Resistance of Kanamycin-and Neomycin-Producing Streptomycetes to Aminoglycoside Antibiotics

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Streptomyces fradiae NRRL B1195 and *Streptomyces kanamyceticus* IFO 13414 are highly resistant to the antibiotics they produce. The ribosomes of these organisms are found to be susceptible to the antibiotics, but the cell free extract of *S. fradiae* is found to contain a phosphotransferase and an acetyltransferase which inactivate kanamycin and neomycin, and that of *S. kanamyceticus* an acetyltransferse which inactivates kanamycin and neomycin. The resistance of these organisms against streptomycin is found to be due to the resistant ribosomes; actually streptomycin activates their ribosomal systems for the synthesis of polyphenylalanine.

Introduction

Many aminoglycoside antibiotics are produced by actinomycetes and considerable attention has been paid to the resistant mechanisms of the aminoglycoside antibiotic producing orgainsms. Many organisms have been proved to possess inactivating enzymes for the aminoglycoside anibiotics, which phosphorylate or acetylate the antibiotics at the specific sites of the molecules.[1,2] Recently, some of the antibiotic producing organisms have been found to have resistant ribosomes against the antibiotics they produce. The ribosomes of the streptomycete strains producing istamycin,[3] nebramycins[4] and kanamycin[5,6] have been found not to be susceptible to the antibiotics they produce. Also, *Micromonospora purpurea* is reported to possess ribosomes resistant to gentamicin. During the study of resistant mechanisms, we have noticed some characteristic multiple resistances among aminoglycoside antibiotic producing organisms and want to report some of the characteristic patterns of resistance against aminoglycoside antibiotics in *Streptomyces kanamyceticus* and *Streptomyces fradiae*.

Experimental

Organisms. *S. fradiae* NRRL B1195 was donated by A. J. Lyons at Northern Regional Research Laboratory in Peoria, ILL, U. S. A. and *S. kanamyceticus* IFO 13414 was purchased from the Institute for Fermentation in Osaka.

Antibiotics. Kanamycin sulfate and neomycin sulfate were obtained from Jeil Pharmaceutical Co., tobramycin and gentamicin sulfate from Dong Wha Pharmaceutical Co. and amikacin was from Dong-A Pharmaceutical Co.. Streptomycin sulfate was purchased from Fluka Co..

Determination of antibiotic resistance. The level of anitibiotic resistance was determined by streaking aerial mycelia on ISP No. 4 agar (Difco) plates containing two fold diluted aminoglycoside antibiotics. The plates were incubated at 28℃ for 5 days and positive or negative growth of each microorganism was determined.

***In vitro* polyphenylalanine synthesis.** Poyl-U directed polyphenylalanine synthesis was carried out according to the method previously described.[7]

Preparation of the crude enzyme solution. The mycelia of *S. kanamyceticus* grown in tryptic soy broth (TSB, Sigma) were collected at the mid-logarithmic growth phase and washed with buffer A (10 mM Tris-HCl, pH 7.8, 60 mM NH_4Cl, 10 mM magnesium acetate, and 6 mM 2-mercaptoethanol). The packed mycelia were then disrupted by grinding with alumina and extracted with buffer A. After removal of the alumina and the cell debris by centrifugation, the supernatant was centrifuged at 150,000×g for 2 hours. The 150,000 ×g supernatnat was used as the S150 fraction.

Enzyme assay. A similar method as those described by Benvenisite *et al.*,[8] Davies *et al.*,[9] and Haas and Davies[10] was empolyed. Phosphorylation of an antibiotic by the crude enzyme solution (S150 fraction) was carried out in a reaction mixture (100 μl) containing 80 mM Tris-HCl (pH 7.0), 2 mM magnesium acetate, 2 mM dithiothreitol, 4 mM γ-^{32}P-ATP (3.36 mCi/mmol), 20 μl S150 and 100 μg/ml antibiotic. Acetylation was examined in a reaction mixture comprising 760 nmoles UL-^{14}C-sodium acetate (7.9 mCi/mmol) and 9.8 nmoles coenzyme A. After incubation at 30℃ for 60 min, 20 μl of the reaction mixture was dropped onto a piece of phosphocellulose paper (Whatman P81, 1.5×1.5 cm).[11] The paper was then immersed into water at 85℃ for 2 min and washed three times with a large volume of deionized water. The radioactivity retained on the paper was counted in a liquid scintillation counter. The background value obtained from a control reaction mixture which did not contain the antibiotic was substracted.

Results

The resistance of antibiotic producers against aminoglycoside antibiotics. A neomycin producer, *S. fradiae* was highly resistant against aminoglycoside antibiotics and thus, showed growth on the agar plates containing a very high concentration of neomycin (500 μg/ml) or streptomycin (250 μg/ml) but was pretty susceptible to kanamycin to show inhibition of the growth at the concentration higher than 62.4 μg per ml in the agar plate (Table 1). A kanamycin producer, *S. kanamyceticus* grew at high concentrations of kanamycin (1000 μg/ml) and streptomycin (125 μg/ml).

Analysis of aminoglycoside antibiotic inactivating

Table 1. The concentration (μg/ml) of aminoglycoside antibiotics* allowing the growth of aminoglycoside antibiotic producing streptomycetes

	SM* μg/ml	NM* μg/ml	KM* μg/ml	GM* μg/ml	TM* μg/ml
S. fradiae NRRL B1195	250	500	31.2	7.8	7.8
S. kanamyceticus IFO 13414	125	3.8	1000	0	7.8

*SM: streptomycin; NM: neomycin; KM: kanamycin; GM: gentamicin; TM: tobramycin.

Table 2. Radioactivities (dpm) of phosporlyated aminoglycoside antibiotics* by the cell free extracts of streptomycetes when they are incubated with γ-^{32}P-ATP

	SM* dpm	NM* dpm	KM* dpm	GM* dpm	TM* dpm
S. fradiae NRRL B1195	26	43767	3356	222	726
S. kanamyceticus IFO 13414	194	33015	737	99	231

*SM: streptomycin; NM: neomycin; KM: kanamycin; GM: gentamicin; TM: tobramycin.

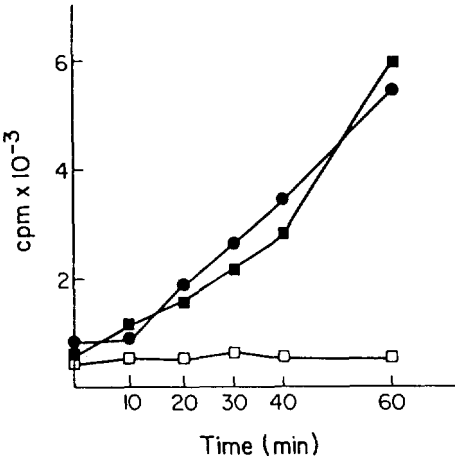

Figure 1. Incorporation of ^{14}C-acetate to neomycin (●) and kanamycin (■) by the cell free extract of *S. fradiae* NRRL B1195 relative to the control (□).

enzyme. When the cell free extract of *S. fradiae* or *S. kanamyceticus* was incubated with several aminoglycoside antibiotics in the presence of γ-^{32}P-ATP and in the presence of ^{14}C-acetate, CoA and ATP, strong activities for the phosphorylation and the acetylation of neomycin and kanamycin were observed (Table 2). The phosporylation had been shown to be specific to the antibiotic (Table 2) but the acetylation activity of the cell-free extracts did not show specificity to the antibiotics. As shown in Figures 2 and 3, both neomycin and kanamycin were acetylated by the cell-free extracts of both organisms. Aminoglycoside acetyltransferase and phosporyltransferase were found to play an important role in the resistance of *S. kanamyceticus* against kanamycin and

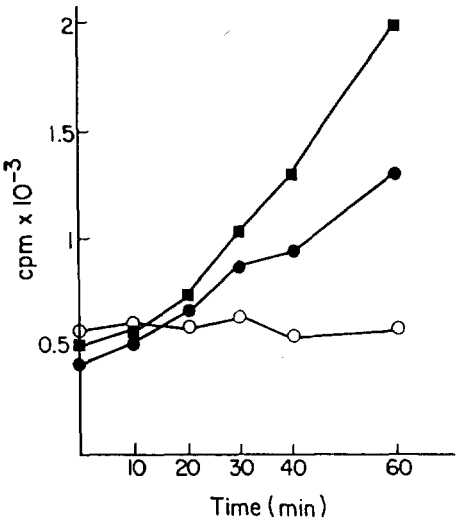

Figure 2. Incoporation of ^{14}C-acetate to kanamycin (■) and neomycin (●) by the cell free extract of *S. kanamyceticus* IFO 13414 relative to the control (○).

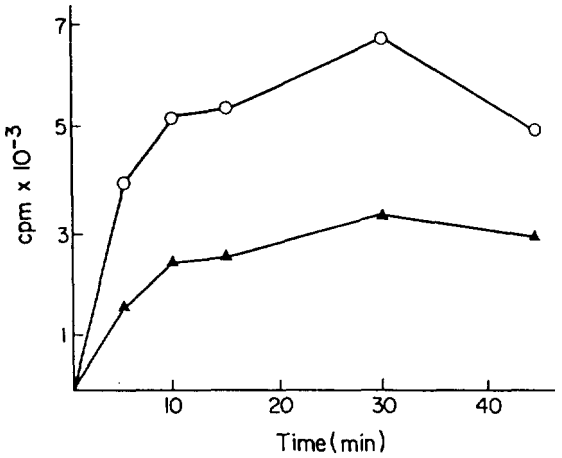

Figure 3. Effect of neomycin on polyphenylalanine synthesis by the cell free ribosomal system prepared from mid-exponential growth phase cells of *S. fradiae* NRRL B1195: the reaction was carried out with the reaction mixture without the addition of neomycin (○) or with addition of 100 μg of neomycin per ml ( ). The ribosomal fraction and the S150 fraction contained 80 μg and 260 μg of protein, respectively.

S. fradiae against neomycin.

Ribosomal resistance to aminoglycoside antibiotics. Examination of the resistance of the ribosomal systems of *S. fradiae* and *S. kanamyceticus* against aminoglycoside anitibiotics they produce indicated that they were clearly sensitive to the antibiotics they produce. Approximately 50% of the polyphenylalanine synthesis of the ribosomal fraction in *S. fradiae* was inhibited by 100 μg of neomycin per ml (Figure 3) and 80% in *S. kanamyceticus* by 100 μg of kanamycin per ml (Figure 4). The sensitivity of the ribosomes of *S. fradiae* and *S. kanamyceticus* to other aminoglycoside antibiotics was studied. Both orgaisms were found to be resistant to streptomycin; streptomycin was actually found to activate their poly-U dependent polyphenylalanine

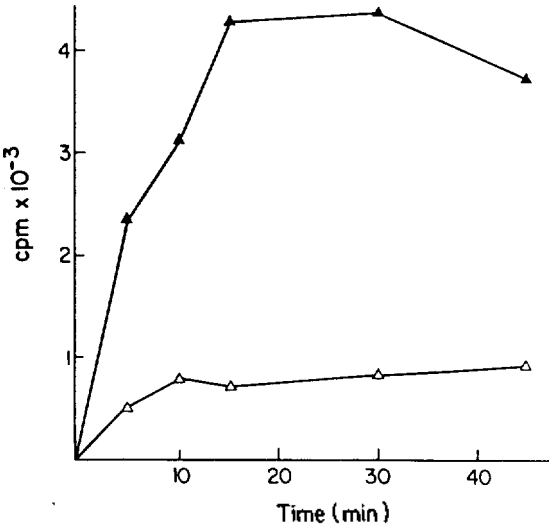

Figure 4. The effect of kanamycin on polyphenylalanine synthesis by the cell free ribosomal system prepared from mid-exponential growth phase cells of *S. kanamyceticus* IFO 13414: the reaction was carried out with the reaction mixture without the addition of kanamycin (▲), or with addition of 100 μg kanamycin per m*l* of the reaction mixture (△). The ribosomal fraction and the S150 fraction contained 80 μg and 260 μg of protein, respectively.

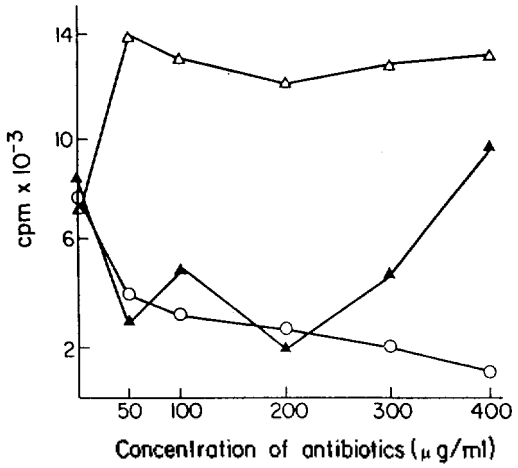

Figure 5. Effects of kanamycin (▲), neomycin (○), and streptomycin (△) on the polyphenylalanine synthesis of *S. fradiae* NRRL B1195.

synthesis at low concentrations and its activating activity was sustained even at very high concentrations. Kanamycin seemed to block much of the ribosomal activity to inhibit the protein synthesis at low concentrations. But the inhibition activity was reversed pretty much at high concentrations (Figure 5 and 6).

Discussion

A strain of kanamycin producing *S. kanamyceticus* and a strain of neomycin producing *S. fradiae* were highly resistant to the antibiotics they produce. From the present study, it was revealed that the ribosomes of the two strains were

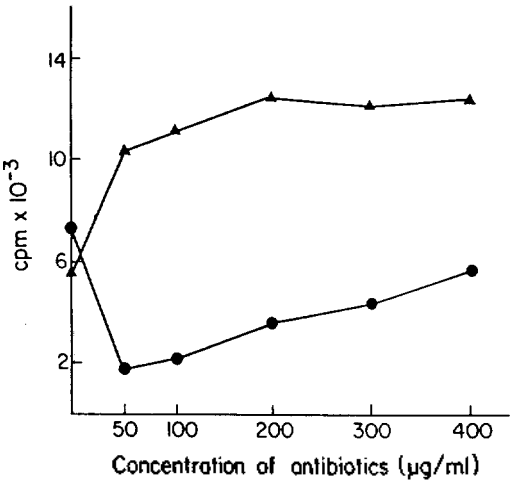

Figure 6. Effects of kanamycin (○) and streptomycin (▲) on the polyphenylalanine synthesis of *S. kanamyceticus* IFO 13414.

sensitive to the anibiotics they were producing and their apparent resistance seemed to be due to the presence of the inactivating enzymes, phosphotransferase and acetyltransferase. These results indicated that the antibioitic inactivating enzymes played an important role in their resistance mechanisms. Recently, the resistance of *S. kanamyceticus* against kanamycin was reported to be due to the resistant ribosomes,[5,6] but we could not confirm the ribosomal resistance to the kanamycin in two kanamycin producing strains (IFO 13414 and NRRL 2535). Sugiyama *et al.* also reported that the synthesis of polyphenylalanine by the ribosomes obtained from a streptomycin producing strain of *Streptomyces griseus*[12] or a neomycin producing strain of *S. fradiae*[13] were markedly inhibited by a streptomycin and neomycin, respectively. *S. fradiae* and *S. kanamyceticus* were resistant to several other aminoglycoside antibiotics. In *S. fradiae*, aminoglycoside acetyltransferase should be responsible for the resistance to kanamycin. The resistance of *S. kanamyceticus* and *S. fradiae* against streptomycin was found to be due to the resistant ribosomes. Polyphenylalanine synthesis by the ribosomal fraction was significantly activated by streptomyin. Streptomycin was not inactivated by any of the aminoglycoside antibiotics modifying enzymes in the S150 fractions of the two strains.

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References

1. Davies, J.; Smith, D. I. *Ann. Rev. Microbiol.* **1978**, *32*, 469.
2. Phillips, I.; Shannon, K. *Brit. Medical. Bull.* **1984**, *40*, 28.
3. Yamamoto, H.; Hotta, K.; Okami, Y.; Umezawa, H. *Biochem. Biophys. Res. Commun.* **1981**, *100*, 1396.
4. Yamamoto, H.; Hotta, K.; Okami, Y.; Umezawa, H. *J. Antibiot.* **1982**, *35*, 1020.
5. Murakami, T.; Nojiar, C.; Toyama, H.; Hayashi, E. *J. Antibiot.* **1983**, *36*, 1305.
6. Nakano, M.; Mashiko, H.; Ogawara, H. *J. Antibiot.* **1984**, *157*, 79.
7. Yamamoto, H.; Hotta, K.; Okami, K.; Umezawa, H. *J. An-*

tibiot. **1981**, *34*, 824.
8. Benveniste, R.; Yamada, T.; Davies, J. *Infect. Immuno.* **1978**, *1*, 109.
9. Davies, J.; Brazerzinska, M.; Benveniste, R. *Ann. N. Y. Acad. Sci.* **1971**, *182*, 226.
10. Haas, M. J.; Davies, J. *Antimicrob. Agents Chemother.* **1973**, *4*, 497.
11. Ozanne, B.; Benveniste, R.; Tipper, D.; Davies, F. *J. Bacteriol.* **1969**, *100*, 1144.
12. Sugiyama, M.; Kobayashi, H.; Nimi, O.; Nomi, R. *FEBS Lett.* **1980**, *110*, 250.
13. Sugiyama, M.; Nimi, P.; Nomi, R. *J. Gen. Microbiol.* **1980**, *121*, 477.

Photoreactivity of ReH_5(Cyttp) (Cyttp = $PhP(CH_2CH_2CH_2PCy_2)_2$) with CO, CO_2 and PMe_3

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The photoreactions of ReH_5(Cyttp) (**1**) (Cyttp = $PhP(CH_2CH_2CH_2PCy_2)_2$) with CO, CO_2 and PMe_3 has been investigated to find the differences in reactivities from those of trismonophosphine analog. Irradiation of **1** under CO, CO_2 and excess PMe_3 in benzene results in the formation of the complexes, $ReH(CO)_2$(Cyttp) (**2**), $ReH_2(\eta^2\text{-}HCO_2)$(Cyttp) (**3**) and $ReH_3(PMe_3)$(Cyttp) (**4**), respectively. The resulting products suggest that photoreactions of ReH_5(Cyttp) proceed by photoextrusion of H_2 giving a phototransient species "ReH_3(Cyttp)" which can be trapped by CO, CO_2 and PMe_3. The structures of **2**, **3** and **4** are inferred based on ^1H, ^{31}P NMR and I. R. spectroscopy.

Introduction

The irradiation of metal polyhydrides usually results in photoinduced loss of H_2 from the complex.[1,2] The resulting unsaturated species can react with various substrates[1a,3-5] and may be active as catalysts in such processes as alkene isomerization and hydrogenation.[5,6] Although the extrusion of H_2 from metal polyhydrides is generally the primary photochemical process, irradiation of $[ReH_5L_3]$ (L = PMe_2Ph, $PMePh_2$, PPh_3) results in loss of the phosphine instead of H_2.[7-10] Thus, photolysis of $[ReH_5(PMe_2Ph)_3]$ yields a new polyhydride dimer, $[Re_2H_6(PMe_2Ph)_5]$, by losing coordinated phosphines.[3] In this context, we were interested in comparing the photochemical reactivity of $[ReH_5L_3]$ with that of rhenium pentahydride complex containing chelating triphosphine ligand, ReH_5(Cyttp). Herein we report our results on the photoreactions of **1** with CO, CO_2 and excess PMe_3 and the characterization for the resulting complexes by spectroscopic method.

Experimental

General Procedures. All reactions and sample manipulations were carried out under an argon atmosphere using either Schlenk techniques or a Vacuum Atmospheres HE43 drybox equipped with Mo-40 catalyst system.[11] Reagent grade solvents were distilled over argon from appropriate drying agents immediately before use.[12] Solutions were transferred by the use of an argon flushed syringe, or stainless steel cannulae. Irradiations were conducted by using a 450-W Hanovia medium-pressure Hg lamp through a quartz photolysis vessel (method A) or through a pyrex Schlenk flask with *ca.* 20 cm distance from a light source (method B) under argon or appropriate reagent gas. The irradiation time for each experiment was determined to give an optimum yield of the described product by monitoring the ^{31}P resonances of crude mixtures.

Proton T_1 values were measured by the inversion/recovery method at 250 MHz with a 180°-τ-90° pulse sequence and calculated *via* a program provided by the manufacturer. Deuterated solvents were degassed *via* the freeze-thaw method.

Materials. The preparation of compound **1** has been described previously.[13] The tridentatephosphine ligand Cyttp was prepared by use of slightly modified literature procedures.[14] The other chemicals were purchased from commercial sources.

Instrumentation. Infrared spectra were recorded on a Perkin-Elmer Model 283B grating spectrophotometer as Nujol mulls between KBr plates and were calibrated with polystyrene film. NMR specra were collected on solutions in CD_2Cl_2 or C_6D_6 with a Bruker AM-250. Residual proton resonances in deuterated solvents were used as internal standards for the ^1H-NMR spectra. Phosphorus chemical shifts were referenced to external 85% H_3PO_4. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. in U. S. A.

Photoreaction of 1 with CO. Irradiation (by method A) of deoxygenated benzene solution (150 m*l*) of **1** (0.30 g, 0.39 mmol) was conducted under a slow, continuous CO purge for 12 h. The reaction mixture was then transferred