MECHANISM OF INCREASED KANAMYCIN-RESISTANCE GENERATED BY PROTOPLAST REGENERATION OF *STREPTOMYCES GRISEUS*

I. CLONING OF A GENE SEGMENT DIRECTING A HIGH LEVEL OF AN AMINOGLYCOSIDE 3-*N*-ACETYLTRANSFERASE ACTIVITY

KUNIMOTO HOTTA, JUN ISHIKAWA, MASARU ICHIHARA, HIROSHI NAGANAWA† and SATOSHI MIZUNO

Department of Antibiotics, National Institute of Health, 2-10-35 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan
†Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication April 21, 1987)

The genetic and biochemical basis of a 200-fold increase in kanamycin (KM)-resistance shown in *Streptomyces griseus* SS-1198PR generated by protoplast regeneration was investigated. A 15-kb *Bcl* I-DNA segment responsible for the KM-resistance was cloned into pIJ61 with *Streptomyces lividans* TK21 as host. The KM-resistance segment was then subcloned into pIJ702 as a 1.8-kb *Bam*H I-*Bgl* II fragment with a *Bam*H I site essential for the KM-resistance. Both *S. lividans* TK21 containing the cloned segments and *S. griseus* SS-1198PR showed multiple resistance to KM, dibekacin and gentamicin C complex. Cell free extracts from these strains inactivated the antibiotics in the presence of acetyl CoA in agreement with their resistance pattern. The structure of the inactivated KM-A was determined as 3-*N*-acetyl-KM-A indicating acetylation by an aminoglycoside acetyltransferase, AAC(3). The substrate range of the enzyme was unique and was designated AAC(3)-V. No genetic linkage was found between the cloned 15 kb *Bcl* I segment and the separately cloned streptomycin resistance gene (*str*) segment (3.8 kb *Sph* I fragment). The *str* genes cloned from both the parent (SS-1198) and the strain SS-1198PR were identical in their size, restriction site and function. In addition, these strains showed no significant difference in the total DNA digestion pattern. These results indicate that protoplast regeneration may cause a critical change in a specific region of DNA resulting in a high activity of an AAC(3) with a novel substrate range.

Development of gene technology in *Streptomyces* has enabled the cloning and analysis of the structure and regulation of antibiotic biosynthesis genes and antibiotic resistance genes. Consequently, several gene clusters consisting of antibiotic biosynthesis genes and self-resistance genes have been discovered[1~7] and the promoter region of several genes identified[8~11]. Two forms of RNA polymerase holoenzyme have also been identified[11].

In the course of studies on antibiotic resistance generated through interspecific protoplast fusion between mutants of *Streptomyces griseus* and *Streptomyces tenjimariensis* that had lost the ability to produce streptomycin (SM) and istamycin (IS), respectively[12~14]. YAMASHITA *et al.*[14] found that protoplast regeneration of *S. griseus* strains resulted in the generation of clones with a dramatically enhanced kanamycin (KM)-resistance. While the *S. griseus* strains that had not undergone protoplast regeneration treatment were sensitive to 5 μg/ml of KM, the resistant clones grew in the presence of 500~1,000 μg/ml of KM. To our knowledge, such a dramatic phenotypic change seems to be unique,

although it has been known that protoplast regeneration of *Streptomyces* strains caused a variety of phenotypic and genetic changes such as morphological variations[15], increase[16] or loss[17] of antibiotic production, reversion of auxotrophy[15] and antibiotic resistance[18], and curing of plasmids[19].

Because the KM-resistance seemed to be dependent on an inactivating enzyme whose expression is often proportional to levels of antibiotic resistance[13], we reasoned that the gene(s) involved in the KM-resistance could be a good model to learn what kinds of genetic changes (*e.g.* promoter or structural gene mutations or gene amplification) would result in a specific change in phenotype. We cloned the KM-resistance gene (*kan*) from a KM-hyper-resistant derivative (SS-1198PR) of *S. griseus* and analyzed the biochemical mechanism of the resistance. In this report we show that the cloned gene segment directs a novel aminoglycoside 3-*N*-acetyltransferase, AAC (3), which makes its host strains highly resistant to KM, dibekacin (DK) and gentamicin (GM) C complex.

Materials and Methods

Strains and Plasmids

Streptomyces griseus strains are listed in Table 1. *Streptomyces lividans* TK21 and plasmids pIJ61 and pIJ702 were used as host and vectors for gene manipulation.

Preparation of DNA

Plasmid DNA was extracted from *S. lividans* TK21 by the method of BIRNBOIM and DOLY[20] and cccDNA was electroeluted from an agarose gel after electrophoresis by the method of GIRVITZ *et al.*[21]. Total DNAs were prepared by the method of OKANISHI and GREGORY[22] with the following slight modification: $CHCl_3$ - isoamylalcohol (1 : 1) was used for elimination of proteins after cell lysis with lysozyme, Pronase E and SDS.

Transformation of *S. lividans* with Plasmids

S. lividans TK21 protoplast cells were prepared and transformed with plasmid DNA according to the method described by CHATER *et al.*[23]. To make protoplasts, strain TK21 was aerobically grown in YEME medium containing sucrose 34%, $MgCl_2$ 5 mM and glycine 0.3% at 28°C for 2 days, washed with P medium and then incubated at 28°C in P medium containing 3 mg/ml of lysozyme (Sigma). To transform protoplasted cells, a suspension (50~100 μl) containing about 4×10^9 protoplasts was mixed with 20 μl of DNA solution followed by 0.4 ml of 25% PEG1000 in T medium (Nakarai) and kept at room temp for 1 minute. The transformation procedure was terminated by addition of 5 ml of P medium and the suspension plated out onto R2YE agar plates and incubated for 16~24 hours at 28°C. The plates were then overlaid with 3 ml of soft R2YE agar medium containing 50 μg/ml of thiopeptin (related to thiostrepton; Fujisawa Pharmaceutical Co., Ltd., Japan) and incubated at 28°C to select transformants. When the *Bam*H I site of the plasmid pIJ61 was used for cloning, ribostamycin (RM) was used instead of neomycin (NM) to confirm the insertional inactivation since the *aph* gene in pIJ61 confers a higher level of resistance to RM than NM in this host (see Table 3).

Manipulation of DNA

To digest DNAs with restriction endonucleases, a buffer consisting of 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT was employed unless otherwise indicated. Ligation of DNAs was performed in a solution containing 66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and T4 ligase (1~5 units; Toyobo, Japan). The ligation mixture (100 μl in total) containing 1 μg of a vector plasmid and 5 μg of donor DNA cleaved with appropriate restriction endonucleases was incubated at 15°C overnight. Digestion and ligation of DNA species were monitored by agarose gel electrophoresis using ethidium bromide (0.5 μg/ml)-containing 0.8% agarose (Seakem ME) in TAE buffer (40 mM Tris-acetate - 2 mM EDTA, pH 8.0). DNA fragments were extracted from agarose gels by the method of GIRVITZ *et al.*[21].

Determination of Antibiotic Resistance Level

Strains of *S. griseus* and *S. lividans* grown on ISP No. 2 medium (yeast extract - malt extract agar; Difco) were incubated on ISP No. 2 medium supplemented with a series of concentrations of an aminoglycoside antibiotic according to the method described previously[24].

In Vitro Inactivation of Antibiotics by Cell Free Extracts

KM-resistant strains of *S. griseus* and *S. lividans* were cultured in TSB liquid medium without addition of KM. Cell free extracts (S30) were prepared by the method described by YAMAMOTO *et al.*[25]. To check inactivation of various aminoglycoside antibiotics in the presence of acetyl CoA, the S30 fractions (10 μl) were mixed with 90 μl of a buffer/cofactor solution consisting of 110 mM Tris-HCl (pH 7.8), 11 mM magnesium acetate, 0.55 mM acetyl CoA, 16 mM ATP disodium salt (in 0.8% $NaHCO_3$) and 110 μg/ml of an aminoglycoside antibiotic, and incubated at 37°C for 90 minutes. The reaction was terminated by heating at 90°C for 5 minutes. Antibiotic activity remaining in the mixture was bioassayed with *Bacillus subtilis* PCI 219 as the test organism.

The inactivated product of KM-A from a reaction mixture containing 20 mg of KM-A, 2 ml of the S30 from *S. lividans* containing pANT3-2 and 2.5 mM acetyl CoA was placed on a column of Amberlite IRC-50 (25 ml; NH_4^+ type) and eluted with 1 N NH_4OH. The eluate (5 ml fractions) was monitored by ninhydrin color reaction following TLC using Silica (Merck 57) and BuOH - MeOH - $CHCl_3$ - NH_4OH (4 : 5 : 2 : 5) as the developing solvent. The ninhydrin positive fractions were applied to a second column of Amberlite CG-50 Type 1 (25 ml; NH_4^+ type). The adsorbed substances were separated by eluting with a NH_4OH gradient (0.05 ~ 0.25 N) and the ninhydrin positive fractions with Rf 0.29 were evaporated (the Rf value of KM-A was 0.22). The residue was dissolved in a small volume of H_2O and its purity examined by TLC. The structure of the purified derivative was determined by ^{13}C NMR, ^1H NMR, ^1H-^1H correlated spectroscopy (COSY), ^{13}C-^1H COSY and nuclear Overhauser effect spectroscopy (NOESY) using Jeol JNM-GX400.

Results

Cloning of Kanamycin-resistance Gene

As shown in Table 1, protoplast regeneration of a SM-producing *S. griseus* SS-1198 and its non-producing mutant NP1-1 yielded KM-resistant clones (SS-1198PR and NP1-1PR, respectively) at a frequency of 10^{-6} without a change in SM-resistance. Phenotypically, the two KM-resistant strains seemed to be identical or very similar. However, digestion of total DNAs with restriction endonucleases revealed substantial differences in their DNA fingerprints as shown in Fig. 1. While the total DNA from the strain SS-1198PR gave substantially the same fingerprint as that of its parent strain SS-1198, specific amplified DNA segments were clearly observed in the total DNAs from strain NP1-1PR. Total DNA of *S. griseus* SK4-3[13] (KM-hyper-resistant) produced by interspecific protoplast

Table 1. Basic properties of the *Streptomyces griseus* strains used.

Strain	Resistance[a]		Production[b]		Amplified segment	Source
	SM	KM	B	HUT		
SS-1198	200	<5	325	125	−	Soil isolate (wild type)
NP1-1	200	<5	0	0	−	UV irradiation of SS-1198
SS-1198PR	200	1,000	0	75	−	PR[c] of SS-1198
NP1-1PR	200	500	0	0	+	PR of NP1-1

[a] Resistance (μg/ml) to streptomycin (SM) and kanamycin (KM) on ISP No. 2 medium.

[b] Titration (μg/ml) as streptomycin in B and HUT media. B: Potato starch 1%, glucose 1%, soy bean meal 1.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1% and NaCl 0.3%. HUT: Glucose 1%, yeast extract 0.2%, meat extract 0.2%, peptone 0.4%, NaCl 0.5% and $MgSO_4 \cdot 7H_2O$ 0.025%.

[c] PR: Protoplast regeneration.

fusion between *S. griseus* NP1-1 and *S. tenjimariensis* NM16, was also found to contain amplified DNA segments different from those observed in strain NP1-1PR. Strain SS-1198PR was chosen as sourse to clone the KM-resistance gene, due to the simplicity of the DNA digestion pattern, although it was recognized that amplified DNA segments might be involved in the resistance.

As shown in Table 2, a 15-kb DNA segment conferring the KM-resistance was first cloned from the *Bcl* I-digested total DNA of the strain SS-1198PR into the *Bam*H I site in *aph* gene of the low copy number plasmid pIJ61[26] with *S. lividans* TK21 as a host. The plasmid was designated as pANT3. Transformants resistant to KM and thiopeptin and with sensitivity to RM were selected. The segment of pANT3 was subcloned into the high copy number plasmid pIJ702[27]. The 4.2-kb *Sph* I fragment and the 1.8-kb *Bgl* II-*Bam*H I fragment were separately subcloned. These plasmids were designated pANT3-1 and pANT3-2, respectively (Fig. 2). The 1.8-kb *Bgl* II-*Bam*H I fragment was completely contained within the 4.2-kb *Sph* I fragment; the *Bgl* II-*Bam*H I fragment inserted in the opposite orientation also conferred KM-resistance. The unique *Bam*H I site in the 1.8-kb *Bgl* II-*Bam*H I fragment was found to be essential for KM-resistance.

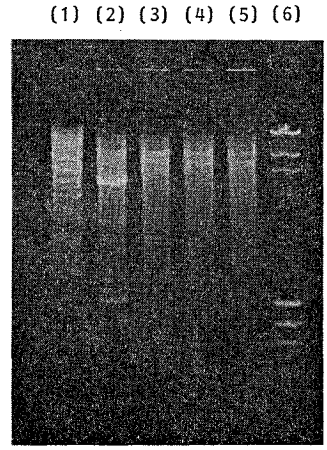
Cloning of the Streptomycin-resistance Gene

In order to verify that the SM-resistance gene was not changed by protoplast regeneration, the SM-resistance genes were also cloned from the total DNAs of strain SS-1198 and the KM-resistant strain SS-1198PR. The total DNAs were cut with *Sph* I and ligated to the *Sph* I site of pIJ702. Transformants were selected as *tsr*/*mel*$^-$ followed by SM-resistance. Thus, the SM-resistance gene was directly cloned from both strains SS-1198 and SS-1198PR into pIJ702 as the 3.8-kb *Sph* I fragment and found to be identical in size, orientation, restriction site and function as shown in Fig. 3 and Table 3. The cloned 3.8 kb *Sph* I fragments were identical to the SM-resistance segments coding for SM-kinase cloned from other strains of *S. griseus*[2,3,28].

No physical linkage was found between the cloned SM-resistance gene segment (or the SM

Fig. 1. Fingerprint of total DNA digested with *Bam*H I.

(1) SK4-3, (2) NP1-1PR, (3) SS-1198PR, (4) NP1-1, (5) SS-1198, (6) size marker.



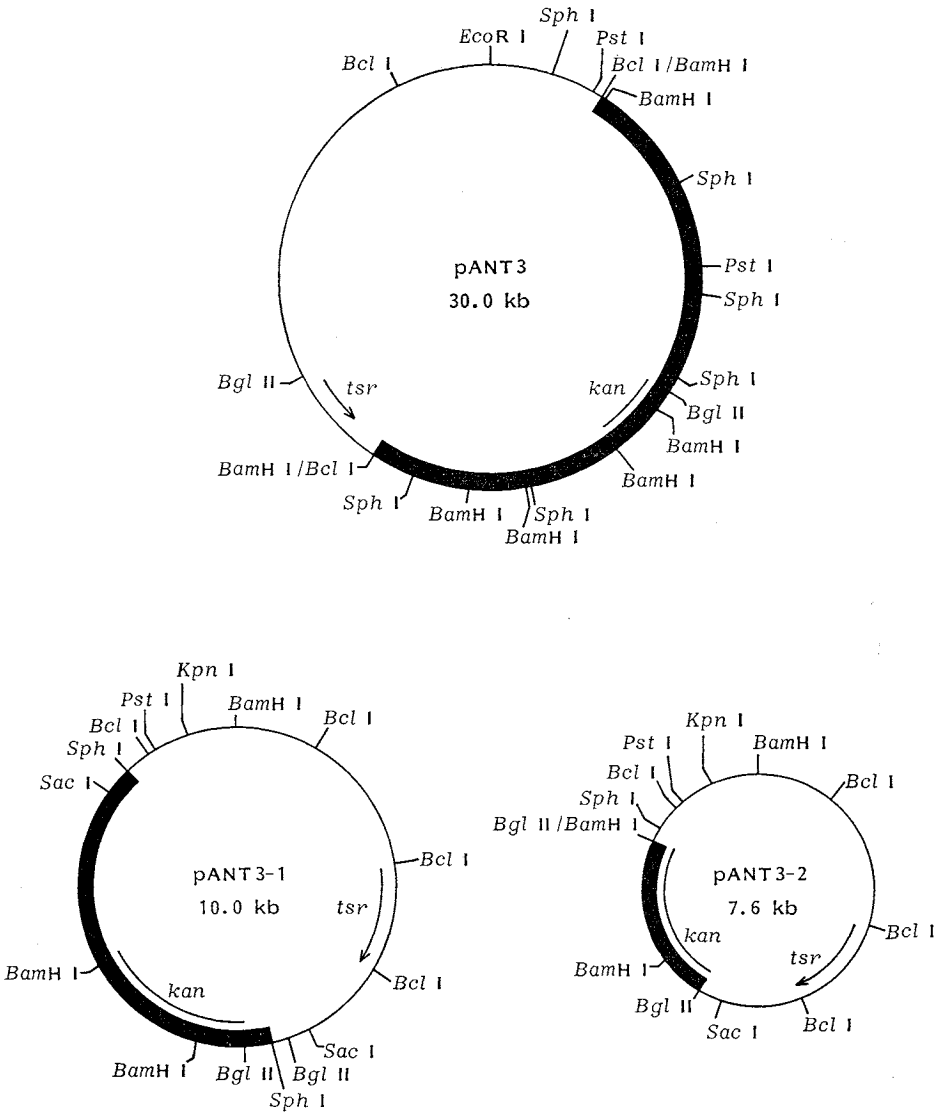
Total DNA (1 μg) from each strain was digested with *Bam*H I, ethanol-precipitated and loaded. *Hin*d III-digested λDNA and *Hae* III-digested ϕX174DNA were used as size marker.

Table 2. Cloning of kanamycin-resistance gene.

Plasmid	Size	Cloned segment	Cloning		Selection of transformant
			Vector	Site	
pANT3	30.0	15.0 kb *Bcl* I-*Bcl* I	pIJ61	*Bam*H I	TS^R, RM^S, KM^R
pANT3-1	10.0	4.2 kb *Sph* I-*Sph* I	pIJ702	*Sph* I	TS^R, MEL$^-$, KM^R
pANT3-2	7.6	1.8 kb *Bgl* II-*Bam*H I	pIJ702	*Bgl* II	TS^R, MEL$^-$, KM^R

TS^R: Thiopeptin (50 μg/ml)-resistance, MEL: melanine formation, KM^R: kanamycin (50 μg/ml)-resistance, RM^S: ribostamycin (50 μg/ml) sensitivity.

Fig. 2. Restriction endonuclease cleavage maps of pANT3, pANT3-1 and pANT3-2.
kan: KM-resistance, *tsr*: thiostrepton-resistance.



gene cluster) and the cloned KM-resistance gene segment.

Antibiotic Resistance Conferred by the Cloned DNA Segments Coding for KM-resistance and SM-resistance

S. lividans TK21 harboring KM-resistance plasmids or the SM-resistance plasmids was examined for levels and patterns of resistance to various aminoglycoside antibiotics, and compared with *S. griseus* strains SS-1198 and SS-1198PR (Table 3). *S. lividans* TK21 containing pANT1 and pANT2 was resistant to SM at 200 μg/ml, the same level of resistance shown by *S. griseus* strains SS-1198 and SS-1198PR. On the other hand, when *S. lividans* TK21 contained pANT3, pANT3-1 or pANT3-2, the strain was multiply resistant to KM, DK and GM at levels comparable to that of the strain SS-

1198PR. The cloned genes conferred multiple aminoglycoside resistance comparable to that of *S. griseus* SS-1198PR, except SM.

It was noted that resistance levels to various aminoglycoside antibiotics were comparable between the strain SS-1198PR and the strain TK21 containing pANT1 or pANT3-2, even though the copy number of the resistance genes should be much higher in plasmid containing TK21 than in strain SS-1198PR. It is possible that the expression of these genes was much lower in *S. lividans* than in *S. griseus*.

Enzymatic Inactivation of Antibiotics by Cell Free Extracts from *S. griseus* SS-1198PR and *S. lividans* TK21 containing pANT1 or pANT3-2

We previously suggested that the resistance mechanism of KM-resistant clones of S. *griseus* generated by protoplast regeneration was not a function of ribosome structure but on enzymatic inactivation by acetyltransferases and a phosphotransferase[13]. Accordingly, cell free extracts (S30) from *S. griseus* SS-1198PR and *S. lividans* TK21 containing pANT1 or pANT3-2 were examined for their ability to inactivate the antibiotics to which these strains showed resistance. As shown in Table 4, a cell free extract from *S. griseus* SS-1198PR inactivated SM in the presence of ATP suggesting phosphorylation. KM, DK, GM and, to lesser extent, NM and paromomycin (PR) were inactivated only in the presence of acetyl CoA, indicating acetylation. Similarly, cell free extracts from *S. lividans* TK21 inactivated SM in the presence of ATP when the strain contained pANT1, and inactivated KM, DK, GM and to lesser extent, NM and PR only in the presence of acetyl CoA when the strain TK21 contained pANT3-2. Thus there was a strict

Fig. 3. Restriction endonuclease cleavage map of pANT1 and pANT2.
str: SM-resistance.

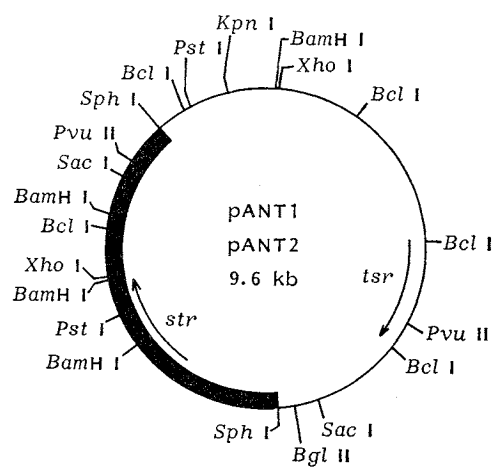


Table 3. Antibiotic-resistance conferred by the cloned resistance genes.

Strain/plasmid	Resistance to aminoglycoside antibiotics (μg/ml)								
	SM	KM	GM	DK	RM	NM	PR	IS	AP
Streptomyces griseus SS-1198	200	—	—	—	—	—	—	—	—
S. griseus SS-1198PR	200	1,000	200	400	25	25	10	—	—
S. lividans TK21	—	—	—	—	—	—	—	5	—
S. lividans TK21/pANT1	200	—	—	—	—	—	—	5	—
S. lividans TK21/pANT2	200	—	—	—	—	—	—	5	—
S. lividans TK21/pANT3	—	400	100	200	25	10	10	5	—
S. lividans TK21/pANT3-1	—	1,000	200	400	5	10	10	5	—
S. lividans TK21/pANT3-2	—	1,000	200	400	5	10	10	5	—
S. lividans TK21/pIJ61	—	—	—	—	200	25	100	5	—
S. lividans TK21/pIJ702	—	—	—	—	—	—	—	5	—

Abbreviations: SM; Streptomycin, KM; kanamycin, GM; gentamicin, DK; dibekacin, RM; ribostamycin, NM; neomycin, PR; paromomycin, IS; istamycin and AP; apramycin.
—: Sensitive to 5 μg/ml of antibiotics tested.

Table 4. Inactivation of aminoglycoside antibiotics by cell free extracts of *Streptomyces griseus* SS-1198PR and *Streptomyces lividans* TK21/pANT3-2.

Antibiotics	*S. griseus* SS-1198PR		*S. lividans* TK21/pANT3-2	
	APH	AAC	APH	AAC
Streptomycin	+++++	−	−	−
Kanamycin A	−	+++++	−	+++++
Dibekacin	−	++++	−	++++
Gentamicin	−	++++	−	++++
Ribostamycin	−	−	−	−
Neomycin	−	++	−	++
Paromomycin	−	++	−	++
Istamycin	−	−	−	−

APH: Phosphorylation, AAC: acetylation.

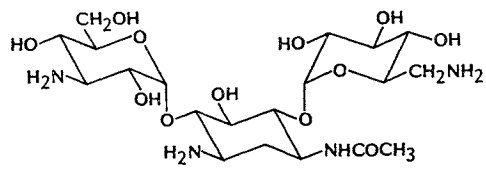
Inactivation of each aminoglycoside antibiotic (100 μg/ml) was scored; +++++ (100%), ++++ (90%), ++ (30%) and − (little or no).

Table 5. ^{13}C NMR chemical shifts of the inactivated kanamycin A.

Carbon assignment	Inactivated KM-A	KM-A	Carbon assignment	Inactivated KM-A	KM-A
1	51.1	51.2	1″	100.8	100.8
2	34.8	36.1	2″	72.5	72.6
3	48.9	49.7	3″	55.1	55.1
4	81.8	87.3	4″	69.9	70.0
5	75.7	74.9	5″	73.0	72.6
6	88.0	88.5	6″	61.1	61.0
1′	99.4	99.8	C=O	174.3	—
2′	72.6	72.6	CH_3	23.2	—
3′	73.6	73.6			
4′	71.0	71.9			
5′	72.0	72.9			
6′	41.5	42.1			

correlation between the resistance patterns and enzymatic inactivation and the biochemical resistance mechanism in the strains was attributed to inactivating enzymes. Antibiotics such as RM and IS, to which the strains were sensitive, were not inactivated.

Fig. 4. Structure of acetylated kanamycin.



3-*N*-Acetylkanamycin A

Structure of the Inactivated KM-A

In order to determine the position of acetylation, the structure of the inactivated KM-A extracted from the reaction mixture containing acetyl CoA was determined by physico-chemical methods: ^{13}C NMR, ^{1}H NMR, ^{13}C-^{1}H COSY, ^{1}H-^{1}H COSY and NOESY in comparison with the data available for 1-acetyl KM-A, 6′-acetyl KM-A and KM-A[29].

As shown in Table 5, signals in the ^{13}C NMR spectrum of the inactivated KM-A were assigned. The signals assigned to the C-2 and C-4 of the 2-deoxystreptamine (DOS) moiety of the substance were shifted to the higher field by 1.3 and 5.5 ppm, respectively, compared to those of KM-A. These chemical shifts reflect the presence of *N*-acetyl group at C-3 of the DOS moiety. The assignment of

the chemical shift of 81.8 ppm to C-4 (not to C-6) was confirmed as follows. The proton signals of the DOS moiety were also assigned by ^{1}H-^{1}H COSY; H-4 was discriminated from 6-H by the NOE between 1′-H and 4-H, and 1″-H and 6-H observed by NOESY (data not shown). Pairing of ^{13}C and ^{1}H by ^{13}C-^{1}H COSY confirmed the assignment of ^{13}C-chemical shifts shown in Table 5. Thus, it was concluded that the inactivated KM-A was 3-*N*-acetyl KM-A (Fig. 4) and accordingly, the acetyltransferase involved in the inactivation of KM-A was of the AAC(3) type. Compared to the substrate specificity of the known AAC(3)[30~34] this enzyme has a unique substrate range (Table 4), thus it is designated AAC(3)-V.

Discussion

Protoplast regeneration of *S. griseus* SS-1198 and its derivative strain NP1-1 yielded KM-hyper-resistant strains, SS-1198PR and NP1-1PR, respectively. In this report, a DNA fragment (designated *kan*) encoding an inactivating enzyme — aminoglycoside 3-*N*-acetyltransferase, AAC(3) — was cloned from strain SS-1198PR into *S. lividans* TK21. Since there was a good correlation between the substrate specificity of the AAC(3) enzyme and the antibiotic resistance range acquired by strain SS-1198PR on protoplast regeneration, the activity of this enzyme must play the major role in the acquired antibiotic resistance.

The AAC(3) enzyme directed by the *kan* gene seems to be unique in its substrate specificity because while KM, DK, GM and, to a lesser extent, NM and PR were inactivated by acetylation at the 3-amino group of their DOS moiety, RM which also contains DOS was not inactivated. This was supported by comparison with the substrate specificity[30~34] and the genes[35,36] of the known AAC(3) enzymes [AAC(3)-I, -II, -III and -IV]. AAC(3)-I is discriminated from the others by its unique and narrow substrate range (GM and fortimicin). The *kan*-directed AAC(3) did not inactivate this group of antibiotics (IS). AAC(3)-II favors GM and KM, but it does not inactivate NM and PR. AAC(3)-III and -IV have a wide substrate range (GM, KM, NM, PR and RM); furthermore AAC(3)-IV inactivates apramycin. Based on these differences as well as the difference in size and restriction endonuclease cleavage of corresponding DNA, the *kan*-directed AAC(3) enzyme was designated as AAC(3)-V. Kinetic studies with the enzyme are in progress.

No significant difference in DNA fingerprint was observed between SS-1198 (parent) and SS-1198PR in spite of the generation of KM-hyper-resistance in the latter strain. This suggested that protoplast regeneration caused a mutation resulting in the expression of a high AAC(3) activity with a novel substrate range in the strain SS-1198PR. This hypothesis was supported by the characterization and comparison of the cloned *kan* genes from these strains in terms of size, restriction sites and function to be described in a subsequent report[37].

No linkage was found between the cloned 15 kb *Bcl* I segment containing the *kan* gene and the streptomycin-resistance gene (*str*) segment (3.8 kb *Sph* I fragment). The *str* genes cloned from both of the strains were identical in size, restriction site and function with each other. This may indicate that the location of the region sensitive to change as a result of protoplast regeneration is distant from the *str* region.

It was of special interest that the *str* gene was identical with those cloned from different strains of *S. griseus* by different research groups. These strains were isolated from soil samples collected at different locations around the world. Studies on hybridization have revealed that the gene cluster for SM biosynthesis and SM-resistance was highly conserved in SM-producing strains of *S. griseus*, while genes homologous to the *kan* gene are distributed specifically in *S. griseus*, independent of SM production[38].

Acknowledgments

We are grateful to Professor D. A. HOPWOOD for *S. lividans* TK21 and plasmids pIJ61 and pIJ702 and to Mr. T. KINAMI for technical assistance. We also express our deep thanks to the late Professor H. UMEZAWA,

and Professor Y. OKAMI for their encouragement and to Professor Y. KOYAMA and Dr. M. YAGISAWA for valuable discussion and advice. This work was supported by a grant from Japan Antibiotics Research Association-Eli Lilly and Company.

References

1) MALPARTIDA, F. & D. A. HOPWOOD: Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in a heterologous host. Nature 309: 462~464, 1984
2) MALPARTIDA, F. & D. A. HOPWOOD: Physical and genetic characterization of the gene cluster for the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2). Mol. Gen. Genet. 205: 66~73, 1986
3) CHATER, K. F. & C. J. BRUTON: Resistance, regulatory and production genes for the antibiotic methylenomycin are clustered. EMBO J. 4: 1883~1892, 1985
4) OHNUKI, T.; T. IMANAKA & S. AIBA: Self-cloning in *Streptomyces griseus* of an *str* gene cluster for streptomycin biosynthesis and streptomycin resistance. J. Bacteriol. 164: 85~94, 1985
5) DISTLER, J.; K. MANSOURI & W. PIPERSBERG: Streptomycin biosynthesis in *Streptomyces griseus*. II. Adjacent genomic location of biosynthetic genes and one of two streptomycin resistance genes. FEMS Microbiol. Lett. 30: 151~154, 1985
6) MURAKAMI, T.; H. ANZAI, S. IMAI, A. SATOH, K. NAGAOKA & C. J. THOMPSON: The bialaphos biosynthetic genes of *Streptomyces hygroscopicus*: Molecular cloning and characterization of the gene cluster. Mol. Gen. Genet. 205: 42~50, 1986
7) STANZAK, R.; P. MATSUSHIMA, R. H. BALTZ & R. N. RAO: Cloning and expression in *Streptomyces lividans* of clustered erythromycin biosynthesis genes from *Streptomyces erythreus*. Biotechnology 4: 229~232, 1986
8) JANSEN, G. R.; M. J. BIBB, C. P. SMITH, J. M. WARD, T. KIESER & M. J. BIBB: Isolation and analysis of *Streptomyces promoters*. *In* Microbiology — 1985. *Ed.*, L. LEIVE, pp. 392~396, American Society for Microbiology, Washington, D.C., 1985
9) BIBB, M. J.; G. R. JANSSEN & J. M. WARD: Cloning and analysis of the promoter region of the erythromycin resistance gene (*erm* E) of *Streptomyces erythraeus*. Gene 41: E357~E368, 1986
10) DENG, Z.; T. KIESER & D. A. HOPWOOD: Expression of a Streptomyces plasmid promoter in *Escherichia coli*. Gene 43: 295~300, 1986
11) WESTPHELING, J.; M. RANES & R. LOSICK: RNA polymerase heterogeneity in *Streptomyces coelicolor*. Nature 313: 22~27, 1985
12) YAMASHITA, F.; K. HOTTA, S. KURASAWA, Y. OKAMI & H. UMEZAWA: 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*. J. Antibiotics 38: 58~63, 1985
13) HOTTA, K.; F. YAMASHITA, Y. OKAMI & H. UMEZAWA: New antibiotic-producing streptomycetes, selected by antibiotic resistance as a marker. II. Features of a new antibiotic-producing clone obtained after fusion treatment. J. Antibiotics 38: 64~69, 1985
14) YAMASHITA, F.; K. HOTTA, Y. OKAMI & H. UMEZAWA: The generation of additional antibiotic resistance by protoplast regeneration of a *Streptomyces griseus* strain. J. Antibiotics 38: 126~127, 1985
15) FURUMAI, T.; K. TAKEDA & M. OKANISHI: Function of plasmids in the production of aureothricin. I. Elimination of plasmids and alteration of phenotypes caused by protoplast regeneration in *Streptomyces kasugaensis*. J. Antibiotics 35: 1367~1373, 1982
16) IKEDA, H.; M. INOUE & S. ŌMURA: Improvement of macrolide antibiotic-producing Streptomycete strains by the regeneration of protoplasts. J. Antibiotics 36: 283~288, 1983
17) BALTZ, R. H.; E. T. SENO, J. STONESIFER, P. MATSUSHIMA & G. M. WILD: Genetics and biochemistry of tylosin production by *Streptomyces fradiae*. *In* Microbiology — 1981. *Ed.*, D. SCHLESSINGER, pp. 371~379, American Society for Microbiology, Washington, D.C., 1981
18) SUGIYAMA, M.; T. KATOH, H. MOCHIZUKI, O. NIMI & R. NOMI: Ribosomal resistance to streptomycin in strains regenerated from protoplasts of a streptomycin-susceptible mutant derived from a streptomycin producer. J. Ferment. Technol. 61: 347~351, 1983
19) HOPWOOD, D. A.: Genetic studies with bacterial protoplasts. *In* Annual Review of Microbiology. Vol. 35. *Ed.*, M. P. STARR *et al.*, pp. 237~272, Annual Reviews Inc., California, 1981
20) BIRNBOIM, H. C. & J. DOLY: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513~1525, 1979
21) GIRVITZ, S. C.; S. BACCHETTI, A. J. RAINBOW & F. L. GRAHAM: A rapid and efficient procedure for the purification of DNA from agarose gels. Anal. Biochem. 106: 492~496, 1980

22) OKANISHI, M. & K. F. GREGORY: Methods for determination of deoxyribonucleic acid homologies in *Streptomyces*. J. Bacteriol. 104: 1086~1094, 1970
23) CHATER, K. F.; D. A. HOPWOOD, T. KIESER & C. J. THOMPSON: Gene cloning in *Streptomyces*. Curr. Topics in Microbiol. Immunol. 96: 69~95, 1981
24) HOTTA, K.; A. TAKAHASHI, N. SAITO, Y. OKAMI & H. UMEZAWA: Multiple resistance to aminoglycoside antibiotics in actinomycetes. J. Antibiotics 36: 1748~1754, 1983
25) YAMAMOTO, H.; K. HOTTA, Y. OKAMI & H. UMEZAWA: Self-resistance of a *Streptomyces* which produces istamycins. J. Antibiotics 34: 824~829, 1981
26) THOMPSON, C. J.; T. KIESER, J. M. WARD & D. A. HOPWOOD: Physical analysis of antibiotic-resistance genes from *Streptomyces* and their use in vector construction. Gene 20: 51~62, 1982
27) KATZ, E.; C. J. THOMPSON & D. A. HOPWOOD: Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. 129: 2703~2714, 1983
28) TOHYAMA, H.; T. SHIGYO & Y. OKAMI: Cloning of a streptomycin resistance gene from a streptomycin producing streptomycete. J. Antibiotics 37: 1736~1737, 1984
29) TODA, S.; S. NAKAGAWA, T. NAITO & H. KAWAGUCHI: Carbon-13 NMR analysis of amikacin and related compounds. Tetrahedron Lett. 1978: 3913~3916, 1978
30) DAVIES, J. & D. I. SMITH: Plasmid-determined resistance to antimicrobial agents. *In* Annual Review of Microbiology. Vol. 32. *Ed.*, M. P. STARR *et al.*, pp. 469~518, Annual Reviews Inc., California, 1978
31) BRZEZINSKA, M.; R. BENVENISTE, J. DAVIES, P. J. L. DANIELS & J. WEINSTEIN: Gentamicin resistance in strains of *Pseudomonas aeruginosa* mediated by enzymatic N-acetylation of the deoxystreptamine moiety. Biochemistry 11: 761~766, 1972
32) LE GOFFIC, F.; A. MARTEL & J. WITCHITZ: 3-N-Enzymatic acetylation of gentamicin, tobramycin, and kanamycin by *Escherichia coli* carrying an R factor. Antimicrob. Agents Chemother. 6: 680~684, 1974
33) BIDDLECOME, S.; M. HAAS, J. DAVIES, G. H. MILLER, D. F. RANE & P. L. J. DANIELS: Enzymatic modification of aminoglycoside antibiotics: a new 3-*N*-acetylating enzyme from a *Pseudomonas aeruginosa* isolate. Antimicrob. Agents Chemother. 9: 951~955, 1976
34) DAVIES, J. & S. O'CONNOR: Enzymatic modification of aminoglycoside antibiotics: 3-*N*-acetyltransferase with broad specificity that determines resistance to the novel aminoglycoside apramycin. Antimicrob. Agents Chemother. 14: 69~72, 1978
35) BRÄU, B.; U. PILZ & W. PIEPERSBERG: Genes for gentamicin-(3)-N-acetyltransferase III and IV. I. Nucleotide sequence of the AAC(3)-IV gene and possible involvement of an IS140 element in its expression. Mol. Gen. Genet. 193: 179~187, 1984
36) ALLMANSBERGER, R.; B. BRÄU & W. PIEPERSBERG: Genes for gentamicin-(3)-N-acetyltransferase III and IV. II. Nucleotide sequences of three AAC(3)-III genes and evolutionary aspects. Mol. Gen. Genet. 198: 514~520, 1985
37) ISHIKAWA, J.; Y. KOYAMA, S. MIZUNO & K. HOTTA: Mechanism of increased kanamycin-resistance generated by protoplast regeneration of *Streptomyces griseus*. II. Mutational gene alteration and gene amplification. J. Antibiotics 41: 104~112, 1988
38) HOTTA, K. & J. ISHIKAWA: Strain- and speices-specific distribution of the streptomycin gene cluster and *kan* related sequences in *Streptomyces griseus*. J. Antibiotics, to submitted, 1987