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

II. MUTATIONAL GENE ALTERATION AND GENE AMPLIFICATION

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Genetic mechanisms involved in the kanamycin (KM)-hyper-resistance of *Streptomyces griseus* SS-1198PR and NP1-1PR generated by protoplast regeneration were investigated. Southern hybridization of *Sph* I-digested genomic DNA with a KM-resistance gene (*kan*) probe revealed that the strain SS-1198PR and its KM-sensitive parent (SS-1198) had the same copy number of a 4.2-kb *Sph* I fragment hybridizing to the *kan* probe, while the *kan* gene of the strain NP1-1PR was located on a highly amplified DNA sequence (100~200 copies/chromosome) consisting of the 6.7 kb amplifiable unit with *Sph* I site at the junction site region. There was no difference in the restriction endonuclease cleavage map of the *kan* gene region of the *Sph* I fragments from the three strains. However, the level (50 μg/ml) of KM-resistance conferred by the cloned NP1-1PR *kan* gene was much lower than that (1,000 μg/ml) conferred by the cloned SS-1198PR *kan* gene in *Streptomyces lividans* TK21 although the strain TK21 harboring these *kan* genes produced an aminoglycoside acetyltransferase, AAC(3)-V, with the same substrate specificity. It seemed, therefore, likely that a mutational alteration of the *kan* gene and a DNA amplification involving the *kan* region played major roles for the KM-hyper-resistance depending on AAC(3)-V in the strains SS-1198PR and NP1-1PR, respectively.

As reported in our previous paper[1], the protoplast regeneration of a streptomycin-producing *Streptomyces griseus* SS-1198 and its non-producing mutant, NP1-1, yielded kanamycin (KM)-hyper-resistant strains SS-1198PR and NP1-1PR, respectively, whose resistance levels were 100~200-fold higher than the parental strains. These two KM-resistant strains were distinguishable from each other in terms of DNA amplification. We have already cloned the SS-1198PR KM-resistance gene (*kan*) directing an aminoglycoside 3-*N*-acetyltransferase designated as AAC(3)-V[1]. Since no obvious DNA amplification was observed in the strain SS-1198PR by agarose gel electrophoresis of its genomic DNA digest, it was suggested that a high activity of the acetyltransferase was due to a mutational alteration of a specific gene that was cryptic or silent in the parental strain SS-1198.

In the strain NP1-1PR, on the other hand, we observed an obviously amplified DNA segment. The structure of amplified DNA sequences reported so far in streptomycetes[2~11] have been discussed in relation to genetic instability of *Streptomyces*. However, no case has provided any straightforward evidence showing that DNA amplification directed a specific phenotypic change whose biochemical basis was clearly characterized. We were interested in knowing if the amplified DNA of the strain NP1-1PR was responsible for the KM-resistance depending on the AAC(3)-V. In this report, char-

acterization of *kan* genes found in parental and hyper-resistant derivatives by Southern blot hybridization and cloning provide evidence that a mutational gene alteration and a DNA amplification play important roles in the KM-resistance of SS-1198PR and NP1-1PR, respectively.

Materials and Methods

Bacterial Strains and Plasmids

Strains of *S. griseus* SS-1198, SS-1198PR, NP1-1 and NP1-1PR, and *Streptomyces lividans* TK21 were used (see Table 1). Plasmids pIJ61 and pIJ702 were used as cloning vectors.

DNA Manipulations

The buffer employed for restriction endonuclease digestion consisted of 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 50 mM NaCl. Ligation buffer consisted of 66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT and 1 mM ATP; and 1~2 units of T4-ligase (Toyobo Co., Ltd.) was used for ligation.

Preparation of DNA

Total genomic DNA was prepared as described by CHATER *et al.*[12]. Plasmid DNA was prepared by the method of BIRNBOIM and DOLY[13] with slight modification. Plasmid DNA and DNA fragments were electroeluted from agarose gel after electrophoresis as described by GIRVITZ *et al.*[14].

Cloning of *kan* Gene Fragments

Total DNA from NP1-1PR was digested with *Sph* I and electrophoresed on 0.8% agarose gel in TAE buffer. The 4.7-kb *Sph* I-band derived from an amplified DNA sequence was isolated from the gel and ligated into the *Sph* I site of pIJ702. Recombinant plasmid DNA was then introduced into *S. lividans* TK21 protoplasts by the method of CHATER *et al.*[12]. Protoplasts were allowed to regenerate on R2YE and transformants were selected by $Thio^r$ followed by KM^r.

To clone the proto-*kan* gene, total DNA from the parental strain SS-1198 was multiply digested with *Bcl* I, *Eco*R I, *Hin*d III, *Kpn* I, *Pst* I and *Sph* I to minimize fragment species having *Sph* I site at both terminal ends by taking advantage of the absence of sites for these enzymes inside the 4.2 kb *Sph* I fragment containing *kan* gene cloned from the strain SS-1198PR. The digested DNA was ligated to the *Sph* I site of pBR322 and introduced into *Escherichia coli* RR1 by the method of KUSHNER[15]. Transformants containing the target *Sph* I fragment were screened by colony hybridization. Transformants grown on LB agar containing ampicillin (35 μg/ml) were replica plated onto a nitrocellulose filter (BA85, 0.45 μm; Schleicher & Schuell). The filters were subjected to denaturation with 0.5 N NaOH - 1.5 M NaCl followed by neutralization with 0.5 M Tris-HCl (pH 8.0) - 1.5 M NaCl and baked for 2 hours at 80°C. Hybridization with a *kan* gene probe was carried out under the conditions described below.

Southern Blot Hybridization

Since the SS-1198PR *kan* gene in pANT3-1[11] is located at a region spanning the 0.5-kb *Bgl* II-*Bam*H I and 1.3 kb *Bam*H I fragments, the latter fragment was isolated from pANT3-1 by agarose gel electrophoresis. It was labeled by nick-translation with [^{32}P]dCTP using kit provided by Amersham (5000). Specific activity of this probe DNA was 2.3×10^8 cpm/μg.

Total DNA from *S. griseus* strains was digested with *Sph* I, electrophoresed on 0.8% agarose gel and then transferred onto a nitrocellulose filter (Schleicher & Schuell BA85, pore size 0.45 μm) as described by SOUTHERN[16]. The filter was prehybridized for 20 hours at 56°C in a solution (7 ml) consisting of 6x SET, 10x DENHARDT's solution, 0.1% SDS and 100 μg/ml of sonicated calf thymus DNA in a plastic bag. The solution was replaced with 7 ml of the fresh solution containing the ^{32}P-labeled probe (7.2×10^6 cpm; 4.5 ng/ml) which had been denatured in a boiling water. After hybridization for 46.5 hours at 56°C, the filter was washed with 4x SSC (100 ml) at room temp (rinse) followed by 2x SSC (200 ml) for 30 minutes at 56°C and then 2x SSC (200 ml) for 30 minutes at 65°C, and air-dried. X-Ray film (Fuji X-ray film RX) was exposed to the air-dried filter at −80°C on an intensifying screen.

Antibiotic Resistance

To determine the antibiotic resistance level, 10~20 μl of aerial mycelium suspensions were streaked on yeast extract - malt extract agar (ISP No. 2; Difco) plates containing aminoglycoside antibiotics at various concentrations. These plates were incubated at 28°C for 3 to 5 days, and the growth was scored.

Inactivation of Antibiotics

Cell free extracts (S30) were prepared from mycelium of *S. griseus* strains and *S. lividans* containing pANT6 by sonication followed by centrifugation. Antibiotic inactivation reactions were carried out at 37°C for 2 hours in a reaction mixture containing cell free extract (25 μl), 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 0.5 mM acetyl CoA, 100 μg/ml of an antibiotic and 1 mM DTT. Antibiotic activity remaining was assayed as described previously[1].

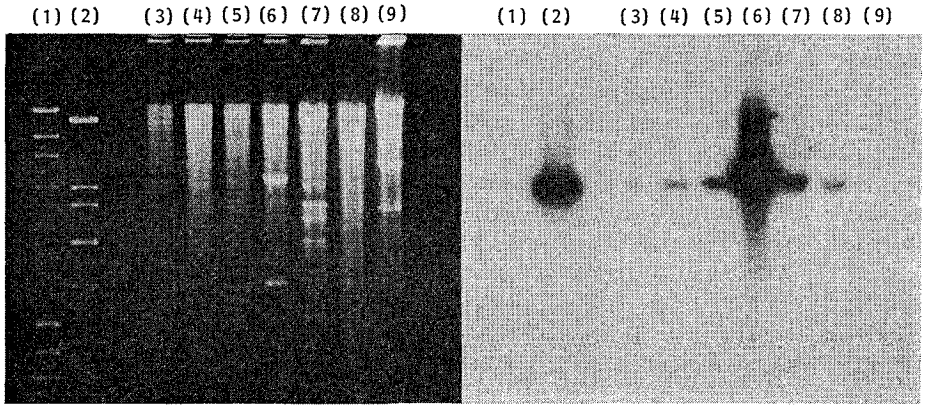
Results

Detection of DNA Sequence Hybridizing to *kan* Gene Segment

In order to characterize gene segments of *S. griseus* SS-1198 and its derivative strains, genomic DNAs from these strains were first subjected to Southern blot hybridization by using the nick-translated 1.3 kb *Bam*H I fragment of pANT3-1 containing the *kan* gene as the probe. It was expected from the restriction map of the 15-kb *Bcl* I fragment containing *kan*[1] that the probe would hybridize to a 4.2-kb fragment. As expected, all strains except NP1-1PR contained this band in roughly equivalent amounts (Fig. 1). The signal was much stronger in NP1-1PR and an amplified DNA fragment of the almost same size could also be visualized ethidium bromide staining. This indicated that NP1-1PR *kan* was on an amplified DNA sequence. All other strains had no significant DNA amplification involving the *kan* region and copy number of *kan* was similar to that of the parental strain SS-1198 (presumably one copy per chromosome). In strains SS-1198 and NP1-1 with sensitivity to 5 μg/ml of KM, KM-acetylating activity produced by their *kan* genes was so weak[17] that it would not contribute to confer KM-resistance. In strain SS-1198PR with high KM-resistance (1,000 μg/ml) and high KM-acetylating activity, it seemed thus obvious that their KM-resistance was dependent on mutation resulting in a high level of AAC(3) activity.

Fig. 1. Southern blot hybridization of *Sph* I-digested genomic DNA of *Streptomyces griseus* strains with *kan* probe.

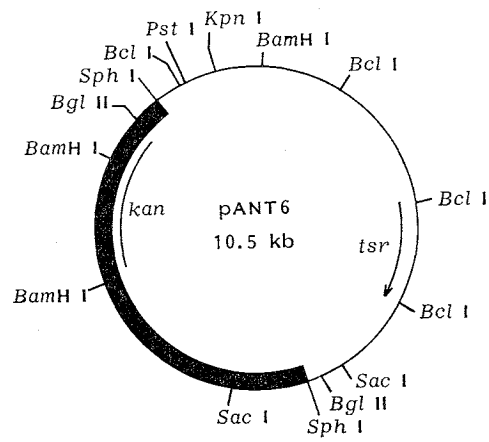
(1) Size marker, (2) pANT3, (3) SS-1198, (4) NP1-1, (5) SS-1198PR, (6) NP1-1PR, (7) SK4-3, (8) SK2-52, (9) TK21/pIJ702.



Cloning and Characterization of *kan* Gene of Strain NP1-1PR

Since the strain NP1-1PR has lower level of KM-resistance than the strain SS-1198PR[1] in spite of the fact that the NP1-1PR *kan* is located on the amplified DNA sequence, it was suggested that KM-hyper-resistance of the strain NP1-1PR was due to a gene dosage effect after amplification of the *kan* region. Subsequently, the *Sph* I fragment (hybridizable to SS-1198PR *kan*) of the amplified DNA segment was electrophoretically purified and cloned into *S. lividans* TK21 with pIJ702. Cleavage with restriction endonucleases of the resultant plasmid, pANT6 (Fig. 2), revealed that the cloned *Sph* I fragment was 4.7 kb in which difference in size and restriction site did not exist in the *kan* gene region but in the *Sac* I-*Sph* I region expanded by 0.5 kb, compared to the 4.2-kb *Sph* I fragment from SS-1198PR. pANT6 was then examined for its ability to confer antibiotic resistance. For comparison, *S. lividans* TK21 containing pANT3-1 that carries the 4.2-kb *Sph* I fragment from the strain SS-1198PR was also examined. As shown in Table 1, *S. lividans* TK21 carrying pANT6 or pANT3-1 was multiply resistant to KM, dibekacin and gentamicin. However, the resistance levels conferred by pANT6 were much lower (1/10~1/20) than that conferred by pANT3-1, indicating that total AAC activity produced by the NP1-1PR *kan* was lower than that produced by the SS-1198PR *kan*. The other fragment (5.35 kb *Bam*H I fragment) cloned into pIJ61 did not confer antibiotic resistance as expected from the lack of the essential region for the resistance (pANT5 in Fig. 3). Antibiotic-inactivating activity requiring acetyl CoA was also examined as shown in Table 2. Cell free extracts from *S. griseus* NP1-1PR and *S. lividans* TK21 carrying pANT6 inactivated KM, dibekacin and gentamicin and to a lesser extent paromomycin and neomycin in a good agreement with their antibiotic resistance patterns and levels. Cell free extracts from strains *S. griseus* NP1-1 and SS-1198 did not inactivate these antibiotics under these conditions.

Fig. 2. Restriction endonuclease cleavage map of pANT6.



Characterization of Amplified DNA Sequence

The amplifiable unit of the amplified DNA

Table 1. Antibiotic resistance conferred by the cloned resistance genes.

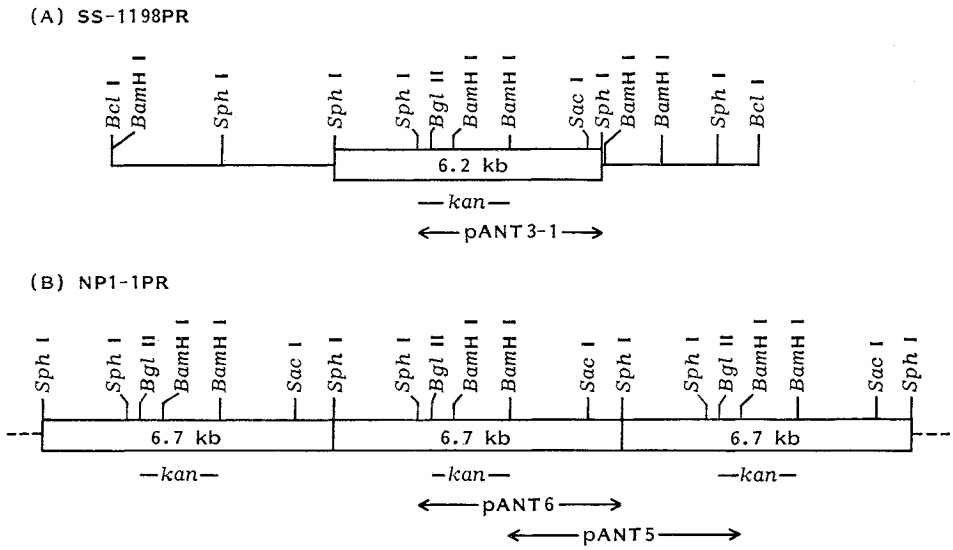
Strain	Antibiotic resistance (μg/ml)								
	KM	GM	DK	RM	PR	NM	IS	AP	SM
Streptomyces griseus SS-1198	—	—	—	—	—	—	—	—	200
S. griseus SS-1198PR	1,000	200	400	25	25	10	—	—	200
S. griseus NP1-1	—	—	—	—	—	—	—	—	100
S. griseus NP1-1PR	400	25	100	5	10	5	—	—	100
S. lividans TK21/pIJ702	—	—	—	—	—	—	5	—	—
S. lividans TK21/pANT3-1	1,000	200	400	5	10	10	5	—	—
S. lividans TK21/pANT6	50	25	50	5	5	5	5	—	—

Abbreviations: KM; Kanamycin A, GM; gentamicin C complex, DK; dibekacin, RM; ribostamycin, PR; paromomycin, NM; neomycin, IS; istamycin, AP; apramycin, SM; streptomycin.

—: Sensitive to 5 μg/ml of antibiotic.

sequence was determined by single and double digestion with restriction endonucleases of the genomic DNA of the strain NP1-1PR. Digestion with *Sph* I yielded a 1.95-kb fragment in addition to 4.7 kb fragment which hybridized to the SS-1198PR *kan* probe as shown in Fig. 1. These two fragments are expected from the restriction map of the 15-kb *Bcl* I fragment of pANT3 that contained SS-1198PR *kan* (Fig. 3). Digestion with *Bam*H I and *Bgl* II yielded 5.35 kb, 1.3 kb, and 6.7 kb fragments, respectively. These results indicated that the amplifiable unit was 6.7 kb long. Furthermore, double digestion with *Bam*H I and *Sph* I of the 5.35-kb *Bam*H I fragment yielded 0.8 kb, 1.95 kb and 2.6 kb fragments. All of the fragments obtained by single and double digestion would be expected only if the 6.7-kb *Sph* I segment was tandemly amplified or rearranged as illustrated in Fig. 3. Thus the amplifiable unit consisted of a 6.7-kb sequence with *Sph* I site at the junction site region. It was noted that the distance between this *Sph* I site and adjacent *Sac* I site was expanded by 0.5 kb compared to the corresponding region of the 4.2 kb *Sph* I fragment from SS-1198PR. The copy number of the

Fig. 3. Rearrangement of genomic DNA in the strain NP1-1PR.



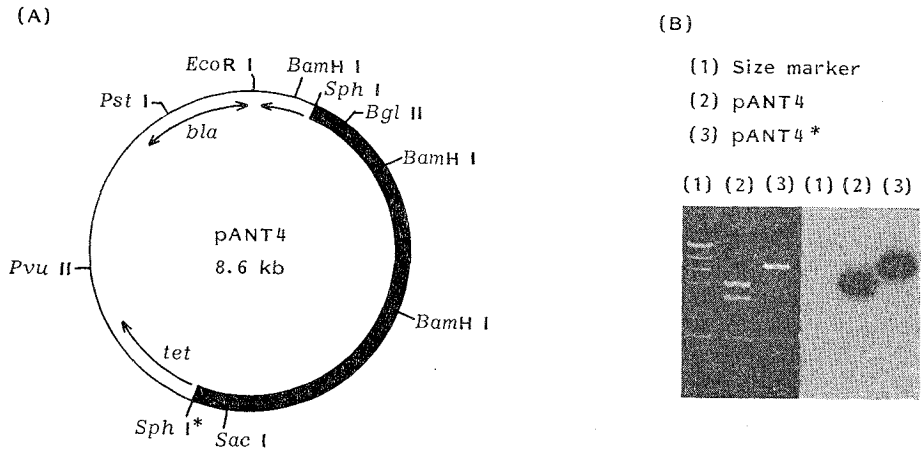
(A) 15 kb *Bcl* I fragment cloned from the strain SS-1198PR. (B) Tandemly repeated 6.7 kb *Sph* I fragment present in the strain NP1-1PR. pANT5 and pANT6 refer to the inserted 5.35 kb *Bam*H I fragment into pIJ61 and the inserted 4.7 kb *Sph* I fragment into pIJ702, respectively, from the amplified DNA segment.

Table 2. Inactivation of aminoglycoside antibiotics by cell free extract.

Antibiotics	*Streptomyces griseus* NP1-1	*Streptomyces griseus* NP1-1PR	*Streptomyces lividans* TK21/pANT6
Kanamycin	−	+++	+++
Dibekacin	−	+++	+++
Gentamicin	−	+++	+++
Neomycin	−	++	++
Paromomycin	−	++	++
Ribostamycin	−	−	−
Istamycin	−	−	−

Inactivation of each antibiotic (100 μg/ml) in the presence of acetyl CoA was scored; +++ (100%), ++ (60%) and − (little or no).

Fig. 4. Restriction endonuclease cleavage map of pANT4 and pANT4*.



(A) Cleavage map: *Sph* I site with an asterisk refers to the site not present in pANT4*. (B) Southern blot hybridization with *kan* probe: Plasmids were double digested with *Pst* I and *Sph* I. Lack of one *Sph* I site in pANT4* was discriminated by the hybridization of the probe to the longer fragment.

amplified sequence was estimated 100~200 per chromosome.

The stability of the amplified DNA was also examined by comparing the intensity and restriction pattern of amplified DNA bands of a series of subcultures of the strain NP1-1PR. There was no substantial difference between the initial subculture and the 7th subculture, indicating that the amplified DNA was stably maintained regardless of the presence of KM as a pressure.

Cloning and Characterization of Parental *kan* Gene

The 4.2-kb *Sph* I fragment of the parental strain SS-1198 was also cloned. Based on the restriction map of the 4.2-kb *Sph* I fragment of the strain SS-1198PR, the total DNA from the strain SS-1198 was subjected to multiple digestion with *Bcl* I, *Eco*R I, *Hin*d III, *Kpn* I, *Pst* I and *Sph* I in order to minimize fragments having *Sph* I site at both terminal ends. The digest was ligated to the *Sph* I cut pBR322 and the ligation mixture was used to transform *Escherichia coli* RR1. Two positive transformants were selected by colony hybridization and confirmed to contain the target fragment by digestion with restriction endonucleases as shown in Fig. 4. However, double digestion with *Sph* I and *Pst* I revealed the presence of two species of plasmids. As shown in Fig. 4, one plasmid designated as pANT4 provided three fragments (1.3, 3.1 and 4.2 kb) while the other plasmid pANT4* provided two fragments (1.3 and 7.3 kb). *Bam*H I digestion of both plasmids, on the other hand, provided the same three fragment (1.0, 1.3 and 6.3 kb). These results indicated that the orientation of insertion of the 4.2 kb *Sph* I fragment was same between pANT4 and pANT4* and, however, in pANT4* one *Sph* I site was eliminated. This *Sph* I site which was not found in pANT4* corresponded to the site located at the junction between the repeated DNA sequences. Although this might indicate an instability associated with this junction region, it might have also been a random event. There was no difference in restriction sites and size of the *kan* region among strains SS-1198 (pANT4), SS-1198PR (pANT3-1) and NP1-1PR (pANT6).

Subsequently, we tried to subclone the SS-1198 4.2 kb *Sph* I fragment from *E. coli* RR1 into

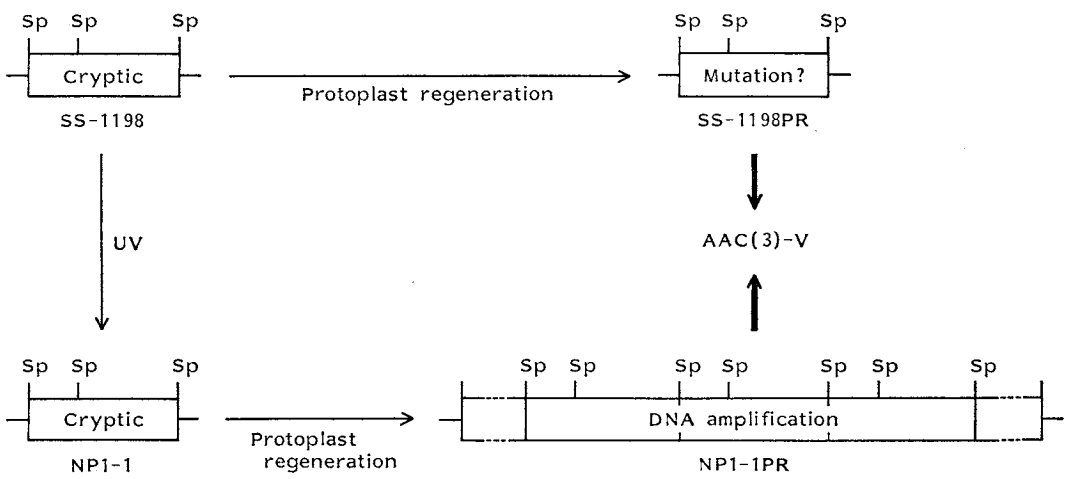
S. lividans. Repeated attempts were unsuccessful except in one case where a single transformant was obtained. The recombinant plasmid contained a cloned fragment which was easily deleted during cultivation even in the presence of selective pressures such as thiopeptin and/or low concentration of KM. Therefore, the level of antibiotic resistance could not be measured. *E. coli* RR1 carrying pANT4 was also sensitive to KM (5 μg/ml) as well as the other antibiotics.

Discussion

In order to characterize genetic mechanisms contributing to KM-resistance, a gene coding for KM acetyltransferase was cloned from *S. griseus* hyper-resistant strains, SS-1198PR and NP1-1PR. *Sph* I fragments containing the *kan* gene were cloned from these strains and the parental strain, SS-1198, and compared with respect to DNA arrangement, restriction site and capability of conferring antibiotic resistance. There was no substantial difference in the restriction maps and size between strain SS-1198PR and its parent SS-1198 and they showed a comparable copy number of the *kan* gene with each other. In contrast, the *kan* gene region of the strain NP1-1PR was found to be located in an highly amplified DNA sequence. The copy number of the *kan* gene was 100~200-fold higher in the strain NP1-1PR than in the other strains. No marked difference in DNA restriction sites was found between the parental strain and KM-hyper-resistant strains except for the expanded *Sac* I-*Sph* I region in the strain NP1-1PR. However, the abilities of SS-1198PR *kan* and NP1-1PR *kan* to confer antibiotic resistance were markedly different. Although both the antibiotic resistance and the antibiotic inactivation pattern was almost the same, the antibiotic resistance level conferred by the NP1-1PR *kan* was only 1/10~1/20 of that conferred by the SS-1198PR when compared under the same condition in *S. lividans*. Based on these evidence, it seemed highly likely that the KM-hyper-resistance of the strain SS-1198PR was dependent on a mutational gene alteration (*e.g.* base substitution) resulting in the expression of high AAC(3) activity, and that DNA amplification involving *kan* region would play an important role for the KM-hyper-resistance of the strain NP1-1PR as illustrated in Fig. 5. We are now studying whether the difference in antibiotic resistance level conferred by *kan* genes reflect the difference in transcriptional or translational level or the specific activity of AAC(3) enzyme itself. Furthermore, comparison of DNA sequence of *kan* genes will reveal the precise difference.

What is the direct product of the *kan* gene? It is highly probable that *kan* encodes AAC(3) itself. However, we can not rule out the possibility that a regulatory gene may positively control the expression of a cryptic gene present in both *S. griseus* and *S. lividans*. We hope to answer this question by comparing the nucleotide sequence of the cloned segment with the amino acid sequence of the

Fig. 5. Genetic changes generated by protoplast regeneration.



purified AAC(3) enzyme.

Although it was critical to compare the function and potential of *kan* genes under the same conditions, we were unable to clone the parental *kan* gene contained on a 4.2-kb *Sph* I fragment into *S. lividans* in spite of repeated attempts. Alternatively, we also attempted to clone the *Bgl* II-*Sac* I fragment (3.6 kb) internal to the *Sph* I fragment. These attempts were also unsuccessful with the exception of one plasmid which was unstable in *S. lividans*. It might be possible that the expression of the parental *kan* gene had toxic effects.

DNA amplification generated by protoplast regeneration or interspecific fusion of *S. griseus* occurred at least at two different regions. In the strain NP1-1PR, a homogeneous amplification of 6.7 kb DNA sequence involving *kan* region was determined. Hybridization with the *kan* gene probe showed that the obviously amplified DNA sequence of the strain SK4-3 did not involve the *kan* region (probably the entire 6.7 kb sequence) although digestion with restriction endonucleases of total DNA yielded many amplified bands suggesting several events (see Fig. 1). Therefore, it seemed highly likely that DNA amplification in the strain SK4-3 occurred in at least some different region(s) from the amplified region of the strain NP1-1PR. In addition, hybridization studies using the cloned streptomycin-resistance gene probe showed that neither amplifications involved the gene cluster region for SM-biosynthesis and SM-resistance (data not shown). These observations suggest that amplified DNA segments generated in these strains may reflect a "regional instability" as described by HASEGAWA *et al.*[10] In this context, it was notable that one terminal region (*Sac* I-*Sph* I region) of the 6.7-kb amplifiable unit in the strain NP1-1PR was expanded by 0.5 kb, compared to the corresponding region of the strains, SS-1198PR and SS-1198. It might be possible that a DNA rearrangement inside of the *Sac* I-*Sph* I region produced a specific sequence promoting amplification.

Occurrence of such intensively amplified DNA sequence have been well known in mutant strains of various *Streptomyces* species[2~11] and discussed in relation to genotypic instability. No cases, however, have been demonstrated to be associated with any biochemically characterized phenotype change. Therefore, to our knowledge, the DNA amplification occurred in the strain NP1-1PR is the first case in this regard.

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