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

II. FEATURES OF A NEW ANTIBIOTIC-PRODUCING CLONE OBTAINED AFTER FUSION TREATMENT

KUNIMOTO HOTTA, FUMIO YAMASHITA, YOSHIRO OKAMI and HAMAO UMEZAWA

Institute of Microbial Chemistry
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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A new antibiotic-producing *Streptomyces* strain SK2-52 obtained by a protoplast fusion treatment between *Streptomyces griseus* NP1-1 and *S. tenjimariensis* NM16 showed taxonomical features identical with those of *S. griseus*. The strain was resistant to wider range of aminoglycoside antibiotics than the parental strains. This multiple resistance corresponded to the activities of streptomycin kinase and acetyltransferase which were probably derived from *S. griseus* NP1-1. Clones with fast-growth and reduced antibiotic productivity frequently segregated from strain SK2-52, while their antibiotic resistance was stable. The results suggest that the fusion treatment caused a genetic change in *S. griseus* which enhanced the expression of genes for unique multiple resistance to aminoglycoside antibiotics and also induced new antibiotic production.

As reported in a previous paper[1], an interspecific fusion treatment between *Streptomyces griseus* NP1-1 and *S. tenjimariensis* NM16 resulted in the generation of a new antibiotic-producing clone designated SK2-52.

In this paper, the strain SK2-52 is characterized in terms of taxonomy, antibiotic resistance and stability of antibiotic productivity.

Materials and Methods

Strains Used

Streptomyces strains SK2-52 and SK4-3 were obtained by interspecific fusion treatment of *S. griseus* NP1-1 and *S. tenjimariensis* NM16 as previously described[1].

Taxonomy

Taxonomical characterization was carried out following the ISP (International Streptomyces Project) procedure[2]. Strains were classified with reference to the 8th edition of BERGEY's Manual of Determinative Bacteriology[3] and ISP descriptions[4].

Antibiotic Resistance

Antibiotic resistance to various aminoglycoside antibiotics (AGs) was examined by the method described in a previous paper[1].

In Vitro Polyphenylalanine Synthesizing System

According to the previously described method[5], mycelium grown in Tryptic Soy Broth (Difco) was disrupted with alumina and centrifuged at $100,000 \times g$ to separate the S-100 fraction from the ribosomal fraction. The S-100 fraction was combined with the ribosomal fraction to reconstitute an *in vitro* polyphenylalanine synthesizing system.

Table 1. Taxonomic properties of the strain SK2-52.

	SK2-52, NP1-1 and SS-1198	*S. griseus* ISP5236
Morphology		
Aerial mass color	"Yellow" series (light olive gray)	"Yellow" series (pale greenish yellow)
Spore chain	Rectiflexibiles	Rectiflexibiles
surface	Smooth	Smooth
Special structure	Absent	Absent
Physiology		
Melanoid formation	+ (ISP No. 7)	+ (ISP No. 7)
Soluble pigment formation	−	−
Starch hydrolysis	+	+
Nitrate reduction	+	+
Milk peptonization	+	+
coagulation	−	+
Gelatin liquefaction	−	+
Carbon utilization:		
Positive	Glucose, xylose, mannitol, fructose	Glucose, xylose, mannitol, fructose
Negative	Arabinose, sucrose, inositol, rhamnose, raffinose	Arabinose, sucrose, inositol, rhamnose, raffinose

Phosphorylation and Acetylation of Aminoglycoside Antibiotics

The S-100 fraction was tested for activity to phosphorylate or acetylate AGs according to the method described previously[6].

Stability of Antibiotic Productivity

A culture of the strain SK2-52 on ISP No. 4 agar slant was successively transferred to a fresh ISP No. 4 agar slant after a 10-day incubation at 27°C. Aerial mass cultures after the 1st, 3rd and 5th transfers were scraped and suspended in 0.85% NaCl and spread onto ISP No. 4 agar plates. Colonies grown on the plates were picked up at random and examined for their antibiotic productivity by the agar cylinder method using *Bacillus subtilis* PCI219 as the test organism as described previously[1].

Results

Taxonomical Features

As summarized in Table 1, aerial mycelium of strain SK2-52 was light olive gray to pale greenish yellow (yellow color series) in its mass color on ISP No. 2, 3, 4 and 5 media, and formed straight to flexeous chains of conidial spores with a smooth surface. These morphological properties were consistent with those of *S. griseus* represented by *S. griseus* IFO12875 (ISP5236). The physiological properties also showed close similarity to those of *S. griseus* with a few exceptions. In addition, no significant difference was observed between strain SK2-52, *S. griseus* SS-1198 and its non-antibiotic producing derivative strain NP1-1. Therefore all of these strains were classified as *Streptomyces griseus*.

Antibiotic Resistance

As reported previously[1], the strain SK2-52 selected as a SM^rKM^r clone showing resistance levels of 400 μg/ml to SM and 20~50 μg/ml to KM. In order to determine the origin of the resistance, the pattern and mechanism of resistance of the strain were characterized.

In addition to SM and KM, the strain exhibited resistance to 6 out of 9 other AGs (50 μg/ml) tested

Table 2. Patterns of resistance to various kinds of aminoglycoside antibiotics.

Organisms		Concentration (μg/ml)	Resistance* to										
			SM	KM	DK	GM	RM	BT	NM	PR	LV	IS	NE
S. griseus	SS-1198	10	++		++		++	++		±			++
		50	++										
	NP1-1	10	++		++		++	+		±			++
		50	++										
	SK2-52	50	++	++	++	±	++	+		+	+		++
	SK4-3**	50	++	++	+	+	±		+	+	+		+
S. tenjimariensis	SS-939	50		++	++		++	++				++	++
	NM16	50		++	++		++	++				++	++

* SM: Streptomycin, KM: kanamycin A, DK: dibekacin, GM: gentamicin C complex, RM: ribostamycin, BT: butirosin A, NM: neomycin B, PR: paromomycin, LV: lividomycin A, IS: istamycin B, NE: neamine. Concentration as free base.
++: Good growth, +: retarded growth, ±: very weak growth, no indication: no growth.
** A SM^rKM^r strain with resistance level of 400 μg/ml to KM.

as shown in Table 2. The other SM^rKM^r clones with the same resistance levels to both SM and KM as those of SK2-52 also showed almost the same pattern of resistance. A different pattern of multiple AG resistance was obtained by another SM^rKM^r clone, designated SK4-3, which exhibited a resistance level of 400 μg/ml to both SM and KM. These two resistance patterns have not been observed previously in naturally occurring actinomycete strains[7].

Table 3. Effect of SM and KM on *in vitro* polyphenylalanine synthesis.

Antibiotic added (μg/ml)	Poly U directed polyphenylalanine synthesis*		
	SS-1198	SK2-52	SK4-3
None	100.0% (13,532 dpm)	100.0% (7,530 dpm)	100.0% (5,349 dpm)
SM (100)	53.6	64.5	63.1
KM (50)	8.4	10.9	14.2

* Reaction mixtures (100 μl) containing ribosomes and S100 fractions from each strain were incubated at 37°C for 60 minutes and 20 μl portions were removed to determine incorporation (dpm) of [^{14}C]phenylalanine into TCA insoluble fractions.

Phenotypically, the resistance pattern of strain SK2-52 seemed to be a summation of the patterns of *S. griseus* NP1-1 and *S. tenjimariensis* NM16. However, strain SK2-52 was sensitive to IS (5 μg/ml) to which *S. tenjimariensis* NM16 was resistant. If the KM resistance of strain SK2-52 were derived from *S. tenjimariensis* NM16, strain SK2-52 should be resistant to IS because the strain NM16 showed resistance to both IS and KM, which depended on ribosomal resistance[8]. Thus, it was postulated that the resistance to KM of the strain SK2-52 did not originate from *S. tenjimariensis*.

The resistance mechanisms of the strains SK2-52 and SK4-3 then were characterized by the use of *in vitro* polyphenylalanine synthesizing systems. As shown in Table 3, the systems were resistant to SM as highly as that of *S. griseus* SS-1198, which is the wild type strain of the strain NP1-1. The result suggested the involvement of SM kinase in SM resistance as in the case of SM-producing *S. griseus* ISP 5236[9]. On the other hand, all the systems were strongly inhibited by KM, indicating their ribosomes were sensitive to KM. However, inhibition by KM seemed to be weaker in strains SK2-52 and SK4-3 than in strain SS-1198. A similar phenomenon was observed in the *in vitro* polyphenylalanine synthesizing system of KM-producing *S. kanamyceticus* although acetyltransferase plays a major role in KM resistance of this organism[9,10]. Subsequently, the S-100 fraction of strains SK2-52 and SK4-3 were examined for the activities of phosphorylation and acetylation (Table 4). The S-100 fractions phos-

Table 4. Phosphorylation of SM and acetylation of KM by cell free extract.

	Strain	Resistance* (μg/ml)		Phosphorylation** (dpm)		Acetylation** (dpm)	
		SM	KM	SM	KM	SM	KM
Expt 1	NP1-1	400	5	12,568	210	0	1,321
	SK2-52	400	20~50	10,835	121	0	3,844
Expt 2	SS-1198	400	5	6,243	0	0	1,860
	SK4-3	400	400	6,339	16	321	20,089

* Upper limit of resistance to SM and KM in Tryptic Soy Broth (Difco).

** Incorporations (dpm) of γ-[^{32}P]ATP and [^{14}C]acetyl CoA into SM and KM (100 μg/ml) by S-100 fractions (1 mg/ml) were counted as activities of phosphorylation and acetylation, respectively.

Table 5. Acetylation of various aminoglycoside antibiotics by cell free extract.

Antibiotics	SK2-52		SK4-3	
	Resistance*	dpm	Resistance*	dpm
Streptomycin	R	0	R	321
Kanamycin A	R	5,757	R	20,089
Dibekacin	R	4,754	R	17,076
Gentamicin C	S	885	R	14,203
Ribostamycin	R	3,813	S	2,023
Butirosin A	WR	859	S	238
Neomycin B	S	866	R	16,613
Paromomycin	R	3,808	R	28,943
Lividomycin A	R	2,549	R	nt**
Neamine	R	2,759	R	nt
Istamycin B	S	217	S	nt

Incorporation of [^{14}C]acetyl CoA (dpm) into an antibiotic (100 μg/ml) was measured after reaction mixtures were incubated at 37°C for 60 minutes. Blank values were subtracted.

* Resistance *in vivo* to 50 μg/ml of antibiotics. R: Resistant, WR: weakly resistant, S: susceptible.

** Not tested.

phorylated SM but not KM, and acetylated KM significantly but not SM. The phosphorylating activity seemed to be the same in all of the S-100 fractions while the acetylating activity varied. The S-100 fractions of strains SK2-52 and SK4-3 had higher acetylating activity than those of their parental strains *S. griseus* NP1-1 and SS-1198. The acetyltransferase activity of strains SK2-52 and SK4-3 was further correlated with their multiple resistance to various AGs as shown in Table 5. The results suggested the involvement of acetyltransferases in the multiple AG resistance of the strain SK2-52 and SK4-3.

Instability of Antibiotic Productivity

Stability of antibiotic productivity of strain SK2-52 was examined by successive transfers to fresh ISP No. 4 medium. As shown in Photo 1, the population of the slant culture after the first transfer consisted of homogeneous small colonies when spread on agar plate medium. However, successive subculture resulted in the segregation of fast-growing colonies (large colonies in the picture).

About 100 colonies were randomly picked from the plates after the 1st, 3rd and 5th transfers and examined for their antibiotic productivity. As shown in Fig. 1, colonies after the 1st transfer provided a normal curve of antibiotic producer population with a peak inhibition zone of 20 mm. However, the peak level of antibiotic productivity was reduced in the colonies after 3rd and 5th transfers. This correlated clearly to the increased segregation of fast-growing colonies. The slant culture after the 5th

Photo 1. Natural selection of fast-growing clones by subculturing of the strain SK2-52.

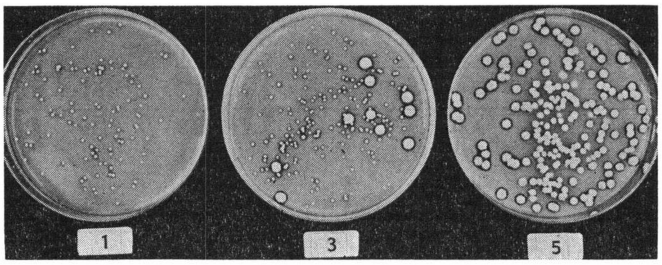
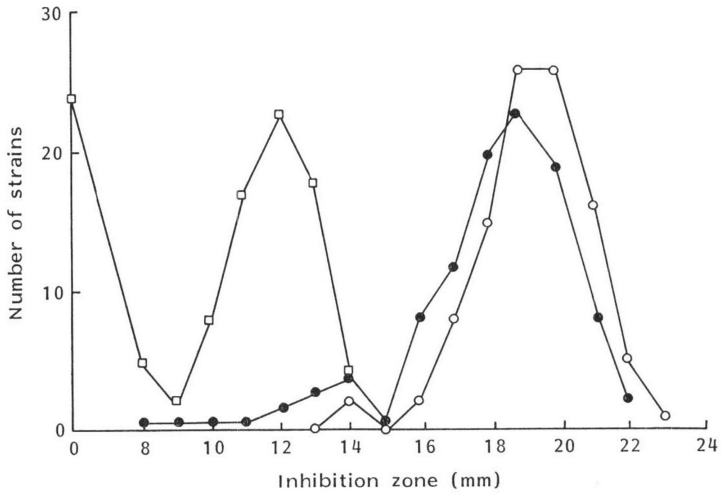


Fig. 1. Antibiotic productivity of clones segregated by subculturing of the strain SK2-52.
Transfer number: 1 ○, 3 ●, 5 □.



transfer was found to be occupied by the fast-growing clones having no or low antibiotic productivity, while their multiple resistance did not change.

The antibiotic productivity of strain SK2-52 could be kept stable for at least one year and a half when the suspension of its aerial mass was kept at −80°C in 20% glycerol.

Discussion

A new antibiotic-producing strain SK2-52 selected as a SM^rKM^r clone after interspecific fusion treatment between *S. griseus* NP1-1 and *S. tenjimariensis* NM16 showed taxonomical features of *S. griseus* and a unique multiple resistance pattern to a wide range of AGs. Characterization of the mechanism of the resistance indicated the probable involvement of phosphotransferase for SM and acetyltransferase for the other AGs to which the strain SK2-52 was resistant.

As reported previously[7,9], SM-producing strains of *S. griseus* show resistance to only SM which depends on SM kinase. Istamycin-producing strains of *S. tenjimariensis* exhibit a multiple resistance to KM, IS and other AGs which depends on ribosomal resistance[7,8]. The strain SK2-52 was found to be sensitive to IS. Thus it seems unlikely that this strain had a gene derived from *S. tenjimariensis* NM16 for its multiple AG resistance. On the other hand, *S. griseus* NP1-1 was found to have a high phosphorylating activity for SM and a weak acetyltransferase activity for KM. Furthermore, a self-fusion treatment of *S. griseus* NP1-1 provided SM^rKM^r clones with the same resistance pattern as that of strain SK2-52 as described previously[1]. Therefore, it seemed likely that the multiple AG resistance of strain SK2-52 exclusively originated from *S. griseus* NP1-1.

In regard to the antibiotic productivity of strain SK2-52, the following circumstantial evidence indicates that fusion between *S. griseus* NP1-1 and *S. tenjimariensis* NM16 was needed to get SM^rKM^r clones having definitive antibiotic productivity, because self-fusion treatment of *S. griseus* NP1-1 provided only SM^rKM^r clones without antibiotic productivity. The antibiotic productivity of strain SK2-52 was so unstable that clones with reduced levels of antibiotic productivity segregated easily by successive subculture, while its multiple AG resistance was stable. These facts imply that generation of antibiotic productivity was not caused by the same genetic change as that resulting in the generation of antibiotic resistance.

In conclusion, it seems likely that the interspecific fusion treatment between *S. griseus* NP1-1 and *S. tenjimariensis* NM16 caused specific genetic changes in the former strain to enhance the expression of acetyltransferase gene and also induce the expression of gene(s) for antibiotic production.

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