NEW ANTIBIOTIC-PRODUCING STREPTOMYCETES, SELECTED BY ANTIBIOTIC RESISTANCE AS A MARKER

I. NEW ANTIBIOTIC PRODUCTION GENERATED BY PROTOPLAST FUSION TREATMENT BETWEEN *STREPTOMYCES GRISEUS* AND *S. TENJIMARIENSIS*

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A novel antibiotic was found after performing an interspecific fusion treatment between *Streptomyces griseus* and *S. tenjimariensis* by the selection of clones with a unique antibiotic resistance. Nonantibiotic-producing mutants of streptomycin (SM)-producing *S. griseus* SS-1198 with resistance to SM and istamycin (IS)-producing *S. tenjimariensis* SS-939 with resistance to kanamycin (KM) were protoplasted, mixed with polyethyleneglycol and regenerated. Resistant clones to both SM and KM were found among spores of the regenerated culture at a frequency of 10^{-6}. Their growth appearance was identical with that of *S. griseus*. Antibiotic productivity was found only in clones resistant to both 20~50 μg/ml of KM and 400 μg/ml of SM. The antibiotic produced by a selected strain, SK2-52, proved to be different from SM and IS.

In our previous papers[1,2], we reported that antibiotic-producing strains of actinomycetes have individual patterns of antibiotic resistance, depending on the type of antibiotic they produce. In addition, strains with multiple resistance to a wide range of antibiotics were found to have a high probability of antibiotic production. It was therefore expected that the selection of new actinomycete clones with new patterns of resistance to wide ranges of antibiotics might result in the discovery of new antibiotics.

In native resistance pattern to streptomycin (SM) and kanamycin (KM), istamycin (IS)-producing *Streptomyces tenjimariensis* SS-939 was found to be reciprocal with SM-producing *S. griseus* SS-1198[2]. By taking advantage of this native difference between two strains, antibiotic non-producing mutants derived from both strains were subjected to an interspecific protoplast fusion treatment. This resulted in SM^rKM^r clones with new antibiotic productivity.

This paper describes the generation of such clones and the partial characterization of the antibiotic produced by a selected SM^rKM^r strain SK2-52.

Materials and Methods

Parent Strains

Antibiotic non-producing strains, *S. tenjimariensis* NM16 and *S. griseus* NP1-1 were obtained from IS-producing *S. tenjimariensis* SS-939 and *S. griseus* SS-1198, respectively, by UV irradiation. The former was resistant to KM (3,000 μg/ml) but sensitive to SM (2 μg/ml). The latter was resistant to SM (400 μg/ml) but sensitive to KM (5 μg/ml).

Protoplast Formation

S. tenjimariensis NM16 was cultivated in a medium consisting of glucose 5.0 g, Polypepton 2.5 g,

yeast extract 0.5 g and glycine 5.0 g in 1,000 ml of 1/2 × Jamarine artificial sea water (pH 7.2, Jamarine Ltd., Osaka, Japan). *S. griseus* NP1-1 was cultivated in Tryptic Soy Broth (Difco) supplemented with 0.9% of glycine. Cultivation was carried out at 27°C on a rotary shaker (180 rpm). Mycelia of the two strains at logarithmic growth phase were protoplasted according to the method of OKANISHI *et al.*[3] with a minor modification. *S. tenjimariensis* NM16 was incubated with 1.5 mg/ml of lysozyme at 32°C for 90 minutes to obtain about 2×10^9 protoplast cells from 50 ml cultured broth. *S. griseus* NP1-1 was incubated with 1.0 mg/ml of lysozyme at 32°C for 60 minutes to obtain about 6×10^{10} protoplast cells from 50 ml cultured broth.

Fusion Treatment

Protoplasted cells (approximately 1×10^9 for each) from the two strains were suspended in 0.1 ml of medium P[3] and incubated for 3 minutes at room temperature after mixing with 0.9 ml of PEG 4000 solution. The solution consisted of 4.0 g of PEG 4000 dissolved in 6 ml of DMSO - medium P′ (15:85) in which 25 mM 3-cyclohexylimino-1-propanesulfonic acid (pH 10.5) was used instead of 25 mM 2-[tris-(hydroxylmethyl)methylamino]-1-ethanesulfonic acid (pH 7.2) in medium P. The PEG 4000-treated protoplast cells were then washed with 9 ml of medium P and resuspended in 1 ml of medium P.

Regeneration after Fusion Treatment

Protoplast regeneration was carried out according to the method of SHIRAHAMA *et al.*[4] but using a modified regeneration medium (R3M) in which 0.555 M sodium succinate and 0.4% of Low Melting Point agarose (B.R.L.) in R3 medium were substituted with 0.4 M sucrose and 0.6% of Low Gelling Temperature agarose (Marine Colloids), respectively. The protoplast suspension after the fusion treatment was serially diluted before plating on R3M medium and incubated for 14 days at 27°C.

Antibiotic Resistance

Spores of regenerated cultures were suspended in 0.85% NaCl solution and spread on ISP No. 4 agar medium (Difco) containing 10 μg/ml of SM and/or KM. After 5 days incubation at 27°C, colonies grown on the media were transferred to fresh ISP No. 4 medium containing both SM and KM to confirm their resistance.

Antibiotic Productivity

Clones which showed resistance to both SM and KM were inoculated on agar cylinders (6 mm in diameter and 4 mm in height) of ISP No. 4 medium supplemented with 0.2% yeast extract. After 7-day incubation at 27°C, antibiotic activity was checked by placing the agar cylinders on the regular antibiotic assay plate seeded with *Bacillus subtilis* PCI219 as the test organism.

Characterization of the Antibiotic Produced by the Strain SK2-52

The selected strain SK2-52 was cultured under rotary shaking (180 rpm) for 48 hours at 27°C in medium M consisting of glycerol 20 g, dextrin 20 g, yeast extract 3 g, Bacto Soyton 10 g, $(NH_4)_2SO_4$ 2 g and $CaCO_3$ 2 g in 1,000 ml of deionized water (pH 7.4). The filtrate of the cultured broth was chromatographed on XAD-2 and eluted with 40% acetone. This was followed by chromatography on CM-Sephadex eluted with a linear gradient of 0.1 ~ 0.5 M NaCl. The active fractions were desalted with XAD-2 and concentrated under vacuum. The crude fraction thus obtained was subjected to high voltage paper electrophoresis[5] to compare with the crude extracts of antibiotics produced by *S. tenjimariensis* SS-939 and *S. griseus* SS-1198. Bioautography was carried out on an agar plate seeded with *B. subtilis* PCI219.

Results

Generation of Clones with Resistance to SM and KM by Protoplast Fusion Treatment

After interspecific fusion treatment between *S. griseus* NP1-1 and *S. tenjimariensis* SS-939 (1×10^9 protoplast cells of each), about 1.3×10^8 colonies grew up and sporulated on R3M medium. Spores (1.2×10^9) of the regenerated colonies were scraped and plated on ISP No. 4 agar medium containing SM and KM (10 μg/ml each). As shown in Table 1, $SM^r KM^r$ colonies appeared at a frequency of about

Table 1. Generation of SM^{r}KM^r clones by protoplast fusion treatment.

Antibiotic added ($10^?$ μg/ml)	Protoplast fusion treatment*					
	NP1-1+NM16		NP1-1		NM16	
	Colonies appeared	Frequency	Colonies appeared	Frequency	Colonies appeared	Frequency
None	1.2×10^9	1	2.1×10^9	1	1.4×10^7	1
SM	1.2×10^9	1	NT**		NT	
KM	2.5×10^3	2.1×10^{-6}	NT		NT	
SM+KM	2.6×10^3	2.2×10^{-6}	2.3×10^3	1.1×10^{-6}	0	—

* Spores of the regenerated cultures after interspecific fusion treatment between *S. griseus* NP1-1 and *S. tenjimariensis* NM16 or self-fusion treatment of each strain were plated.

** Not tested.

Table 2. Antibiotic productivity of SM^{r}KM^r clones.

Strains* used for fusion treatment	Resistance**		Resistant		Antibiotic producer***	
	SM	KM	No./Total	%	No./Total	%
NP1-1+NM16	400	10	176 / 1,045	16.8	0 / 176	0
	400	20~50	831 / 1,045	79.6	604 / 831	72.7
	400	400	38 / 1,045	3.6	0 / 38	0
NP1-1	400	10	259 / 736	35.2	0 / 259	0
	400	20~50	440 / 736	59.8	0 / 440	0
	400	400	37 / 736	5.0	0 / 37	0

* NP1-1: *S. griseus* NP1-1; NM16: *S. tenjimariensis* NM16.

** Upper limit of resistance to streptomycin and kanamycin A (μg/ml).

*** SM^{r}KM^r strains which showed 10.0 mm or larger inhibition zone against *B. subtilis* PCI219 by agar cylinder method.

2×10^{-6}. The same numbers of colonies were observed on the medium containing only KM. They also proved to be resistant to SM by replica plating.

On the other hand, protoplasted cells of each of *S. griseus* NP1-1 and *S. tenjimariensis* NM16 were separately subjected to a self-fusion treatment. Interestingly, SM^{r}KM^r colonies were obtained from the regenerated culture of *S. griseus* NP1-1 at almost the same frequency as in the case of interspecific fusion. Self-fusion treatment of *S. tenjimariensis* NM16 did not provide any SM^{r}KM^r clone. No colonies were observed when about 1×10^9 spores of parental strains without protoplast fusion treatment or protoplast regeneration treatment were plated onto the medium containing KM and SM.

All the SM^{r}KM^r clones obtained by the interspecific fusion treatment showed a growth appearance identical with that of *S. griseus* NP1-1.

Antibiotic Productivity of SM^{r}KM^r Clones

Out of SM^{r}KM^r clones obtained by interspecific fusion treatment, 1,045 clones were selected at random and examined for the upper limit of resistance to both SM and KM and antibiotic productivity. The resistance level to SM was the same in all clones (400 μg/ml), while they showed different resistance levels to KM. Based on a resistance level to KM of 10, 20~50, or 400 μg/ml, they were roughly divided into three groups with distribution of about 17%, 80% and 4%, respectively (Table 2). Interestingly, obvious antibiotic production with a 10 to 22 mm inhibition zone by the agar cylinder method was observed only in the group with 20~50 μg/ml resistance to KM at a high frequency (72.7%). The other

Fig. 1. Time course of antibiotic production by the strain SK2-52.

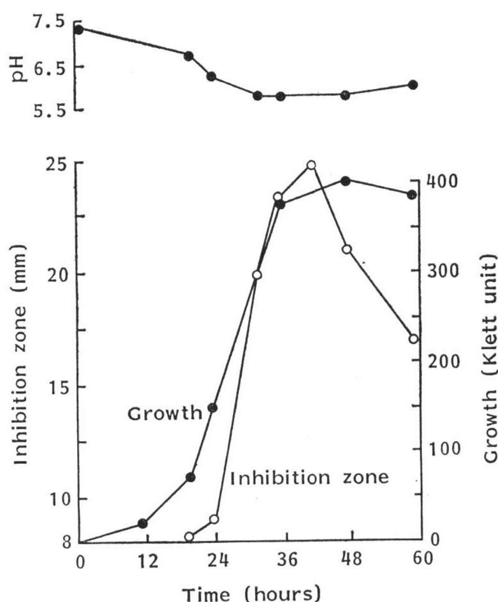


Table 3. Antibacterial activity of the filtrate of the cultured broth of the strain SK2-52.

Test organisms	Inhibition zone (mm)
Staphylococcus aureus 209P	16.0
Bacillus subtilis PCI219	25.0
Escherichia coli K12	24.0
E. coli JR66/W677	0
Mycobacterium smegmatis ATCC 607	18.0

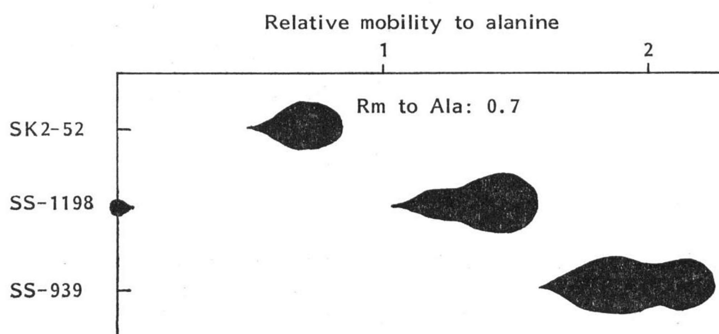
two groups of clones did not show antibiotic production. As will be described in the next paper[6], the difference in resistance level to KM corresponded to a difference in the pattern of multiple resistance to aminoglycoside antibiotics.

The SM^{r}KM^r clones obtained by the self-fusion treatment of *S. griseus* NP1-1 also fell into the same three resistance groups with similar polarity of distribution as above. However, no antibiotic production was found. Furthermore, 280 colonies sensitive to 10 μg/ml of KM were also picked up after self-fusion of *S. griseus* NP1-1 and examined for their antibiotic productivity. None of the clones showed antibiotic production.

New Antibiotic Production by a SM^{r}KM^r Strain, SK2-52

Among the antibiotic producing SM^{r}KM^r strains, strain SK2-52 proved to be the most stable in terms of antibiotic productivity; therefore its antibiotic substance was characterized. When the strain was incubated in medium M, antibiotic production began intensively after about a 24-hour incubation. Accumulation of the antibiotic reached a peak after a 36~40-hour incubation and then abruptly decreased (Fig. 1). The filtrate of the cultured broth was active against certain ranges of Gram-positive and Gram-negative bacteria (Table 3). To characterize the active principle, the filtrate was first chromatographed on an Amberlite IRC-50 (NH_4^+ or Na^+) column which was conveniently used for the extraction of aminoglycoside antibiotics such as IS and SM produced by *S. tenjimariensis* SS-939 and *S. griseus*

Fig. 2. Bioautogram of the crude antibiotic extracts.



SS-1198, respectively. However, the active principle was not eluted from the column by this method, suggesting that the antibiotic is not an aminoglycoside. The antibiotic principle was extracted by chromatography using Diaion XAD-2 and CM-Sephadex C-25 columns as described in "Materials and Methods". The crude extract thus obtained was compared with those from the cultured broth of *S. tenjimariensis* SS-939 and *S. griseus* SS-1198 by high voltage paper electrophoresis. As shown in Fig. 2, the bioautography revealed that their active principles were clearly distinguishable from each other. The structure determination of the antibiotic from strain SK2-52, which is a new antibiotic structure having no similarity to IS and SM, is described in a separate paper[7].

Discussion

On the basis of observations on antibiotic resistance of antibiotic-producing actinomycetes[1,2], it was expected that selection of strains with unique multiple antibiotic resistance might result in the discovery of new antibiotics. As described in this paper, selection of clones with resistance to both SM and KM after an interspecific-fusion treatment between protoplasts of *S. griseus* NP1-1 and *S. tenjimariensis* NM16 successfully resulted in the discovery of a novel antibiotic by the strain SK2-52. On the other hand, SM^rKM^r clones obtained by self-fusion treatment of the above two strains did not result in antibiotic production. Furthermore, selection of prototrophic clones after interspecific fusion treatment between auxotrophic mutants of *S. griseus* SS-1198 and *S. tenjimariensis* SS-939 did not yield new antibiotic production (data not shown). Therefore, it can be concluded that selection using antibiotic resistance after interspecific fusion treatment was effective in obtaining a novel antibiotic-producing clone.

As reported previously[1], naturally occurring yellow streptomycete strains rarely show resistance to both SM and KM. Since SM^rKM^r clones generated after interspecific fusion treatment had a yellow colored growth appearance identical with that of *S. griseus*, unique resistance patterns to aminoglycoside antibiotics were expected. In fact, the multiple resistance patterns expressed by the SM^rKM^r clones were not previously observed by naturally occurring streptomycete strains. Therefore, the antibiotic produced by the strain SK2-52 was predicted to be a novel one.

There have been reports on the selection of novel antibiotic-producing clones using auxotrophic markers after an interspecific cross between *Streptomyces* species[8,9]. The antibiotics identified were derivatives of the ones produced by the parental strains. In contrast, the approach described here has provided a novel antibiotic which has no structural similarity to the ones produced by the parental strains as shown in a separate paper[7]. This approach might be useful for the selection of other novel antibiotics.

The phenotype of the strain SK2-52 caused by the interspecific fusion treatment is characterized in the following paper[6].

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