[J. Ferment. Technol., Vol. 61, No. 4, p. 347-351, 1983]

Ribosomal Resistance to Streptomycin in Strains Regenerated from Protoplasts of a Streptomycin-Susceptible Mutant Derived from a Streptomycin Producer

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When protoplasts of streptomycin-sensitive Streptomyces griseus KSN were regenerated to the mycelial form, streptomycin-resistant cultures appeared at a high frequency (approximately 1%). The resistance mechanism of these regenerated cultures was different from that of ancestral streptomycin-producing Streptomyces griseus HUT 6037, from which the parent S. griseus KSN was derived. While the resistance to streptomycin of S. griseus HUT 6037 was due mainly to streptomycin-phosphorylating activity of streptomycin 6-kinase produced in the cells, the streptomycin-resistant cultures regenerated from protoplasts produced no streptomycin 6-kinase but the ribosomes changed and became resistant to streptomycin. In highly streptomycin-resistant cultures regenerated, one of the 30S ribosomal proteins was altered in electrophoretic mobility as compared to the corresponding protein of S. griseus KSN.

We reported previously that while the ribosomes of a streptomycin (SM) producer were substantially susceptible to its own antibiotic product, the protein-synthesizing system could be protected from the lethal effect of SM as a result of inactivation of the drug by SM 6-kinase.¹⁻⁴⁾ Not only did the phosphate ester of SM not inhibit protein synthesis,4) but also the ribosome-bound SM was liberated by phosphorylation with SM 6-kinase.³⁾ An SM 6-kinase-defective mutant, S. griseus KSN, isolated from SMproducing S. griseus HUT 6037, was in fact susceptible to SM and this character never changed on successive transfer of the culture.

In the present study, it was shown that when protoplasts from S. griseus KSN were regenerated to the mycelial form, SMresistant colonies appeared at the high frequency of approximately 1%. Although these colonies did not produce SM 6-kinase like the parent KSN, their ribosomes changed and became resistant to SM. These strains seem to be the first ribosomal mutants obtained for the Streptomycetes.

Materials and Methods

Microorganisms SM-producing Streptomyces griseus HUT 6037 and SM-non-producing S. griseus KSN derived from HUT 6037 were used. The latter strain did not produce SM 6-kinase and was susceptible to $SM^{2,3}$

Medium FB medium consisted of 1% fructose, 0.2% Polypepton, 0.1% yeast extract, 0.1% meat extract and 2% agar, pH 7.0. P medium for washing protoplasts and R2 medium for regeneration of protoplasts to the mycelial form were prepared according to the methods of Okanishi *et al.*⁵) except that Tris/HCl (pH 7.2) was used in place of TES buffer (pH 7.2).

Protoplast formation and regeneration of protoplasts to mycelium S. griseus KSN was grown in 1% glucose-meat extract-peptone (GMP) medium⁶) supplemented with 0.6% glycine at 28°C. A 50 ml portion of the culture broth at the transient growth phase was centrifuged at $2200 \times g$ for 6 min at 4°C. The harvested mycelium was washed twice with 20 ml of SES solution [0.3 M sucrose, 0.1 M EDTA-Na₂. 2H₂O and 0.15 M NaCl, pH 8.0], suspended in 10 ml of the same solution and then mixed with 5 ml of lysozyme solution [20 mg lysozyme (Sigma no. 6876) per ml of SES solution]. The suspension was incubated for 30 to 60 min at 32°C with gentle shaking. When

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formation of protoplasts could be microscopically confirmed, the suspension was filtered through cotton wool, and then through a $3 \mu m$ Nuclepore filter. A 4 ml portion of the filtrate was centrifuged at $4530 \times g$ for 15 min at 4°C. The resulting pellets were washed twice with 2 ml P medium by centrifugation at $1700 \times g$ for 6 to 10 min at 4°C and then suspended in 1 ml of the same medium. A 0.2 ml portion of this protoplast suspension was spread on R2 medium, followed by incubation for 3 to 5 days at 28°C.

Selection of SM-resistant colonies Colonies which appeared on R2 medium were transferred on FB medium by random selection. After incubation at 28°C, the grown colonies were examined for SM resistance by replica-plating on FB medium supplemented with 50 μ g/ml of SM. The SM-resistant strains obtained were examined for the maximal SM concentration which permitted normal growth, and then for productivities of SM and SM 6-kinase.

Preparation of ribosomes and S-150 fractions, assays of polyphenylalanine synthesis *in vitro* and SM 6-kinase in cell-free extracts, and binding of [³H]dihydrostreptomycin to ribosomes These were all performed by the same methods as described previously.³)

Two-dimensional electrophoretic analysis of ribosomal proteins The ribosomal proteins were extracted according to the method of Hardy *et al.*⁷) Two-dimensional polyacrylamide gel electrophoresis of the ribosomal proteins was performed according to the method of Kaltschmidt and Wittmann.⁸) The gel was stained with 0.2% Coomassie Brilliant Blue in 10% acetic acid-30% ethanol solution, followed by destaining.

Results and Discussion

SM resistance induced by regeneration of protoplasts to mycelium Protoplasts pepared from mycelium of SMsusceptible S. griseus KSN were spread on the R2 medium. From among numerous colonies regenerated, 1325 colonies were randomly selected and examined for the resistance to 50 μ g/ml of SM. Twelve colonies (0.9× 10^{-2}) were found to be resistant. On the other hand, when 3×10^5 spores of S. griseus KSN were spread on FB medium containing 50 μ g/ml of SM, only 2 resistant colonies (6.7×10^{-6}) appeared. These results seemed to indicate that regeneration of protoplasts to the mycelial form gave rise to spontaneous mutation with a high frequency. Ten cultures selected from the above twelve were

Strain	Level of SM resistance (µg/ml)
HUT 6037	200
KSN	< 20
PSR-1	230
PSR-2	350
PSR-3	180
PSR-4	180
PSR-5	300
PSR-6	75
PSR-7	120
PSR-8	100
PSR-9	100
PSR-10	120

named S. griseus PSR-1 to 10, and examined for the maximal SM concentration which permitted normal growth. Also SM and SM 6-kinase productivities of the PSR strains were determined. As shown in Table 1, the SM-tolerances of PSR strains were 350 to 75 μ g/ml, whereas the SM-tolerances of S. griseus KSN and HUT 6037 were less than 20 μ g/ml and 200 μ g/ml, respectively. None of the PSR strains produced SM or SM 6-kinase.

Influence of SM on cell-free protein synthesis of PSR strains The extents of inhibition by SM of polyphenylalanine synthesis in cell-free extracts were examined using four PSR strains (PSR-1, PSR-2, PSR-4 and PSR-5) and compared with those of HUT 6037 and KSN. Polyphenylalanine synthesis in PSR-2, PSR-5 and HUT 6037 was not inhibited by 0.5 μ g/ml of SM, whereas the synthesis in KSN was strongly inhibited by the same concentration of SM (Fig. 1). Strains PSR-1 and PSR-4 showed an intermediate pattern of SM inhibition between those of HUT 6037 and KSN.

Binding of [³H]dihydrostreptomycin to ribosomes of PSR strains To elucidate the mechanism of SM resistance in PSR strains, the affinity of ribosomes for [³H]dihydrostreptomycin ([³H]DSM) was determined. While about 0.7 pmol of [³H]-

Table 1. SM resistance in strains obtained by regeneration of protoplasts from *S. griseus* KSN.





Fig. 1. Inhibition of polyphenylalanine synthesis by SM in *in vitro* protein-synthesizing systems prepared from HUT 6037, KSN and PSR strains. The inhibition (%) with a given concentration of SM was expressed as the ratio of the reduced amount of polyphenylalanine synthesis to that synthesized in the respective SM-free reaction mixture. Ribosomes (60 μ g) and the S-150 fraction (240 μ g as protein) from late exponential phase cells were used. Symbols: KSN (\blacktriangle), HUT 6037 (\bigcirc), PSR-1 (\odot), PSR-2 (\square), PSR-4 (\bigtriangleup) and PSR-5 (\blacksquare).

DSM bound to 1 pmol of ribosomes from the KSN strain, no binding was observed with the ribosomes from PSR-2 and PSR-5 (Fig. 2). Ribosomes from PSR-1 and PSR-4 showed approximately 30 and 70% affinity for



Fig. 2. Binding of [3H]DSM to ribosomes isolated from KSN and PSR strains.

Ribosomes from late exponential phase cells were used. The reaction mixtures were made up of 50 mM Tris-HCl (pH 7.65), 16 mM magnesium acetate, 77.1 pmol ribosomes, 695 pmol [³H]-DSM (1.8 Ci/mmol) and the indicated concentrations of potassium chloride. Since the binding affinity of ribosomes for [³H]DSM is affected by monovalent cations such as K⁺ and NH₄⁺,¹⁰) the binding was examined with various concentrations of potassium chloride. The reaction mixtures were incubated at 28°C for 20 min. The symbols are the same as Fig. 1. [*H]DSM, respectively, as compared with the ribosomes from KSN. These results show that the ribosomes from PSR strains are more resistant to SM than those from KSN.

Ribosomal resistance of PSR strains In order to confirm the ribosomal resistance of PSR strains (PSR-1, PSR-2, PSR-4 and PSR-5), hybrid protein-synthesizing systems were prepared from the ribosomes of PSR strains and the S-150 fraction from KSN, and the inhibition of protein synthesis by SM was determined. Since strain KSN does not produce SM 6-kinase, the degree of inhibition will depend on SM susceptibility of the ribosomes used. The ribosomes from S. griseus HUT 6037 and KSN were used as references. The influence of SM on polyphenylalanine synthesis with the hybrid systems is shown in Fig. 3. The ribosomes of HUT 6037 and KSN are susceptible to SM,¹⁻³⁾ so the polyphenylalanine synthesis was inhibited by SM. However, the ribosomes of PSR-2 and PSR-5 were resistant to SM, because no inhibition of polyphenylalanine synthesis was observed. In the case of PSR-1 and PSR-4, the inhibition was significantly lower than that of HUT 6037. These results seemed to indicate that the regeneration of protoplasts gave rise to mutation in ribosomal resistance to SM.





Ribosomes of HUT 6037 and KSN strains were used as reference. The inhibition (%) by SM was expressed as in Fig. 1. Ribosomes ($60 \mu g$) and the S-150 fraction (240 μg as protein) from late exponential phase cells were used. Ribosomes from KSN, HUT 6037, PSR-1, PSR-2, PSR-4 and PSR-5 strains are indicated by the same symbols as in Fig. 1.

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Fig. 4. Two-dimensional polyacrylamide gel electrophoresis of the 70S ribosomal proteins from KSN and PSR strains.

The arrow indicates the protein shifted to the left as compared to the corresponding protein from S. griseus KSN.

Analysis of ribosomal proteins from PSR strains Possible mutational alteration of ribosomal proteins of PSR-2 and PSR-5 were examined by two-dimensional gel electrophoresis. The results are shown in Fig. 4. One of the 70S ribosomal proteins from *S. griseus* PSR-2 and PSR-5 seemed to be altered in electrophoretic mobility as compared with that of *S. griseus* KSN. To



Fig. 5. Two-dimensional polyacrylamide gel electrophoresis of a mixture of the 70S ribosomal proteins from two strains.
Ribosomes from S. griseus PSR-2 or PSR-5 were mixed with some from S. griseus KSN and subjected to co-electrophoresis. The arrow indicates the protein from S. griseus PSR-2 or PSR-5 which was shifted to the left in electrophoretic mobility as compared with the corresponding protein from S. griseus KSN.

confirm the above alteration, the 70S ribosomal proteins of S. griseus KSN and PSR-2, or S. griseus KSN and PSR-5 were mixed and co-electrophoresed. Figure 5 shows that one of the 70S ribosomal proteins of PSR-2 was clearly shifted from the corresponding protein of KSN. This is also the case for PSR-5. In addition, the same protein was altered in S. griseus PSR-2 and PSR-5. Electrophoretic analysis of the 30S ribosomal proteins from S. griseus HUT 6037 indicated that the altered protein belonged to the 30S subunit (Fig. 6).

For *Escherichia coli*, several SM-resistant mutants have been examined as to alteration of protein components of the 30S ribosomal subunit and S12 the protein was found to be altered.⁹⁾ The above *S. griseus* ribosomal

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Acknowledgement

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(Received January 13, 1983)



Fig. 6. Two-dimensional polyacrylamide gel electrophoresis of the 30S ribosomal proteins from S. griseus HUT 6037.

The protein indicated by the arrow corresponds to that in Figs 4 and 5.

protein may therefore correspond to the S12 protein in E. coli.

The results obtained here seem to be the first case of ribosomal mutants in the Streptomycetes.