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Effects of Natural Selection, Mutagenesis, and Protoplast Formation and Cell Wall Regeneration on the Production of Aminoglycoside Antibiotics

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Abstract□High producers or blocked mutants of aminoglycoside antibiotic-producing *Streptomyces* spp. were selected by application of an agar plug method and by culturing individual colonies in broth. The productivities of aminoglycoside antibiotic producing organisms were increased by selection of a high producer from colonies obtained by spreading spores of wild strain, or survived from treatment of a mutagen or from the colonies regenerated from protoplast-formation and cell-wall regeneration. Some mutagen treated colonies lost the ability to produce antibiotics (5-8%). Some A-factor negative and deostreptamine or streptidine negative mutants were obtained by N-methyl-N'-nitro-N-nitrosomethylguanidine (MNNG) treatment. Many of the survivors from the MNNG treatment lost the ability to produce antibiotics. Major colonies produced less amount of antibiotics; only few survived colonies produced more antibiotics than the parent. Resistance of *Streptomyces* spp. against the antibiotics produced by itself was also markedly affected by mutagen treatment.

Keywords□Selection of high producing strains, *S. griseus, S. fradiae, S. kanamyceticus,* aminoglycoside antibiotics, antibiotic blocked mutants.

We are interested in the development of high producers of aminoglycoside antibiotics. Selection of blocked or analog resistant mutants gave high yielding strains of primary metabolites. However, for the secondary metabolites, such as antibiotics it is usually hard to apply the same methodology for the development of high yielding strains due to the lack of knowledge on their biosynthetic pathways or on their regulation mechanisms.

Increase of the antibiotic productivity has been achieved by mutation. Frequently an auxotroph obtained by treatment of mutagen produces a higher amount of antibiotics. Genetic recombination between complementary auxotrophs was used for the development of a high producer of kasugamycin[1]. Mutants were also employed to study the biosynthetic pathway[2], to produce new antibiotics[3] and to improve the fermentation process[4]. Antibiotic-producing strains of Actinomycetes have individual patterns of antibiotic resistance depending on the type of antibiotic they produce[5-7]. Increased production of aminoglycoside antibiotics has been found to be associated with the resistance against the antibiotic[8], and seems to be related with the cell wall synthesis. We have undertaken to study various effects on the productivity of the aminoglycoside antibiotic producing strains and want to report the results in this paper.

MATERIAL AND METHODS

Microorganisms

Streptomyces fradiae NRRL 1195 and *Streptomyces griseus* NRRL B-2682 were donated by A.J. Lyons, at Nothern Regional Research Laboratory (NRRL) in Peoria, ILL, USA. *Streptomyces kanamyceticus* IFO 13414 was purchased from the Institute for Fermentation in Osaka, Japan. *Bacillus subtilis* ATCC 6633 was purchased from the American Type Culture Collection in USA and was used as the test organism for the quantitative determination of antibiotics.

Growth condition and culture media

Streptomyces spp. were stock-cultured on V-8-agar slants. Spore suspensions were prepared from spores grown on V-8 agar plates[9] and spores were stored in 20% sterile glycerol at -20 °C. Colonies of *S. kanamyceticus* and *S. fradiae,* and colonies of *S. griseus* were transferred on to nutrient agar (Difco) plugs (1/4 inch) and on to agar plugs (1/4 inch) of the Chucken medium[9], respectively. The inoculated agar plugs were incubated at 28 °C for 4.5 days under humid conditions. Liquid cultivations were performed for *S. kanamyceticus* and *S. fradiae* in 2 m*l* of the V-8 medium[9] in test tubes and for *S. griseus* in 5 m*l* of the Chucken medium[9].

Antibiotic production

The antibiotic productivity of each colony was examined by the agar-plug method using *B. subtilis* ATCC 6633 as the test organism[1]. The antibiotic productivity was also examined for the individual colony by inoculation in broth. The incubated brothes were examined for the contents of antibiotics by a paper disk diffusion assay. Standard curves were prepared with authentic antibiotics.

Mutagenesis

Spores derived from *S. griseus, S. fradiae* or *S. kanamyceticus* were treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 3 mg/m*l*) for 1-2 hrs to give 99.9% or more killing. Survived hundreds of colonies formed on the nutrient agar (Difco) plate were tested for their antibiotic productivity to select high yielding mutants and antibiotics-blocked mutants. The antibiotic-blocked mutants were tested for the antibiotic production by culturing near the parent strain to find out A-factor negative mutants[9], or by culturing on the agar plate supplemented with deoxystreptamine or streptidine to identify deoxystreptamine or streptidine-negative mutants. The mutagen treated colonies were examined for the growth on ISP No. 4 agar plates containing two fold diluted antibiotics.

Preparation of protoplasts and cell-wall regeneration

A spore suspension was inorculated in Medium S[10] and incubated for 48 hours. The culture was used as an inorculum and transferred to the same medium supplemented with 4% of glycine to give 2% inorculation. After incubation again for 48 hrs at 28 °C, the mycelial suspension (25 m*l*) was harvested by centrifugation and washed with sucrose solution (10.3%). The washed mycelia was centrifuged and resuspended in medium[10]. The suspension was centrifuged again and resuspended in the same medium containing lysozyme (1 mg/m*l*) and incubated at 30 °C for 1 hr. The lysozyme treated cell suspension was centrifuged and filtered through cotton wool to remove intact cells. The protoplast-suspension was diluted in Medium P and plated on the regeneration medium, R2YE medium[11].

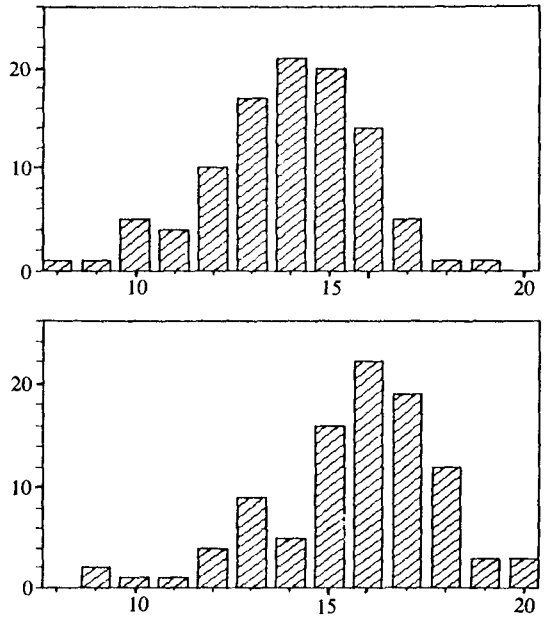

Fig. 1. The Distributions of the antibiotic productivity for the colonies of *S. fradiae* obtained from a wild strain (a) and those from the highest producing colony of the wild strain (b).

RESULTS

Strain improvement by natural selection

The distribution of kanamycin productivity of the original strain, *S. kanamyceticus* is shown in Fig. 2-a. To determine whether the strain contains more high antibiotic-producting variants, the spore suspension of the highest yielding colony was spread on a nutrient agar plate. Examination of the antibiotic productivity of about 200 colonies gave the results shown in Fig. 2-b. Comparison of the distribution plot of the high yielding colony with that of the parent strain indicated clearly the increase of kanamycin-productivity. In the case of *S. fradiae* which produces neomycin, similar results shown in Fig. 1 were obtained. This implied that similar significant increase of neomycin-producti-

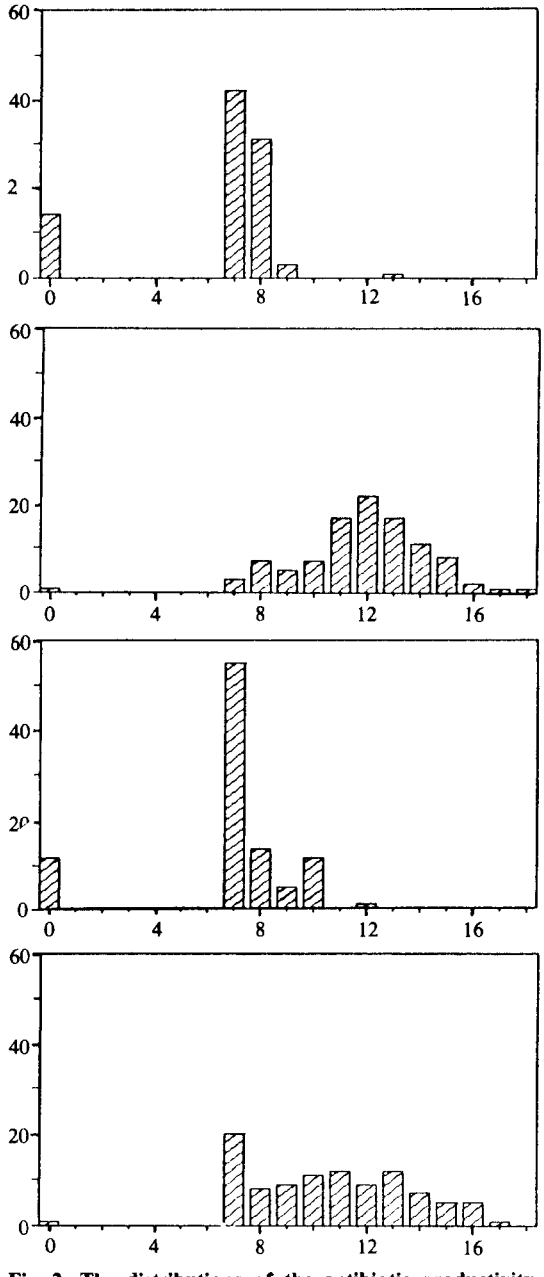

Fig. 2. The distributions of the antibiotic productivity for the colonies of *S. kanamyceticus* obtained from a wild strain (a,c) and from those from the highest producing colonies (b, d).

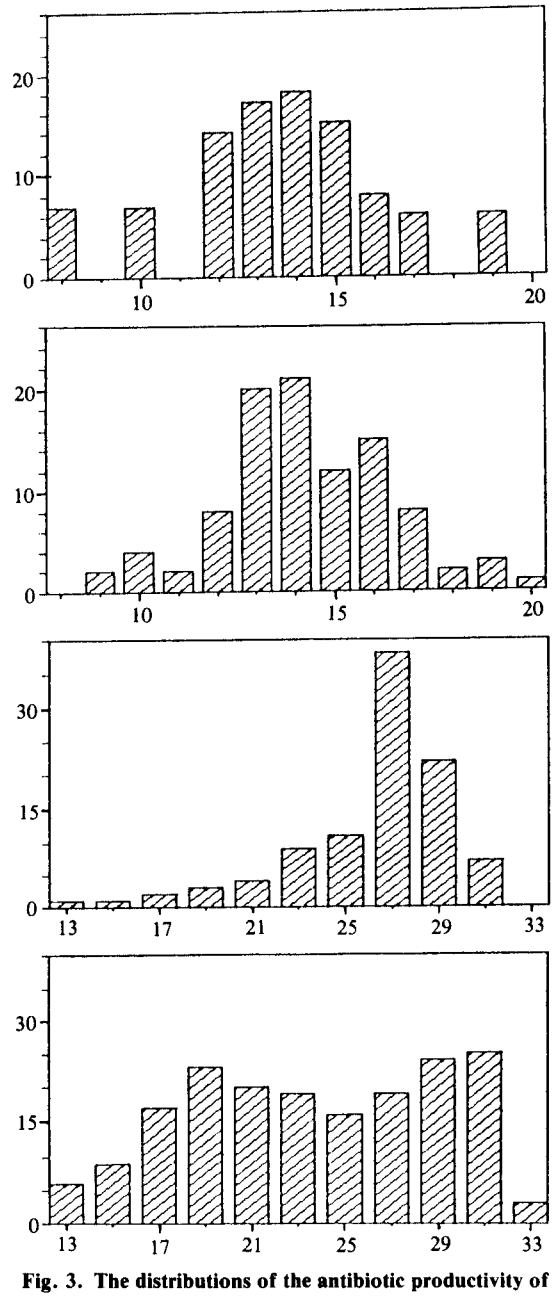

Fig. 3. The distributions of the antibiotic productivity of the colonies obtained from the protoplast formation and the cell wall regenerations of *S. kanamyceticus* (b) and *S. griseus* (d).
The distributions of the antiiotic productivity of the original strains are shown in a and c.

vity could be achieved by natural selection.

The effects of mutagenesis on the antibiotic production

After MNNG was treated on aminoglycoside-antibiotic producing strains, most of the colonies survived showed decreased productivity of antibio-

Table I. Mutants obtained from Streptomycetes by treatment of MNNG

Strains	Colonies	A-factor negative mutants	Deoxystreptamine or Streptidine blocked mutants	Unidentified blocked mutants
S. fradiae	310	4(1.3%)	9(2.9%)	13(4.2%)
S. kanamyceticus	362	6(1.7%)	10(2.8%)	5(1.3%)
S. griseus	629	15(2.5%)	3(0.5%)	20(2.9%)

tics, 8.7% of the colonies of *S. kanamyceticus,* 11.0% of the colonies of *S. fradiae,* and 5.9% of the colonies of *S. griseus,* survived from the MNNG treatment showed no antibiotic production; some of the nonproducers seemed to be A-factor negative mutants and others were deoxystreptamine or streptidine-blocked mutants as shown in Table I.

Effects of protoplast-formation and regeneration of cell-walls on the production of antibiotics

The purified protoplast suspension diluted in Medium P was plated on the regeneration medium. The colonies formed were examined for the antibiotic productivity. The results were shown in Fig. 3. In case of *S. fradiae,* many regenerated colonies showed higher productivity than the parent strain and also the number of higher yielding colonies was increased. In case of *S. kanamyceticus* the antibiotic productivity seemed not to be markedly affected by protoplast-formation and cell-wall regeneration. When *S. griseus* was similarly treated, many colonies showed higher productivity of streptomycin than the parent strain.

DISCUSSION

Development of high yielding strains of antibiotic producing microorganisms is very important to achieve industrial production antibiotics. Selection of natural variants of antibiotic producing microorganisms seems to be important for the maintainance of antibiotic producing microorganisms. To determine the productivity of antibiotics, we examined 200-600 colonies formed from one parent colony by inorculating them individually on agar plugs, by culturing at 28 °C for 4 days and by examining the inhibition zones of *B. subtilis* around the agar plugs on a seeded agar plate. The agar plugs made with a punch were arranged on a Petri dish individually and were inoculated with toothpickers. The cells grown on each agar plugs were used to inoculate liquid media. Comparison of the amount of the antibiotic produced in agar plugs with that in broth culture did not show consistency; we preferred to chose the results obtained from broth cultures. As shown in Fig. 1, the productivity of an antibiotic was treated statistically and thus, we represented the effects of mutagen treatments or other treatments on the productivity of antibiotic by bars in the graphs.

Treatment of mutagens on the antibiotic producing strains showed marked decrease of antibiotic productivity totally, although a few colonies showed higher yield of antibiotic production than the parent strain. When blocked mutants were examined; some mutants were A-factor negative and some were deoxystreptamine or streptidine negative mutants. Since *Streptomyces* spp. do not accumulate intermediates of antibiotic in media, we assume that from the parent strain highly lypopholic A-factors diffuses to the mutant. On the basis of this assumption, we regarded the colony which produced antibiotics only when it was cultured near the wild strain as A-factor negative mutants. Although all the strains showed stronger resistance against the antibiotic they produced, the survived colonies showed marked decrease of resistance against streptomycin in case of *S. griseus.* Currently we are investigating other blocked mutants to identify the functions blocked.

Biosynthesis of aminoglycoside antibiotics seems to be related with other physiological changes. Since many antibiotic producing microorganisms have been known to start the biosynthesis of antibiotics as the formation of spores starts. Protoplast formation and cell wall regeneration may affect markedly on the productivity of antibiotics. We have obtained similar results for *S. fradiae* and *S. griseus* as Ikeda *et al.*[12] Ikeda *et al.*[13] observed a 2 to 3.6 fold increase of the production of a macrolide antibiotic with regenerated colonies from protoplasts. But Baltz *et al.*[14] reported that regenerated protoplasts of *S. fradiae* produced very low level of tylosine. By the protoplast formation and the cell wall regeneration, we could obtain high antibiotic producers. Generally the increased productivity of regenerated protoplasts were believed to be originated from genetic variation as also do the changes in morphology, the restoration of antibiotic productivity[13] and the elimination of extrachromosomal element[15]. All of which have been observed after regeneration of *Streptomyces* protoplast. The protoplast formation and the cell wall regeneration seem to be also suitable to obtain

high producers of antibiotic in case of *S. fradiae, S. kanamyceticus* and *S. griseus.*

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