EXPRESSION OF THE KANAMYCIN RESISTANCE GENE IN A KANAMYCIN-PRODUCING STRAIN OF *STREPTOMYCES KANAMYCETICUS*

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The previously cloned kanamycin resistance gene (*kmr*) from *Streptomyces kanamycetticus* ISP5500 was shown to modify the 30S ribosomal subunit in a subunit exchange experiment. The *kmr* gene, which was normally repressed in *S. kanamyceticus*, appeared to be induced under growth conditions which activated kanamycin biosynthesis. S1 mapping analysis revealed that the expression of the *kmr* gene was regulated at the transcriptional level. Acetylation of kanamycin is another resistance mechanism in the kanamycin producer. However, unlike *kmr*-mediated resistance, the enzyme which catalyzed acetylation was not regulated coordinately with kanamycin biosynthesis.

The successful molecular cloning of the actinorhodin biosynthesis genes of *Streptomyces coelicolor* and their expression in a heterologous host[1], has stimulated efforts to successfully clone other antibiotic biosynthetic genes. In many cases, the genes required for the biosynthesis of an antibiotic have been shown to be tightly linked to the genes encoding resistance to the same antibiotic and genes involved in regulating the synthesis of the antibiotic[2~5]. Taking advantage of this fact, the erythromycin biosynthetic genes were cloned using the resistance gene as a tool[6]. We are interested in the mechanism by which the expression of clustered biosynthesis genes is controlled, and how the expression of the resistance gene is regulated with respect to biosynthesis genes. Solutions to these problems will throw light on the regulation of secondary metabolism and differentiation in *Streptomyces*. In this report, we describe the characterization of the previously cloned kanamycin resistance gene (*kmr*)[7] and its transcriptional regulation in the kanamycin-producing strain.

Materials and Methods

Bacterial Strains and Media

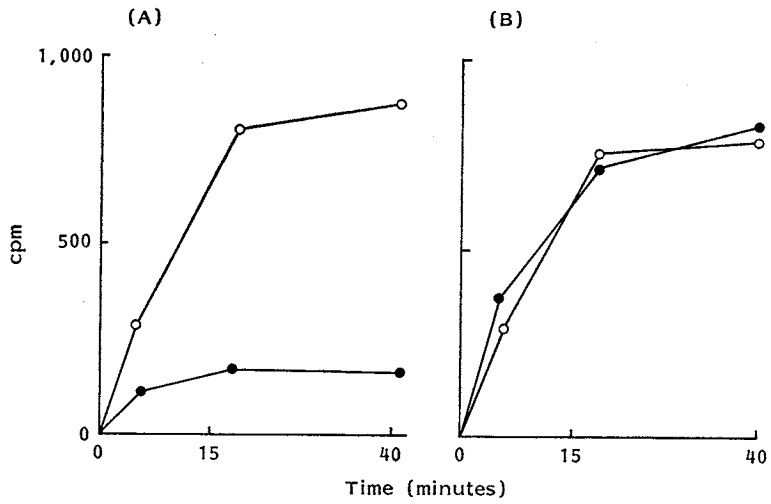
Streptomyces kanamyceticus ISP5500 was the kanamycin producer strain used in the experiments. *Streptomyces lividans* CMA5 and CMA64 were derivatives of *S. lividans* strain 1326 which contained the kanamycin resistance plasmids pMCP5[7] and pMCP64 (the preceding paper[8], Fig. 1), respectively. pKU3 was a gift from H. IKEDA.

Antibiotic non-producing medium was Tryptic Soy Broth (Difco) with 1% glucose (TSBG). Antibiotic producing media were SM and GMYP media. SM medium was comprised of potato starch 2%, maltose 2%, soy bean meal 3%, $NaNO_3$ 1% and $CaCO_3$ 0.01% (pH 7.0). GMYP medium contained glucose 0.1%, meat extract 0.2%, yeast extract 0.2%, Polypeptone 0.4% and $MgSO_4 \cdot 7H_2O$ 20 mM (pH 7.0). Because SM medium contained insoluble materials, GMYP medium was used to culture cells for RNA preparations. Strains were maintained on MM agar plates[9].

Determination of Antibiotic Resistance

Cells were subcultured on MM agar plates containing 100 μg/ml of various aminoglycoside antibiotics. Kanamycin and ribostamycin were obtained from Meiji Seika Kaisha, Ltd., sisomicin from

Fig. 1. Effect of kanamycin on polyU-directed polyphenylalanine synthesis.



(A) 30S particles from *Streptomyces lividans* 1326 and 50S particles from *S. lividans* CMA5; (B) 30S particles from *S. lividans* CMA5 and 50S particles from *S. lividans* 1326. Open circles: Assay without kanamycin, closed circles: assay with kanamycin.

Yamanouchi Pharmaceutical Co., Ltd., gentamicin and tobramycin from Shionogi & Co., Ltd., amikacin from Banyu Pharmaceutical Co., Ltd. Growth was checked after incubation at 28°C for 3 to 5 days.

Preparation of Ribosomal Subunits

S150 fraction and ribosomes were prepared by the method of YAMAMOTO *et al.*[10]. Ribosomes (500~1,000 A_{260} units) were washed with buffer D (Tris-HCl 100 mM, pH 7.8, Mg-acetate 10 mM, NH_4Cl 20 mM, mercaptoethanol 5 mM). One A_{260} unit was defined as the amount of substances which showed an optical absorbance of 1.0 at 260 nm in 1 ml solution. Ribosomes were pelleted by centrifugation at 150,000×*g* for 3 hours and resuspended in buffer F (Tris-HCl 20 mM, pH 7.6, NH_4Cl 0.5 M, Mg-acetate 0.5 mM, mercaptoethanol 5 mM) at a concentration of 1,000 A_{260} units/ml or more, followed by dialysis at 4°C against buffer G (Tris-HCl 20 mM, pH 7.6, NH_4Cl 100 mM, Mg-acetate 1 mM, mercaptoethanol 5 mM) at a concentration of 200~300 A_{260} units/ml. The ribosomes were layered over 0.3~0.9 M linear sucrose gradients in buffer G and centrifuged at 22,000 rpm for 15 hours at 4°C in a Hitachi RPS27 rotor. Fractions containing 50S and 30S particles were monitored and pooled separately. 50S subunits were diluted with an equal volume of buffer E and collected by centrifugation at 150,000×*g* for 15 hours, resuspended in small volume of buffer E containing 50% glycerol and stored at −70°C.

In Vitro PolyU-directed Polyphenylalanine Synthesis

The assay for polyU-directed polyphenylalanine synthesis was previously described[7]. Three A_{260} units of 30S plus one A_{260} unit of 50S subunits were used for each reaction. At various intervals, an aliquot of the reaction mixture was withdrawn for measurement of [^{14}C]phenylalanine incorporation.

Isolation of Mutants Which Expresses the *kmr* Gene Constitutively

S. kanamyceticus ISP5500 was grown in TSBG medium and 2% of the culture was transferred to the same medium with 20 μg/ml of ethidium bromide. After incubation for 5 days, cells were plated onto MM agar medium with 100 μg/ml sisomicin.

Detection of Kanamycin Production and Aminoglycoside Acetyltransferase

Kanamycin production was measured in the culture supernatant fluid by the biological disk assay using *Bacillus subtilis* ATCC 6633 as a test organism. Detection of aminoglycoside acetyltransferase

activity was previously described[7].

DNA, RNA Preparation and S1 Mapping

These procedures were described in the preceding paper[8].

Results

Kanamycin Resistance Mechanism

As previously reported[7], the 70S ribosomes from CMA5, a derivative of *S. lividans* 1326 that harbored the plasmid pMCP5 containing the cloned *kmr* gene of *S. kanamyceticus*, were resistant *in vitro* to kanamycin. In order to determine which subunit was responsible for the resistance, 70S particles were reconstituted by reassociation of 30S and 50S subunits from *S. lividans* 1326 and those from CMA5. The reconstituted ribosome as assayed for polyU-directed polyphenylalanine synthesis in the presence or absence of kanamycin. Only when the 30S subunits from CMA5 were used were reconstituted ribosomes shown to be resistant to kanamycin (Fig. 1). It was concluded that the mechanism of kanamycin resistance involved alteration of 30S ribosomal subunits.

Aminoglycoside Acetyltransferase Activity and Kanamycin Production

We have previously reported that ISP5500 has two mechanisms of kanamycin resistance, inactivation by acetylation and by ribosomal alteration[7]. To know which mechanism is responsible for self-resistance in the producing organism, we examined aminoglycoside acetyltransferase activity in relation to kanamycin produciton. When ISP5500 was cultured in TSBG medium, activity of the acetylating enzyme remained at a high level until very late in exponential growth, even though kanamycin was not produced. On the contrary, in GMYP medium, the acetylating activity reached a maximum level in early exponential phase and decreased rapidly before the onset of antibiotic production in late exponential phase (data not shown). Absence of the acetyltransferase activity in the kanamycin production phase was also reported by SATOH *et al.*[11]. Ribosomes from ISP5500 showed resistance to kanamycin both before and after kanamycin production (data not shown).

Regulation of Ribosomal Kanamycin Resistance and Acetylation of Kanamycin in ISP5500

The above results suggest that the ribosomal resistance gene is responsible for self-resistance of ISP5500. To confirm this, the effects of carbon and nitrogen sources on ribosomal resistance, the activity of the acetylating enzyme, and kanamycin production were examined. ISP5500 was pre-cultured in SM medium and 2% of the culture was transferred to SM medium supplemented with glucose or Polypeptone at different concentrations. After incubation for 48 hours, cells from each culture flask were plated onto SM plates which contained supplements corresponding to those of the liquid medium. The plated cells were challenged with kanamycin, ribostamycin and amikacin. Resistance against ribostamycin and amikacin is due to acetyltransferase and ribosomal alteration, respectively, and both of the resistance mechanisms can confer kanamycin resistance on the cells[7]. Therefore we could detect the presence of acetyltransferase activity and ribosomal resistance by checking the growth of cells on agar plates with these antibiotics. Kanamycin production in each culture was also checked as described in Materials and Methods. As summarized in Table 1, ribosomes from cells grown in the presence of a high concentration of rapidly utilizable nitrogen source like Polypeptone were found to be sensitive to kanamycin and kanamycin was not produced. However, the activity of the acetylat-

Table 1. Effect of medium on kanamycin production, ribosomal resistance and acetyltransferase.

Medium	Kanamycin production	Growth with				Ribosomal resistance	Acetyl-transferase
		No antibiotic	Km	Rm	Am		
Starch - maltose (SM)	+	+	+	+	+	+	+
Tryptic Soy Broth (TSB)	−	+	+	+	−	−	+
SM+0.6% Polypeptone	+	+	+	+	+	+	+
SM+2.0% Polypeptone	−	+	+	+	−	−	+
SM+0.5% glucose	+	+	+	+	+	+	+
SM+1.5% glucose	−	+	+	−	−	−	−

Km: Kanamycin, Rm: ribostamycin, Am: amikacin.

ing enzyme was apparently present under these conditions. Growth in the presence of high glucose concentrations rendered the ribosome sensitive to kanamycin and prevented kanamycin production. Cells grown under these conditions were also sensitive to ribostamycin, indicating that there was little or no acetyltransferase activity. Glucose or Polypeptone, if added at low concentrations, did not affect these functions, presumably because these rapidly-utilizable components were quickly exhaused from the medium. The results indicate that the ribosomal kanamycin resistance and kanamycin biosynthesis were regulated in parallel, suggesting the involvement of the *kmr* gene in the self-resistance of the kanamycin producer.

Table 2. Resistance to various aminoglycoside antibiotics.

Antibiotic (100 μg/ml)	ISP5500	CMA5 (1326/ pMCP5)	SR1 (ISP5500 mutant)
Sisomicin	S	R	R
Ribostamycin	R	S	R
Tobramycin	S	R	R
Paromomycin	S	S	S
Amikacin	S	R	R
Dibekacin	R	R	R
Dihydrostrepto-mycin	S	S	S
Neomycin	S	S	S
Gentamicin	S	R	R
Kanamycin	R	R	R

R: Resistant, S: sensitive.

Isolation of a Mutant of *S. kanamyceticus* Which Constitutively Expresses the *kmr* Gene

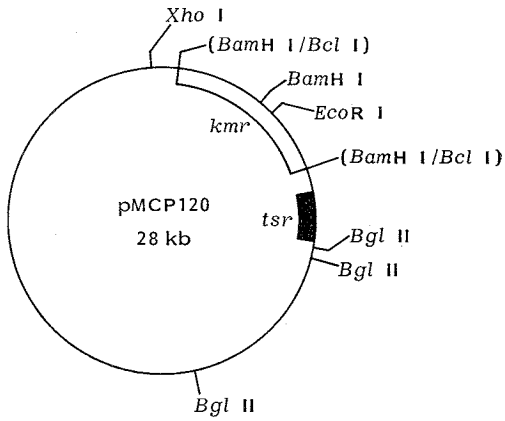
We had shown that the expression of the *kmr* gene was apparently unregulated when it was cloned on a multicopy plasmid such as pIJ702[7]. In *S. kanamyceticus*, however, the gene was inducible, which indicated that ribosomes from *S. kanamyceticus* were kanamycin-resistant only when grown under kanamycin-producing conditions[7]. The ribosomal resistance gene of *S. kanamyceticus* ISP5500 was not expressed when cells were grown on MM agar. For this reason ISP5500 was resistant on MM agar only to ribostamycin and dibekacin as well as kanamycin as shown in Table 2. This resistance is due to an acetylating enzyme since it is known that the acetyltransferase from *S. kanamyceticus* ISP5500 [AAC(6′)] confers resistance to only these three antibiotics among the aminoglycosides listed in Table 2[12]. CMA5 showed ribosome-derived resistance patterns.

We isolated a mutant from ISP5500 which constitutively expressed the ribosomal kanamycin resistance gene by selecting sisomicin-resistant clones on MM agar plates. The resistant clones were further replicated on MM agar plates supplemented with tobramycin, amikacin or gentamicin which are also selective for ribosomal resistance. One of the putative constitutive mutants, named SR1, showed both resistance caused by an acetylating enzyme and a ribosomal alteration (Table 2). This type of mutant could be isolated spontaneously as well (M. M. NAKANO; unpublished result).

Subcloning of the *kmr* Gene onto a Low Copy Number Plasmid

The *kmr* gene of *S. kanamyceticus* is presumed to be located on the chromosome in a single copy. The gene was cloned on multicopy plasmids, in which it existed as several hundred copies per chromosome. To examine the possibility that the difference in the expression of the *kmr* gene in ISP5500 cells and on multicopy plasmids depended on the copy number of the gene, we subcloned the *kmr* gene into the plasmid pKU3. pKU3 is a derivative of SCP2*[13] which is reported to be a low copy number plasmid (1~2 copies per chromosome). pKU3 has two antibiotic resistance markers, neomycin resistance (*aph*) and thiostrepton resistance (*tsr*) (H. IKEDA and S. ŌMURA; unpublished results). The *kmr* gene on a *Bcl* I fragment from plasmid pMCP5, which was the same fragment as the insert in pMCP12 (ref 7, NAKANO *et al.*, the preceding paper, Fig. 1) was cloned into the *Bam*H I site of the *aph* gene, yielding two plasmids, pMCP120 and pMCP121 (Fig. 2), which contained the *kmr* gene in opposite orientations. *S. lividans* 1326 carrying pMCP120 and pMCP121 constitutively expressed the ribosomal resistance gene because they grew on MM agar plates containing sisomicin, tobramycin, amikacin or gentamicin. Readthrough from the plasmid vector (SCP2*) was unlikely because the *kmr* insert rendered constitutively resistant to the cells in both orientations. These results indicate that the difference in expression of the *kmr* gene is not due to the copy number of the gene.

Fig. 2. Restriction enzyme map of pMCP120.

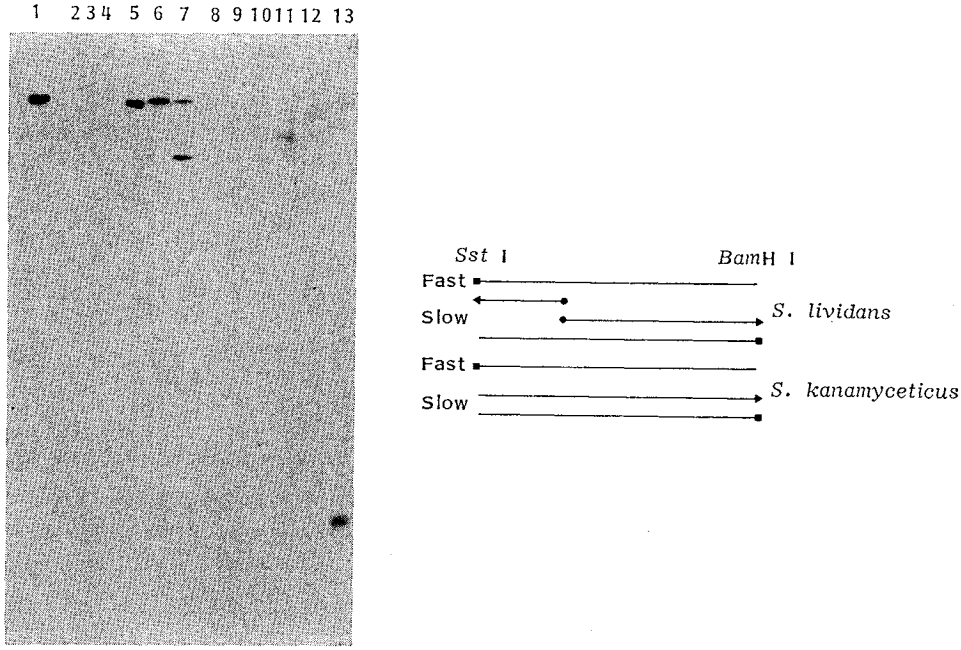


S1 Mapping of the *kmr* Gene in *S. kanamyceticus*

To determine if expression of the *kmr* gene is regulated at the transcriptional level, S1 mapping was done using RNA isolated from ISP5500 cells that were grown in antibiotic-producing medium or in non-producing medium. A *Bam*H I - *Sst* I fragment carrying the *kmr* promoter (fragment a shown in Fig. 4 of the preceding paper) was labeled with ^{32}P at the 5′ ends and was subjected to strand separation electrophoresis. When the slow-migrating fragment a which was labeled at the 5′ *Bam*H I end was used as probe, RNA isolated from *S. lividans* CMA64 (*S. lividans* 1326 carrying the *kmr* plasmid pMCP64) protected a 210 bp fragment (lane 7 in Fig. 3). This corresponds to the *kmr* transcript as shown in Fig. 5A in the preceding paper. A larger fragment was also seen in lane 7 which was also seen in the previous paper (lane 1, Fig. 5A) but probably did not represent a second start site since only one band was seen with the shorter fragment b (lane 1, Fig. 5A in the preceding paper). RNA was isolated from ISP5500 cultured in an antibiotic-producing medium before and after the onset of kanamycin production. Both RNAs protected the slow-migrating fragment a intact (lanes 5 and 6 in Fig. 3); however, the *kmr* transcript was not detected in ISP5500 grown in non-producing medium (lanes 3 and 4). These results indicate that *kmr* gene expression is regulated at the transcriptional level and also show that the transcription start point of the *kmr* gene in ISP5500 is different from that of *S. lividans* containing the plasmid with the *kmr* gene. *kmr* transcription in ISP5500 apparently started upstream of the *Sst* I site in Fig. 2 of the preceding paper.

The other transcript of 90 bp was identified in *S. lividans* CMA64 using the fast-migrating fragment

Fig. 3. S1 mapping analysis of the *kmr* gene.



The *Bam*H I - *Sst* I fragment carrying the *kmr* promoter (fragment a in Fig. 4 of the preceding paper) was 5′ end labeled with ^{32}P and then subjected to strand separation electrophoresis. Lanes 1~7: Slow-migrating fragment a, lanes 8~13: fast-migrating fragment a. Lane 1 shows control without S1 nuclease treatment. Hybridization was performed using tRNA (lanes 2 and 8), RNA from *Streptomyces kanamyceticus* early log phase cells grown in TSBG medium (lanes 3 and 9), late log phase cells grown in TSBG medium (lanes 4 and 10), early log phase cells grown in GMYP medium (lanes 5 and 11), and late log phase cells grown in GMYP medium (lanes 6 and 12). Lanes 7 and 13 show the results using RNA from *Streptomyces lividans* CMA64 cells. Fast- and slow-migrating strands are shown together with the ^{32}P-label as black squares.

a labeled at the 5′ *Sst* I end as the template (lane 13 in Fig. 3). This transcript was not detected in ISP5500 cells grown in TSBG and GMYP media as can been seen in lanes 9~12 in Fig. 3.

Discussion

Two self-resistance mechanisms are known in *S. kanamyceticus*. One is modification of ribosomes as described here and the other is inactivation of kanamycin by 6′-*N*-acetyltransferase[12]. The acetyltransferase gene from *S. kanamyceticus* was also cloned[14] and reintroduction of the gene on a high copy number plasmid vector into *S. kanamyceticus* gave transformants with increased production of kanamycin[15]. We showed that ribosomal resistance, rather than acetyltransferase, was probably responsible for self-resistance in *S. kanamyceticus* from the results described in this report. First, kanamycin production was observed under the same physiological conditions as ribosomal resistance. The presence of acetyltransferase was not always coupled with kanamycin production. Secondly, acetyltransferase activity was not detected when kanamycin was produced; ribosomal resistance, on the other hand, was detected both before and after kanamycin production. The acetyltransferase might participate in kanamycin biosynthesis rather than self-resistance in the kanamycin producer. In fact, a biologically inactive *N*-acetyl derivative of kanamycin, which was produced by the acetyltransferase, was isolated from the fermentation broth of the producing strain[16]. The biologically inactive kanamycin derivative was restored to the active antibiotic by *N*-acetylkanamycin amidohy-

drolase which appeared late in the growth stage[17]. These results suggest that the acetyltransferase may be essential in the biosynthetic process of antibiotic production for storing the modified antibiotic (*N*-acetylkanamycin) temporarily in the cells and that the aminoglycoside-liberating enzyme (*N*-acetylkanamycin amidohydrolase) is necessary for excreting the active antibiotic outside the cells[17].

Recently, the involvement of ribosomal resistance in the self-defense mechanisms of aminoglycoside producers has been reported in *Streptomyces tenjimariensis*, the producer of istamycin[17], *Streptomyces tenebrarius*, the producer of nebramycin complex[18], *Micromonospora purpurea*, the gentamicin producer[19] and several other aminoglycoside-producing *Micromonospora* species[20]. PIENDL *et al.* reported involvement of 16S ribosomal RNA in resistance of *S. tenjimariensis*, *S. tenebrarius* and *M. purpurea*[21]. In addition, methylation of 16S rRNA was shown to be responsible for the resistance in *S. tenjimariensis*[22]. We showed that the 30S subunit confered resistance on ribosomes from *S. lividans* carrying the cloned *kmr* gene. Though we have not yet identified the ribosomal component responsible for kanamycin resistance, it seems quite probable that 16S rRNA renders the ribosome resistant to kanamycin.

The results in Table 1 show that ISP5500 cells were still resistant to kanamycin when high concentrations of glucose repressed the expression of the ribosomal resistance gene and acetyltransferase activity. It remains to be seen if ISP5500 has another kanamycin resistance mechanism, for example, interference with drug transport.

The expression of the acquired ribosomal resistance gene was shown to be unregulated when cloned on multicopy plasmids, but inducible in producer cells. There are at least two possible explanation. One possibility is that the putative repressor molecule is not specified on the plasmid and that the normal amount of repressor molecule is titrated out by the large number of the operator sites on the multicopy plasmids and the gene is therefore overexpressed. Another possibility is that the gene is auto-regulated and the expression of the plasmid-amplified *kmr* gene results in the synthesis of a higher basal level of *kmr* product which causes self-induction of the *kmr* gene. However, these possibility are ruled out because the same fragment subcloned from the multicopy plasmid to the low-copy plasmid allowed constitutive expression of the *kmr* gene. Considering that the ribosomal resistance gene was regulated coordinately with some of biosynthetic genes, and the expression of the resistance gene was regulated at the transcriptional level, it is likely that the resistance gene is closely linked to the biosynthetic genes and that it is regulated under the same transcriptional control as some of the biosynthesis genes. This point will be solved by cloning the kanamycin biosynthetic gene cluster using the *kmr* gene.

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