SELF-RESISTANCE OF A *STREPTOMYCES* WHICH PRODUCES ISTAMYCINS

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Streptomyces tenjimariensis SS-939, a producer of istamycins, is highly resistant to its own antibiotics and grows in Tryptic Soy Broth containing istamycin A or B at 3,000 μg/ml.

No istamycin-inactivating enzyme was detected in extracts of strain SS-939. Polyphenylalanine synthesis in an *in vitro* system, consisting of the S-150 fraction and ribosomes prepared from strain SS-939, was not inhibited by 200 μg/ml of istamycins. Using reciprocally reconstituted systems consisting of S-150 fractions and ribosomes from strain SS-939 and those from *Streptomyces griseus* ISP5236 (istamycin-sensitive strain), ribosomes of strain SS-939 were found to be resistant to istamycins. Thus, ribosomes have the main role in the self-resistance mechanism of *S. tenjimariensis* SS-939.

In general, aminoglycoside antibiotic-producing organisms are resistant to their own antibiotics[1,2]. In order to clarify their self-resistance mechanisms, it is necessary to investigate the sensitivity of their ribosomes to their own antibiotics *in vitro*. However, such studies have been done in only a few cases, due to technical difficulties in establishing *in vitro* protein synthesizing systems with streptomycetes[3~5].

Streptomyces tenjimariensis SS-939, producing the aminoglycoside antibiotics called istamycins, was isolated from coastal sea mud using a kanamycin selection technique; this strain was found to be highly resistant to its own antibiotics[6~9]. Since we have succeeded in establishing an *in vitro* protein synthesizing system from this strain, we report in this paper on the mechanism of resistance of strain SS-939 to its own antibiotics.

Materials and Methods

Strains

Streptomyces tenjimariensis SS-939 which produces istamycin, *Streptomyces griseus* ISP5236 which is sensitive to istamycins, and *Escherichia coli* Q13 were used.

Chemicals

Polyuridylic acid, pyruvate kinase and phenylmethylsulfonylfluoride (PMSF) were purchased from Sigma Chemical Company, tRNA (*E. coli* MRE600) from Boehringer Mannheim GmbH, EDTA-2K-Mg from Nakarai Chemicals Ltd., and ^{14}C-phenylalanine (521 mCi/mM) from Radiochemical Centre, Amersham.

Preparation of S-30 Fraction of *E. coli* Q13 and Poly(U)-directed Polyphenylalanine Synthesis *In Vitro*

E. coli Q13 cells grown in a nutrient broth at 37°C were collected at the middle of the logarithmic growth phase and washed with buffer A [10 mM tris-HCl, pH 7.8, 60 mM NH_4Cl, 10 mM magnesium acetate, 1 mM dithiothreitol (DTT)]. Packed cells were disrupted by grinding with alumina followed by treatment with deoxyribonuclease I and extracted with the same buffer. The extract was centrifuged at 30,000×*g* for 30 minutes and the supernatant (S-30) was stored at −80°C before use. Polyuridylic acid (poly(U))-directed polyphenylalanine synthesis was assayed as follows. Each 300 μl of reaction

mixture contained 50 mM tris-HCl pH 7.8, 60 mM NH_4Cl, 15 mM magnesium acetate, 1 mM ATP, 5 mM phosphoenolpyruvate (pH 7.0), 25 μM GTP, 9 μg pyruvate kinase, 20 μM L-(U-^{14}C)-phenylalanine (25 mCi/mM), 90 μg *E. coli* tRNA, 12 μg poly(U) and 48 μl iS-30 (S-30 preincubated for 7 minutes at 35°C, 1.8 A_{260} units). The reaction mixture was incubated at 35°C. At given intervals, 50 μl was removed and transferred into tubes containing 0.5 ml of 10% (w/v) trichloroacetic acid (TCA). The suspension was heated at 90°C for 15 minutes and filtered through Whatman GF/C glass fibre discs. Radioactivity on dried filters, after washing with 5 ml of 5% (w/v) TCA, was counted in a liquid scintillation counter.

Preparation of S-150 Fractions and Ribosomes of *S. griseus* ISP5236 and *S. tenjimariensis* SS-939

For the preparation of the S-150 fraction and ribosomes of *S. griseus* ISP5236, the procedure described by SUGIYAMA *et al.*[5] was modified as follows. *S. griseus* ISP5236 was grown in Tryptic Soy Broth (TSB, Difco) at 27°C for 24 hours and collected by centrifugation. Cells were washed twice with buffer B [10 mM tris-HCl pH 7.7, 10 mM magnesium acetate, 1 M KCl, 5 mM EDTA-2K-Mg, 1 mM DTT] and twice with buffer C [10 mM tris-HCl pH 7.5, 10 mM magnesium acetate, 30 mM NH_4Cl, 5 mM EDTA-2K-Mg, 3.45 mM phenylmethylsulfonylfluoride (PMSF), 1 mM DTT]. Packed cells (5 g, wet weight) were disrupted by grinding with 11 g of alumina followed by treatment with deoxyribonuclease I. Thereafter, buffer C was added and the crude homogenate was centrifuged at low speed to remove alumina and unbroken cells. The extract was centrifuged at 30,000×*g* for 20 minutes. The supernatant (S-30) was centrifuged at 150,000×*g* for 2 hours and the postribosomal fraction was dialyzed against buffer A at 4°C overnight. The dialyzed fraction (S-150) was stored at −80°C. The crude ribosomes were washed twice with buffer D [100 mM tris-HCl pH 7.8, 10 mM magnesium acetate, 1 M NH_4Cl, 1 mM DTT], and suspended in 2 ml of buffer A and stored at −80°C. The S-150 fraction of *S. tenjimariensis* SS-939 was found to be unstable. EDTA-2K-Mg and PMSF showed no protecting effect against the loss of activity. Therefore, the S-150 fraction of strain SS-939 was prepared as follows. Cells were washed twice with buffer A (instead of buffers B and C), and packed cells (10 g, wet weight) were disrupted by grinding with 22 g of alumina. After removing alumina and unbroken cells, the extract was immediately centrifuged at 150,000×*g* for 2 hours. The supernatant (S-150) was stored at −80°C. Ribosomes of the strain were prepared in the same way as above.

In Vitro Poly(U)-directed Polyphenylalanine Synthesis by S-150 Fractions and Ribosomes of *S. griseus* ISP5236 and *S. tenjimariensis* SS-939

Poly(U)-directed polyphenylalanine synthesis was assayed in a 300 μl reaction mixture containing 50 mM tris-HCl pH 7.8, 60 mM NH_4Cl, 7.5 mM magnesium acetate, 1 mM ATP, 5 mM phosphoenolpyruvate (pH 7.0), 25 μM GTP, 9 μg pyruvate kinase, 20 μM L-(U-^{14}C)-phenylalanine (25 mCi/mM), 180 μg *E. coli* tRNA, 84 μg poly(U), 0.4 mM spermidine, 84 μl S-150 fraction and ribosomes (3.8 A_{260} units). The reaction mixture was incubated at 35°C. The radioactivity of the TCA insoluble fraction in a 50 μl sample was counted in a liquid scintillation counter.

Assay of Istamycin-inactivating Enzyme of Istamycins

S. tenjimariensis SS-939 was grown in TSB medium. Cells were collected at the middle of the logarithmic growth phase and washed with buffer E [10 mM tris-HCl pH 7.6, 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTT]. Packed cells were disrupted by grinding with alumina and extracted with the same buffer. After removing alumina and unbroken cells, the extract was centrifuged at 150,000×*g* for 2 hours. The supernatant fraction was applied to a Sephadex G-200 superfine column (1.6×80 cm) equilibrated with buffer E. The column was eluted with the same buffer. Each fraction of 3 ml was assayed for istamycin-inactivating enzyme activity.

The reaction mixture contained 80 mM tris-HCl (pH 7.8), 10 mM magnesium acetate, 10 mM DTT, 25 μg/ml istamycin A or B, 4 mM ATP or 0.2 mM *S*-acetyl coenzyme A and a portion of each fraction. It was incubated at 37°C for 3 hours, and the residual antibacterial activity was measured by a paper disc method using *Bacillus subtilis* PCI219 as the test organism.

Results

Resistance to Istamycins A and B

As reported in a previous paper[7], the growth of *S. tenjimariensis* SS-939 was not inhibited by 400 μg/ml of istamycins A and B when incubated on Inorganic Salt Starch (ISP No. 4) agar medium[5]. In TSB medium, the organism grew in the presence of up to 3,000 μg/ml of istamycins A and B, but not at 4,000 μg/ml, during incubation for 3 days. As shown in Table 1, the growth of the organism in TSB medium was somewhat suppressed by 1,500 and 3,000 μg/ml of istamycin B. However, the growth later increased to the control level. The growth of *S. griseus* ISP5236, on the other hand, was inhibited by 2 μg/ml of istamycin B.

Effect of Istamycins A and B on Protein Synthesis of *E. coli*

Istamycins A and B inhibited protein synthesis in *E. coli*. The incorporation of ^{14}C-leucine into the acid insoluble fraction was inhibited by the addition of the antibiotics to the culture broths of *E. coli* Q13 (data not shown).

In order to clarify the action mechanism of istamycins A and B, the effect of the antibiotics on a poly(U)-directed protein synthesizing system prepared from *E. coli* Q13 was examined. As shown in Fig. 1, istamycins markedly inhibited the poly(U)-directed polyphenylalanine synthesis by iS-30 fraction of *E. coli* Q13. The activity of istamycin B was stronger than that of istamycin A. These activities correlate with their antibacterial activities[6].

Effect of Istamycins A and B on Polyphenylalanine Synthesis *In Vitro* by S-150 Fractions and Ribosomes of *S. tenjimariensis* SS-939 and *S. griseus* ISP5236

Poly(U)-directed polyphenylalanine synthesizing systems *in vitro* were prepared from *S. tenjimariensis* SS-939 and *S. griseus* ISP5236 and the effect of istamycins A and B was tested. Polyphenylalanine synthesis using S-150 fraction and ribosomes of *S. tenjimariensis* SS-939 was not affected by istamycins A and B. However, syn-

Table 1. Resistance of *S. tenjimariensis* SS-939 to istamycin B.

Istamycin B (μg/ml)	O.D. 660 (Klett units)
0	575
400	575
800	560
1,500	400
3,000	330
4,000	0

Spore suspension (0.1 ml) of *S. tenjimariensis* SS-939 was inoculated into 20 ml of TSB medium containing various concentrations of istamycin B and incubated at 27°C for 3 days. Growth of the organism was measured by Klett-Summerson photoelectric colorimeter.

Fig. 1. Poly(U)-directed polyphenylalanine synthesis by iS-30 fraction from *E. coli* Q13.
Experimental conditions are described in Materials and Methods.

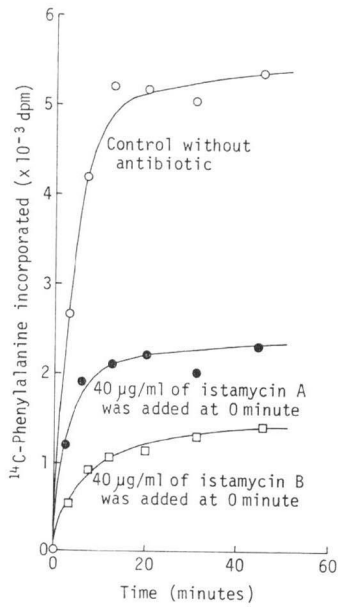


Table 2. Effect of istamycins A and B on *in vitro* polyphenylalanine synthesis by S-150 fractions and ribosomes from *S. tenjimariensis* SS-939 and *S. griseus* ISP5236.

Antibiotic μg/ml		*In vitro* polyphenylalanine synthesis*1, dpm (%)	
		S. tenjimariensis SS-939	*S. griseus* ISP5236
	0	2,503(100)	14,954(100)
Istamycin A	100	2,744(110)	4,075(27.2)
	200	3,192(128)	4,275(28.2)
Istamycin B	100	2,253(90.0)	2,556(17.1)
	200	2,453(98.0)	2,225(14.9)
-poly (U)*2		241	208

*1 A reaction mixture (100 μl) was incubated at 35°C for 45 minutes and radioactivity of TCA insoluble fraction was counted.

*2 A reaction mixture without poly (U) was incubated.

Fig. 2. Poly(U)-directed polyphenylalanine synthesis by reconstituted S-150 fractions and ribosomes from *S. tenjimariensis* SS-939 and *S. griseus* ISP5236.

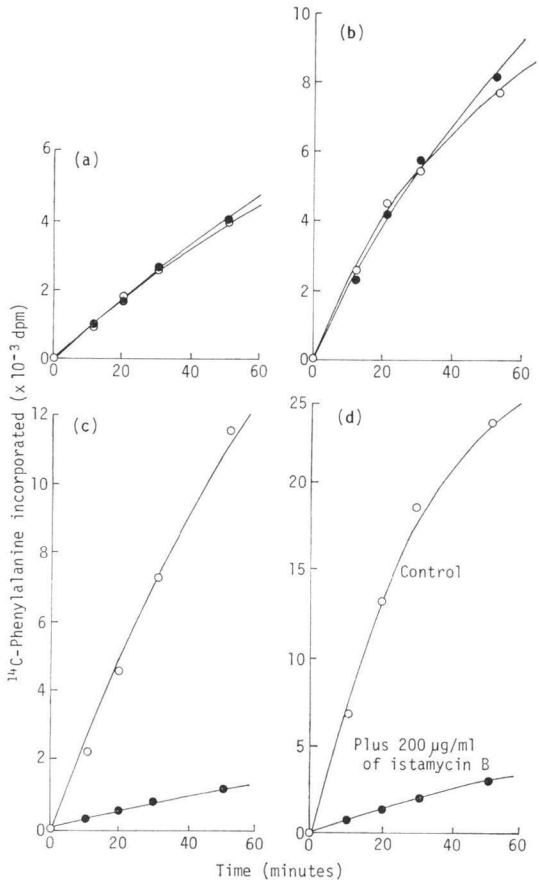
Polyphenylalanine synthesis was assayed as described in Materials and Methods.

(a) *S. tenjimariensis* SS-939 S-150 and *S. tenjimariensis* SS-939 ribosomes;

(b) *S. griseus* ISP5236 S-150 and *S. tenjimariensis* SS-939 ribosomes;

(c) *S. tenjimariensis* SS-939 S-150 and *S. griseus* ISP5236 ribosomes;

(d) *S. griseus* ISP5236 S-150 and *S. griseus* ISP-5236 ribosomes.



thesis in *S. griseus* ISP5236 system was inhibited by istamycins (Table 2).

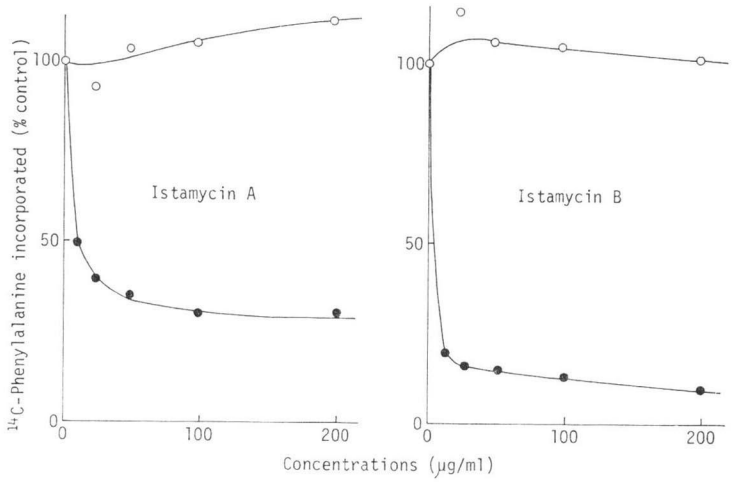
S-150 fractions and ribosomes of *S. tenjimariensis* SS-939 and those of *S. griseus* ISP5236 were combined reciprocally and the sensitivity of the polyphenylalanine synthesis to istamycin B was tested (Fig. 2). Resistance to istamycin B was observed only when ribosomes from *S. tenjimariensis* SS-939 were used for polyphenylalanine synthesis (Fig. 2 a, b). The S-150 fraction of the organism did not affect resistance to istamycin B (Fig. 2 c). Thus, the resistance of *S. tenjimariensis* SS-939 to istamycin B *in vitro* was exclusively shown to be due to its ribosomes.

S. tenjimariensis SS-939 did not produce enzymes inactivating istamycins, *i.e.*, its S-150 fraction showed no effect on the activity of istamycins to inhibit polyphenylalanine synthesis in the system containing ribosomes of *S. griseus* ISP5236. In order to confirm the absence of istamycin-inactivating enzymes, the supernatant of *S. tenjimariensis* SS-939 cells was fractionated by gel filtration on Sephadex G-200 superfine column and the inactivation of istamycins A and B by each fraction was examined. Also in this case, no enzymic inactivation of istamycins was found.

Effect of Concentration of Istamycins A and B on Ribosomes in Polyphenylalanine Synthesis

Ribosomes of *S. tenjimariensis* SS-939 were combined with S-150 fraction of *S. griseus* ISP5236, and the effect of various concentrations of istamycins A and B on polyphenylalanine synthesis was

Fig. 3. Sensitivity of ribosomes of *S. tenjimariensis* SS-939 and *S. griseus* ISP5236 to istamycins A and B.
Poly (U)-directed polyphenylalanine synthesis was assayed as described in Materials and Methods. S-150 fraction from *S. griseus* ISP5236 and ribosomes from either *S. tenjimariensis* SS-939 (○) or *S. griseus* ISP5236 (●) were combined, and control incorporation of ^{14}C-phenylalanine after 45 minutes incubation was 9,260 dpm and 14,345 dpm, respectively.



examined (Fig. 3). The ratio of polyphenylalanine synthesis in the presence of istamycins to that in their absence was 1.12 and 1.0 at 200 μg/ml of istamycins A and B, respectively. On the other hand, polyphenylalanine synthesis by ribosomes from *S. griseus* ISP5236 was drastically decreased by 12.5 μg/ml of istamycins. Maximum inhibition was observed already at about 25 μg/ml of istamycins A and B.

Discussion

It is well known that antibiotic-producing organisms are resistant to their own antibiotics[1,2], but the mechanism of resistance have been studied in detail in only a few cases. Several studies have been done on the mechanisms of self-resistance in streptomycetes producing antibiotics which inhibit protein synthesis. From a biochemical view point, three kinds of mechanism can be considered. The first and second are due to enzymic modification or degradation of the drug, and/or membrane impermeability. These mechanisms have been reported in producers of streptomycin[3,10,11], chloramphenicol[12,13], viomycin and capreomycin[14]. The third mechanism is resistant ribosomes. Ribosomes of *S. erythreus*[15,16], the producer of erythromycin, and *S. azureus*[17], the producer of thiostrepton, have been reported to be resistant to their own antibiotics.

As is well known, almost all aminoglycoside antibiotic-producing streptomycetes so far examined have enzymes inactivating their own antibiotics[18,19]. If the protein-synthesizing systems of these strains are prepared and examined for their sensitivity to their own antibiotics, the inactivating enzyme in the soluble portion of the cell might disturb the action of the drugs on the ribosomes. Therefore, it is necessary to reconstitute protein-synthesizing systems *in vitro* by using S-150 fractions and ribosomes prepared from a producing (resistant) organism and a sensitive organism.

We prepared such *in vitro* protein-synthesizing systems by reciprocal reconstitution of the S-150 fractions and ribosomes prepared from the istamycin producer and *S. griseus* ISP5236. The latter is susceptible to istamycins. Our results (Fig. 2) indicate that the ribosomes of the producer of istamycins are resistant to istamycins and that ribosomal resistance may play the main role in *in vivo* resistance.

This is the first observation of ribosomal resistance among aminoglycoside-producing streptomycetes.

In contrast to the above results, we shall report in the following paper that ribosomes of *S. kanamyceticus* producing kanamycins, *S. fradiae* producing neomycins and *S. griseus* producing streptomycins are all sensitive to their own antibiotics[20].

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References

1) OKAMI, Y.; T. HASHIMOTO & M. SUZUKI: Sensitivity of actinomycetes to antibiotics as a guide to identification. J. Antibiotics, Ser. A 13: 223~227, 1960
2) DEMAIN, A. L.: How do antibiotic-producing microorganisms avoid suicide? Annals New York Acad. Sci. 235: 601~612, 1974
3) CELLA, R. & L. C. VINING: Resistance to streptomycin in a producing strain of *Streptomyces griseus*. Can. J. Microbiol. 21: 463~472, 1975
4) VALU, G. & G. SZABÓ: The effect of endogenous proteolytic activity on the *in vitro* ^{14}C-phenylalanine incorporation in *Streptomyces griseus*. Zentral. Bakteriol., Parasitenkd., Infektionskr. Hyg., Abt. 1, Suppl. 1976 6: 409~413, 1978
5) SUGIYAMA, M.; H. KOBAYASHI, O. NIMI & R. NOMI: Susceptibility of protein synthesis to streptomycin in streptomycin-producing *Streptomyces griseus*. FEBS Lett. 110: 250~252, 1980
6) OKAMI, Y.; K. HOTTA, M. YOSHIDA, D. IKEDA, S. KONDO & H. UMEZAWA: New aminoglycoside antibiotics, istamycins A and B. J. Antibiotics 32: 964~966, 1979
7) HOTTA, K.; N. SAITO & Y. OKAMI: Studies on new aminoglycoside antibiotics, istamycins, from an actinomycete isolated from a marine environment. I. The use of plasmid profiles in screening antibiotic-producing streptomycetes. J. Antibiotics 33: 1502~1509, 1980
8) HOTTA, K.; Y. OKAMI & H. UMEZAWA: Studies on new aminoglycoside antibiotics, istamycins, from an actinomycete isolated from a marine environment. II. Possible involvement of plasmid in istamycin production. J. Antibiotics 33: 1510~1514, 1980
9) HOTTA, K.; M. YOSHIDA, M. HAMADA & Y. OKAMI: Studies on new aminoglycoside antibiotics, istamycins, from an actinomycete isolated from a marine environment. III. Nutritional effects on istamycin production and additional chemical and biological properties of istamycins. J. Antibiotics 33: 1515~1520, 1980
10) CELLA, R. & C. VINING: Action of streptomycin on the growth of *Streptomyces griseus*. Can. J. Microbiol. 21: 463~472, 1975
11) PIWOWARSKI, J. M. & P. D. SHAW: Streptomycin resistance in a streptomycin-producing microorganism. Antimicr. Agents & Chemoth. 16: 176~182, 1979
12) MALIK, V. S. & L. C. VINING: Metabolism of chloramphenicol by the producing organism. Some properties of chloramphenicol hydrolase. Can. J. Microbiol. 17: 1287~1290, 1971
13) MALIK, V. S. & L. C. VINING: Chloramphenicol resistance in a chloramphenicol-producing *Streptomyces*. Can. J. Microbiol. 18: 583~590, 1972
14) SKINNER, R. H. & E. CUNDLIFFE: Resistance to the antibiotics viomycin and capreomycin in the *Streptomyces* species which produce them. J. Gen. Microbiol. 120: 95~104, 1980
15) TERAOKA, H. & K. TANAKA: Properties of ribosomes from *Streptomyces erythreus* and *Streptomyces griseus*. J. Bacteriol. 120: 316~321, 1974
16) GRAHAM, M. Y. & B. WEISBLUM: 23S Ribosomal ribonucleic acid of macrolide-producing streptomycetes contains methylated adenine. J. Bacteriol. 137: 1464~1467, 1979
17) CUNDLIFFE, E. & J. THOMPSON: Ribose methylation and resistance to thiostrepton. Nature 278: 859~861, 1979
18) BENVENISTE, R. & J. DAVIES: Aminoglycoside-antibiotic inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc. Natl. Acad. Sci. U.S.A. 70: 2276~2280, 1973
19) DAVIES, J.; C. HOUK, M. YAGISAWA & T. J. WHITE: Occurrence and function of aminoglycoside-modifying enzymes. *In* Genetics of Industrial Microorganisms (*eds*, SEBEK, O. K. & A. I. LASKIN), pp. 166~169, American Society for Microbiology, Washington, D.C., 1979
20) HOTTA, K.; H. YAMAMOTO, Y. OKAMI & H. UMEZAWA: Resistance mechanisms of kanamycin-, neomycin- and streptomycin-producing streptomycetes to aminoglycoside antibiotics. J. Antibiotics, submitted