THE GENERATION OF ADDITIONAL ANTIBIOTIC RESISTANCE BY PROTOPLAST REGENERATION OF A *STREPTOMYCES GRISEUS* STRAIN

Sir:

As reported previously[1], clones with resistance to both streptomycin (SM) and kanamycin (KM) were generated by a self-fusion treatment of *Streptomyces griseus* NP1-1 which was resistant to only SM. The self-fusion treatment consisted of protoplast formation, polyethyleneglycol (PEG) treatment, and regeneration of mycelium. In this paper, it is shown that the regeneration of the mycelium by incubation of protoplasted cells is critical for the generation of additional resistance.

As shown in Table 1, when spores (about $1\sim2\times10^9$) of *S. griseus* NP1-1 and its parental strain SS-1198 which were sensitive to 5 μg/ml of KM were plated and incubated on ISP No. 4 agar medium containing 50 μg/ml of KM, no colonies were observed after a 14-day incubation at 27°C. Subculturing the strain NP1-1 onto a fresh ISP No. 4 medium did not influence the KM sensitivity of the strain.

Mycelium of *S. griseus* NP1-1 grown in Tryptic Soy Broth (Difco) was then protoplasted and regenerated with or without PEG 4000 treatment according to the method described in a previous paper[1]. Clones resistant to 50 μg/ml of KM were observed among spores ($2\sim3\times10^9$) of the regenerated cultures at a frequency of 1.3×10^{-8} regardless of the PEG treatment. Thus, it was observed that protoplast regeneration treatment without the PEG treatment was sufficient for the generation of KM-resistant clones.

Subsequently, protoplasted cells were challenged to regenerate mycelium in the presence of KM. No colonies were observed when protoplasted cells were plated onto the regeneration medium R3M containing 50 μg/ml of KM. In contrast, colonies developed at a frequency of 10^{-6} when KM was added to the regeneration medium after pinpoint colonies were microscopically detected. The regenerated colonies retained the resistance to SM. Therefore, the results indicate that the incidence of SM^rKM^r clones was neither the selection of KM resistant clones from spores or protoplasted cells, nor the effect of PEG treatment of protoplasted cells but due to the development of the resistance during incubation of protoplasted cells to regenerate mycelium.

The SM^rKM^r clones obtained by the protoplast regeneration treatment were examined for their resistance patterns to 50 μg/ml of 11 different aminoglycoside antibiotics according to the method described previously[2]. The clones predominantly provided two different patterns of resistance to 8 and 7 aminoglycosides. The two

Table 1. Appearance of kanamycin-resistant clones after protoplast regeneration.

Strains	Treatment*	Kanamycin concentration (μg/ml)		
		0	50	
		No. of colonies (A)	No. of colonies (B)	Frequency (B/A)
SS-1198	SP	7.8×10^8	0	0
NP1-1	SP	2.2×10^9	0	0
	1 Transfer-SP	2.1×10^9	0	0
	PR-SP	2.9×10^9	3.8×10	1.3×10^{-8}
	PR (PEG)-SP	2.1×10^9	2.7×10	1.3×10^{-8}
	PR (KM)	1.2×10^8	0	0
	PR (KM overlaid)	1.2×10^8	1.5×10^2	1.3×10^{-6}

* SP: Simple plating of spores onto ISP No. 4 agar medium; 1 Transfer-SP: Spores of the subculture on ISP No. 4 agar medium were plated; PR-SP: Spores of the regenerated mass culture from protoplasts were plated; PR (PEG)-SP: Spores of the regenerated mass culture from the PEG 4000-treated protoplasts were plated; PR (KM): Protoplasts were plated onto a regeneration medium R3M containing 50 μg/ml of KM; PR (KM overlaid): Tryptic Soy Broth (Difco) agar (0.6% Low Gelling Temperature agarose, Marine Colloids) containing KM was overlaid three days after protoplasts were plated onto R3M medium. Final concentration of KM in the regeneration medium was 50 μg/ml.

patterns were commonly resistant to SM, KM, dibekacin (DK), paromomycin (PR) and lividomycin (LV). In addition to these antibiotics, one pattern was resistant to neamine, ribostamycin and butirosin and another one was resistant to the gentamicin C complex and neomycin. Almost the same results were obtained previously by us with the SM^rKM^r strains, SK2-52 and SK4-3, which were obtained by an interspecific fusion treatment between *S. griseus* NP1-1 and *S. tenjimariensis* NM16[2]. The strains SK2-52 and SK4-3 exhibited acetyltransferase activity to the different antibiotics to which each strain acquired resistance. Therefore, it seems likely that protoplast regeneration treatment of *S. griseus* NP1-1 resulted in a genetic change which enhanced the expression of silent genes for at least two different acetyltransferases.

It was reported that resistance to certain antibiotics of certain *Streptomyces* species was induced by direct contact of the organisms with the antibiotics[3]. However, the present experiment indicated that the protoplast regeneration treatment of *S. griseus* NP1-1 resulted in the generation of additional antibiotic resistance without direct contact with the antibiotics.

There have been reports on the generation of streptomycete clones with a changed phenotype caused by protoplast regeneration; *e.g.*, prototrophic revertants from a citrulline-requiring strains of *S. kasugaensis*[4], high yielding clones from strains of macrolide antibiotic producers[5], and clones with an increased resistance to SM in a SM-producing strain of *S. griseus*[6]. These facts would indicate that protoplast regeneration can result in a genetic change in *Streptomyces* species. Accordingly, our observation should be taken into account in transformation and cell fusion of *Streptomyces* where antibiotic resistance is often used for selection of desired clones.

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