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Note

Kanamycin Acetyltransferase Gene from Kanamycin-producing *Streptomyces kanamyceticus* IFO 13414

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A kanamycin producer, *Streptomyces kanamyceticus* IFO 13414 is highly resistant to kanamycin. Cloning of the kanamycin resistance genes in *S. lividans* 1326 with pIJ702 gave several kanamycin resistant transformants. Two transformants, *S. lividans* SNUS 90041 and *S. lividans* SNUS 91051 showed similar resistance patterns to various aminoglycoside antibiotics. Gene mapping experiments revealed that plasmids pSJ5030 and pSJ2131 isolated from the transformants have common resistant gene fragments. Subcloning of pSJ5030 gave a 1.8 Kb gene fragment which showed resistance to kanamycin. Cell free extracts of *S. lividans* SNUS 90041, *S. lividans* SNUS 91051 and subclone a *S. lividans* SNUS 91064 showed kanamycin acetyltransferase activity. The detailed gene map is included.

Key words : Kanamycin, Acetyltransferase gene, Cloning, Resistance, Antibiotic producing organism

INTRODUCTION

Many *Streptomyces* show resistance to the antibiotics they produce (Cundliffe, 1989). Some of them have intrinsically resistant ribosomes to their own antibiotics. Many antibiotics are biosynthesized as inactive forms in producing organisms. Antibiotic-inactivating enzymes are identified in antibiotic producing organisms. Some aminoglycoside antibiotics are biosynthesized as phosphorylated (streptomycin, Sugiyama *et al.*, 1983 paromomycin, Pérez-Gonzàlez *et al.*, 1989) or acetylated (neomycin, kanamycins, etc. Davies *et al.*, 1979; Thompson *et al.*, 1982) forms which do not have antibacterial activities (Goo *et al.*, 1994). A kanamycin producer, *Streptomyces kanamyceticus* shows two kinds of resistant mechanisms, acetyltransferase (Matsuhashi *et al.*, 1984) and ribosomal resistance (Goo, 1994; Hotta *et al.*, 1981; Nakano *et al.*, 1984; Nakano *et al.*, 1989). Currently, several kinds of ribosomal resistance mechanisms against antibiotics are reported (Yamamoto *et al.*, 1981). Methylation of 16S ribosomal RNA by specific methylases (Holmes and Cundliffe, 1991; Piendle *et al.*, 1984), or of 23S rRNA by a RNA-pentose methylase (Cundliffe, 1978; Cundliffe and Thompson, 1979), possession of N_6,N_6-dimethyladenine within 23S rRNA (Skinner and Cundliffe, 1980) or possession of resistant ribosomal protein S12 (Bonny *et al.*, 1991) are known to be responsible for ribosomal resistance against antibiotics. It has been suggested that kanamycin producing organisms express acetyltransferase at early stage (Satoh, 1975), but ribosomal resistance mechanism is suggested to be responsible for self-protection against kanamycin during production of the kanamycin in the kanamycin producer (Nakano *et al.*, 1989). Two different resistance mechanisms may be expressed depending on the concentration of kanamycin during biosynthesis of kanamycin in kanamycin producing organism (Nakano *et al.*, 1989). To understand the control of kanamycin resistance gene expression in producing organisms, we cloned kanamycin resistance genes from a kanamycin producing organism. In the present paper, we wish to report cloning of acetyltransferase gene.

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MATERIALS AND METHODS

Organisms

S. kanamyceticus IFO 13414 was from the Institute for Fermentation in Osaka and *S. lividans* 1326 and *S. lividans* 3131 (pIJ702) were kindly supplied by Dr. Thompson.

Antibiotics

Kanamycin sulfate and neomycin sulfate were from Jeil Pharmaceutical Co., Gentamicin was from Yu-Han Pharmaceutical Co.. Tobramycin and amikacin were from Dong Wha Pharmaceutical Co., and Dong-A Pharmaceutical Co. respectively. Streptomycin sulfate was purchased from Fluka Co..

DNA preparation

Chromosomal DNA was isolated from *S. kanamyceticus* IFO 13413 by employing the method reported by Hopwood (Hopwood *et al.*, 1987). Plasmids pIJ702 and pSJ597 were isolated from *S. lividans* 3131 and *Streptomyces* 8810-597A (Joe, 1992), respectively by using alkaline denaturation method (Kieser, 1984).

Plasmid DNA isolation (small scale)

Plasmid DNA was isolated from *Streptomyces* grown in TSB (tryptic soy broth, Sigma) by the reported method (Joe and Goo, 1994).

Cloning of kanamycin resistance gene

Chromosomal DNA fragments of 3~10 kilobase (Kb) size (200 μg/ml) that was obtained from *S. kanamyceticus* IFO 13414 by treatment of *Sau* 3A1 and with *Bcl* I, followed by sucrose density gradient centrifugation were ligated with pIJ702 which was cleaved with *Bgl* II and dephosphorylated, or with vector pSJ 597 that was cleaved with *Bcl* I, in a ligation mixture (Tris-HCl 50 mM pH 7.8, $MgCl_2$ 10 mM, dithiothreitiol 20 mM, ATP 1 mM, bovine serum albumin 50 μg/ml) with T4 ligase at 4°C overnight. The ligated mixture was precipitated with 2 volumes of cold ethanol and the precipitate was dissolved in 20 μl of TE buffer (Tris-HCl 10 mM pH 8.0, EDTA 1 mM) and mixed with protoplasts (4×10^9) (Okanishi *et al.*, 1974) of *S. lividans* 1326 in a small volume of P medium. Transformation was done by addition of polyethylene glycol 1000 (T medium, 0.5 ml, 20%) (Thompson *et al.*, 1982). Transformed protoplasts were regenerated on R2YE medium at 38°C for 7 days.

Selection of kanamycin resistant transformants

Colonies on the regeneration plates were replicated onto the ISP No 4 medium containing kanamycin sulfate (50 μg/ml). Colonies grown on plates were selected as transformants.

Determination of antibiotic resistance pattern

Antibiotic resistance was determined by streaking aerial mycelia on ESP No. 4 agar (Difco) plates containing aminoglycoside antibiotics. After incubation at 28°C for 5 days, growth of organisms in the plates was examined.

Gene mapping of kanamycin resistant fragment

Plasmids of transformants were isolated by alkaline denaturation method (Kieser, 1984). The size of the plasmid and the DNA fragment inserted in a vector was compared with marker DNAs by agarose gel elctrophoresis. Electrophoresis of restriction fragments on agarose gel gave the size of the restriction fragments (Southern, 1975).

Subcloning of kanamycin resistant fragment.

Plasmid pSJ5031 was isolated from kanamycin resistant transformant *S. lividans* SNUS 90041. pSJ5031 was treated with *Bcl* I and ligated to pIJ702 that was cleaved with *Bcl* I. The protoplasts of *S. lividans* 1326 was transformed with ligated DNA. Regeneration of the cell on R2YE medium 38°C for 7 days and replication of the colonies onto ISP No. 4 plates containing kanamycin sulfate (50 μg/ml) allowed isolation of kanamycin resistant colonies. Plasmids were isolated from the kanamycin resistant colonies and their genes were mapped. Colonies containing smaller resistant fragments were selected as subclones.

RESULTS

Characterization of the kanamycin-resistant transformants

The resistance of *S. kanamyceticus* IFO 13414, host strain *S. lividans* 1326, *S. lividans* 3131 (pIJ702), a wild strain *Streptomyces* spp. 8810-597A (pSJ597), kanamycin resistant transformants *S. lividans* SNUS 90041 and *S. lividans* SNUS 91051 was examined against various aminoglycoside antibiotics (Table I). *S. lividans* 1326, *S. lividans* 3131 and *Streptomyces* 8810-597A are sensitive to the aminoglycoside antibiotics tested in the present study. The kanamycin producer, *S. kanamyceticus* IFO 13414, is highly resistant to kanamycin, moderately resistant to amikacin and streptomycin, but is

Table I. The concentrations of aminoglycoside antibiotics in the agar (ISP No. 4) plates which allowed growth of organisms

Strain (plasmid)	Aminnoglycoid antibiotics* (μg/ml)						
	SM	KM	GM	SI	TM	AK	NM
S. kanamyceticus IFO13414	125	1000	7.8	31.2	3.9	125	31.2
S. lividans 1326	7.8	3.9	7.8	7.8	1.9	-	1.9
S. lividans 3131 (pIJ702)	7.8	7.8	7.8	3.9	1.9	1.9	1.9
Streptomyces sp. 8810-597A (pSJ597)	3.9	-	-	3.9	1.9	1.9	-
S. lividans SNUS 90041 (pSJ5031)	7.8	1000	15.6	31.2	250	250	31.2
S. lividans SNUS 91051 (pSJ2131)	7.8	1000	15.6	31.2	500	250	31.2

*SM: streptomycin; KM: kanamycin; GM: gentamicin; SI: sisomicin; TM: tobramycin; AK: amikacin; NM: neomycin.

sensitive to tobramycin, neomycin, gentamicin and sisomicin. Two transformants *S. lividans* SNUS 90041 and *S. lividans* SNUS 91051 show same resistance patterns to aminoglycoside antibiotics. They are highly resistant to kanamycin, tobramycin, and amikacin, but sensitive to streptomycin, gentamicin, sisomicin and neomycin. Transformation of *S. lividans* 1326, with plasmids pSJ5031 and pSJ2131 which were isolated from *S. lividans* SNUS 90041 and *S. lividans* SNUS 91051, respectively, produced colonies which contained the plasmid DNAs and resistance to kanamycin was observed in these colonies. The resistances against of aminoglycoside antibiotics in these organisms were confirmed to be encoded in their plasmids.

Subcloning of the resistance gene

Plasmid pSJ5031 and pSJ2131 isolated from *S. lividans* SNUS 90041 and *S. lividans* SNUS 91051, respectively, contained a 2.4 Kb gene fragment. Actually pSJ2131 contained a 5.5 Kb insert which had the insert of pSJ5031 (Fig. 1). This result indicated that the kanamycin resistance was coded in a 2.4 Kb fragment. Treatment of 2.4 Kb insert of pSJ5031 with *Bcl* I, ligation with pIJ702 cleaved with *Bcl* I, transformation on *S. lividans* 1326, and selection with media containing kanamycin gave a tranformant, *S. lividans* SNUS 91064. From the transformant, a plasmid pSJ 2030 was isolated. It contained an 1.8 Kb insert in pIJ 702. The restriction map of the 1.8 Kb insert is shown in Fig. 2.

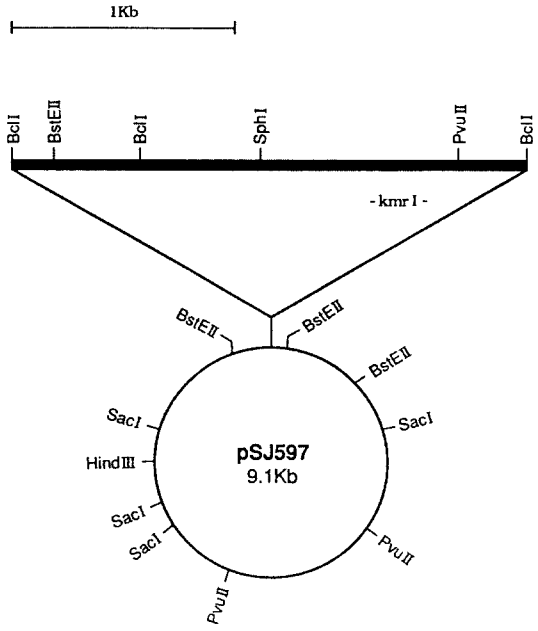

Fig. 1. Restriction map of the kanamycin resistant fragment inserted in pSJ5031 isolated from *S. lividans* SNUS 90041.

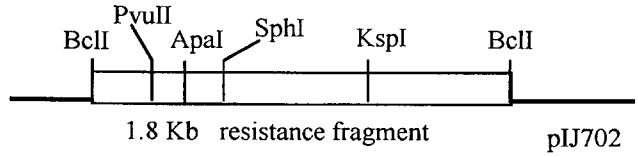

Fig. 2. Restriction map of the kanamycin resistant fragment isolated from subclone *S. lividans* SNUS 91064

DISCUSSION

Cloning of kanamycin resistance genes from a kanamycin producer *S. kanamyceticus* IFO 13414 gave several transformants which showed resistance against kanamycin. Two of the transformants, *S. lividans* SNUS 90041 and *S. lividans* SNUS 91051, showed very similar resistance patterns against aminoglycoside antibiotics. The transformant obtained by Nogaoka group which contained a *BclI* gene fragment of 1.3 Md size showed resistance against kanamycin and ribostamycin, but did not show resistance against neomycin and gentamicin. The gene fragment was confirmed to encode kanamycin-6'-*N*-acetyltransferase (Murakami *et al.*, 1983; Matsuhashi *et al.*, 1984). Their results are very similar with ours. Although the transformants, *S. lividans* SNUS 90041 and SNUS 91051 contained plasmids originated from pSJ597 and pIJ702, respectively, both transformants showed resistance against kanamycin at the concentration of 1000 μg/ml.

When cell free extracts of transformants were examined by the reported method (Goo *et al.*, 1994), *S. lividans* SNUS 90041, and *S. lividans* SNUS 91051 showed strong kanamycin-transacetylation activities. The host strain *S. lividans* 1326 did not show kanamycin-acetyltransferase activity. The results revealed that the transformant possesses an acetyltransferase gene which codes a kanamycin-inactivating enzyme. They contained a common resistance gene fragment. Subclone *S. lividans* SNUS 91064, which contained a 1.8 Kb resistance fragment showed resistance to kanamycin.

Acetylation of kanamycin in kanamycin-producing strain has been reported by Satoh *et al.* (1975), and AAC(6') (6'-*N*-acetyltransferase) was cloned by Matsuhashi *et al.* (1985). Satoh *et al.* (1975) have assumed that kanamycin acetyltransferase is the enzyme associated with kanamycin-biosynthesis. From our previous experiments on resistance mechanism of antibiotic-producing *Streptomyces* (Goo *et al.*, 1994), ribosome of *S. kanamyceticus* IFO 13414 was resistant to kanamycin. Currently, acetyltransferase is may be involved in the biosynthesis of kanamycin, but the enzyme is not to be responsible for self-resistance against kanamycin in the kanamycin-producer (Nakano *et al.*, 1989).

When we compared the resistance patterns of the

transformants with the wild kanamycin producing strain, *S. kanamyceticus* IFO 13414 with transformants, they showed a little different resistance patterns to aminoglycoside antibiotics. The transformants of *S. lividans* containing plasmids with a kanamycin-ribosomal resistance gene cloned from the same kanamycin producer showed strong resistance against gentamicin which was actually very different from those cloned with kanamycin-acetyltransferase (Joe, 1992). The kanamycin producer, *S. kanamyceticus* IFO 13414 seemed to express the kanamycin-acetyltransferase gene only when it is grown in the minimal medium (ISP No. 4) containing kanamycin, but it did not express the ribosomal resistance gene in the same medium although the medium contained kanamycin. It seemed that kanamycin was not good enough to induce the kanamycin-ribosomal resistance gene. Kanamycin-ribosomal resistance gene might be expressed in the kanamycin-producer only with other kanamycin-biosynthesis genes during biosynthesis of kanamycin. The result was very well consistant with those of Hotta *et al.* (1981) and Nakano *et al.* (1984), who reported that the ribosomal resistance was not able to be confirmed biochemically with mycellia of the kanamycin-producer, *S. kanamyceticus* when is was grown in the kanamycin-acetyltransferase liquid media. Satoh *et al.* (1975) observed that the expression of kanamycin-acetyltransferase was increased very rapidly in *S. kanamyceticus* during the lag phase of growth in the kanamycin-production medium, but its activity was decreased abruptly in the log phase of growth when production of kanamycin was started and increased very rapidly.

Many anibiotics producing strains showed presence of multiple resistance (Murakami *et al.*, 1983). *S. kanamyceticus* IFO 13414 also has multiple resistance patterns. We also isolated several other kanamycin-resistant transformants including *S. lividans* SNUS 90041 and *S. lividans* SNUS 91051 from cloning of the resistance genes against kanamycin against *S. kanamyceticus* IFO 13414 (Joe, 1992). The two transformants contained a ribosomal resistance gene to kanamycin. We are currently studing the resistance mechanism of the transformants. Ribosomal resistance of kanamycin producing *S. kanamyceticus* was studied briefly by Nakano *et al.*, (1988). Ribosomal resistance gene is thought to be induced during kanamycin production.

Kanamycin acetyltransferase was cloned from *S. kanamyceticus* (Matsuhashi *et al.*, 1984) and from *S. griseus* SS-1193PR which was isolated during protoplast regeneration (Hotta *et al.*, 1988; Ishikawa *et al.*, 1988). But the gene map of the resistance determinant of *S. kanamyceticus* was not examined in detail. The gene map of the resistance determinant of *S. griseus* SS-1198PR showed no homology with the gene cloned in this study from *S. kanamyceticus.*

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