ISP5500 and *S. griseus* ISP5236 respectively. R refers to resistant to 50 μg/ml of antibiotic and S refers to sensitive.

fradiae ISP5063 are susceptible to all the antibiotics examined and that its S150 fraction determines the antibiotic resistance of *in vitro* polypeptide synthesis.

Neamine, neomycin B, paromomycin and ribostamycin were completely inactivated by incubation with the S150 fraction of *S. fradiae* ISP5063 under the conditions for polypeptide synthesis; kanamycin A and butirosin A were partly inactivated (Table 4). This substrate range correlates exactly with *in vivo* resistance pattern of the strain ISP5063 and also with the substrate range reported for the aminoglycoside 3′-phosphotransferase V of a neomycin-producing strain of *S. fradiae*[17].

Table 4. Inactivation of antibiotics by S150 fractions from *S. kanamyceticus*, *S. fradiae* and *S. griseus*.

Antibiotic*	Inactivation (%)			
	S. kanamyceticus ISP5500		*S. fradiae* ISP5063	*S. griseus* ISP5236
	I*	II*	I*	I*
Kanamycin A	100	100	10	0
Dibekacin	70	100	nt**	0
Butirosin A	70	98	18	0
Neamine	100	100	100	0
Ribostamycin	100	100	100	0
Neomycin B	30	100	100	0
Paromomycin	10	nt**	100	0
Streptomycin	0	0	0	100

* S150 fractions were incubated with each antibiotic (100 μg/ml) under the conditions for protein synthesis *in vitro* (I) and acetylation (II) as described in Materials and Methods.

** Not tested.

Table 3 shows the action of aminoglycoside antibiotics on polyphenylalanine synthesis *in vitro* using ribosomes and S150 fractions of *S. kanamyceticus* ISP5500. Polypeptide synthesis was markedly inhibited by all the antibiotics except streptomycin when added at a concentration of 100 μg/ml. Inhibition by kanamycin A, neamine, dibekacin and butirosin A was reduced when the S150 fraction from *S. kanamyceticus* was used in place of that of *S. griseus* ISP5236. When the S150 fraction from *S. kanamyceticus* ISP5500 was examined for the inactivation of these antibiotics (Table 4), there was a correlation between *in vivo* resistance pattern of the strain and the substrate range of the S150 fraction; no inactivating-enzyme activity was observed for streptomycin. The resistance of *S. kanamyceticus* ISP5500 to streptomycin is therefore suggested to be ribosomally-mediated.

Discussion

Kanamycin-producing *S. kanamyceticus* ISP5500, neomycin-producing *S. fradiae* ISP5063 and streptomycin-producing *S. griseus* ISP5236 were highly resistant to the antibiotics produced by these strains. *In vitro* polyphenylalanine synthesizing system using ribosomes and S150 fractions from each of the above strains also showed resistance to their "own" antibiotics. Analyses by reciprocal exchange of ribosomes and S150 fractions from these strains revealed that the ribosomes of all the strains are sensitive to the antibiotics they produced and the apparent resistance of *in vitro* polypeptide synthesis depends on the presence of inactivating enzymes in their S150 fractions. These results indicate that antibiotic inactivating enzymes play an important role in their self-resistance mechanisms.

In this connection SUGIYAMA *et al.* reported that a polyphenylalanine synthesizing system using ribosomes and S150 fractions from a streptomycin producing strain of *S. griseus*[6] or a neomycin producing strain of *S. fradiae*[14] were markedly inhibited by streptomycin or neomycin respectively. Therefore, their results are not in agreement with ours. SUGIYAMA *et al.* did not consider the streptomycin kinase or neomycin phosphotransferase which should be present in the respective S150 fractions and should have interfered with the action of these antibiotics on *in vitro* polypeptide synthesis. The inactivating enzymes in the S150 fractions used by SUGIYAMA *et al.* may have been reduced in activity perhaps because of the presence of alkaline phosphatase.

S. fradiae ISP5063 and *S. kanamyceticus* ISP5500 were resistant to several aminoglycoside antibiotics other than the antibiotics they produced (Table 1). In these cases also the inactivating enzymes were responsible. In *S. fradiae* ISP5063, aminoglycoside 3′-*O*-phosphotransferase V[17] should be the factor determining resistance because the substrate range of the enzyme exactly correlated with *in vivo* and *in vitro* resistance pattern to aminoglycoside antibiotics. In *S. kanamyceticus* ISP5500, resistance of its polyphenylalanine synthesis *in vitro* to various antibiotics (except streptomycin) was relatively low because the activity of the enzyme inactivating these antibiotics was reduced under the conditions used. However, higher activity of the enzyme (probably kanamycin 6′-*N*-acetyltransferase) was observed when the S150 fraction was incubated with antibiotics under conditions suitable for acetylation of kanamycin (Table 4). Furthermore, there was a correlation between *in vitro* resistance pattern to aminoglycoside antibiotics (except streptomycin) and the substrate range of the inactivating enzyme. These results strongly suggest that the inactivating enzyme is important in the *in vivo* resistance of *S. kanamyceticus* ISP5500 to certain aminoglycoside antibiotics. This is supported by the fact that strain ISP5500 is sensitive to the gentamicin C complex which is not susceptible to kanamycin 6′-*N*-acetyltransferase.

In contrast, the mechanism of resistance of *S. kanamyceticus* ISP5500 to streptomycin appears different from that seen for the other aminoglycoside antibiotics. Polyphenylalanine synthesis *in vitro* was not significantly inhibited by streptomycin (Table 3), and streptomycin was not inactivated by the S150 fraction of strain ISP5500. This suggests that *S. kanamyceticus* ISP5500 has a specific ribosomal resistance to streptomycin.

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