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Cloning of the Kanamycin Resistance Gene from a Kanamycin-Producing *Streptomyces* Species

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A kanamycin-producing strain, *Streptomyces kanamyceticus* ISP5500, is resistant to kanamycin. A kanamycin resistance determinant was cloned from *S. kanamyceticus* into *Streptomyces lividans* 1326, using the plasmid vector pIJ702. The resulting plasmid, pMCP5, could also transform *Streptomyces lavendulae* S985 and *Streptomyces parvulus* 2283 to kanamycin resistance. Transformants carrying pMCP5 were markedly more resistant than *S. kanamyceticus* to the aminoglycoside antibiotics sisomicin, tobramycin, amikacin, and gentamicin. Studies in vitro polyphenylalanine synthesis showed that strains carrying pMCP5 contained kanamycin-resistant ribosomes. However, growing *S. kanamyceticus* contained kanamycin-sensitive ribosomes. Ribosomes from *S. kanamyceticus* grown under kanamycin-producing conditions were kanamycin resistant.

Many streptomycetes which produce antibiotics defend themselves from their own antibiotics. These mechanisms of antibiotic resistance include inactivation of the antibiotics by antibiotic-modifying enzymes (5, 19), alteration of target sites (3, 18, 19), and reduced uptake of antibiotics (13). Recent progress in cloning systems in streptomycetes makes it possible to isolate such antibiotic resistance genes (2). For example, genes determining antibiotic-modifying enzymes such as neomycin acetyltransferase and neomycin phosphotransferase from *Streptomyces fradiae* and viomycin phosphotransferase from *Streptomyces vinaceus* were successfully cloned into *Streptomyces lividans* 1326 (14–17).

Streptomyces azureus and *Streptomyces erythreus* are resistant to their own antibiotics, thiostrepton and erythromycin, respectively. In both cases, the resistance is due to methylation of 23S rRNA which prevents the binding between altered ribosomes and antibiotics. These 23S rRNA methylases from *S. azureus* and *S. erythreus* were also cloned into *S. lividans* (14, 16, 17).

Kanamycin also binds to ribosomes and inhibits protein synthesis. A kanamycin producer, *Streptomyces kanamyceticus* contains an enzyme that acetylates the 6′-amino group of kanamycin (aminoglycoside acetyltransferase, AAC6′). This observation leads to an attractive speculation that resistance determinants in clinical isolates of other bacteria might originate from producing strains (1). Other reports suggested that the kanamycin acetyltransferase was involved in the production of kanamycin (11) and that the enzyme also played an important role in the self-resistance mechanism (5). In this paper we describe the cloning of the kanamycin resistance gene from *S. kanamyceticus* ISP5500. This gene provides resistance against kanamycin by altering ribosomes.

MATERIALS AND METHODS

Restriction enzymes *Bgl*II, *Bam*HI, and *Mlu*I were purchased from Takara Shuzo Co., and *Bcl*I was from Bethesda Research Laboratories. T4 ligase was purchased from Bio Labs. Polyuridylic acid [poly(U)], pyruvate kinase, phenylmethylsulfonyl fluoride, and phenylalanine-specific tRNA (*Escherichia coli*) were purchased from Sigma Chemical Co. L-[*U*-^{14}C] phenylalanine (510 mCi/mmol) was purchased from New England Nuclear Corp. Thiostrepton was a kind gift from R. D. Sykes. Aminoglycoside antibiotics are commercially available.

S. lividans JI1326 and its derivative JI3131 (containing a plasmid cloning vector pIJ702; E. Katz et al., personal communication) and *Streptomyces parvulus* 2283 were kind gifts from D. A. Hopwood. *Streptomyces lavendulae* S985 and *S. kanamyceticus* ISP5500 (K-2J) were gifts from A. Seino and H. Umezawa, respectively.

DNA preparation. Chromosomal DNA from *S. kanamyceticus* ISP5500 was isolated by the method of Marmur (8). Plasmid pIJ702 was isolated from *S. lividans* JI3131 by using an alkaline denaturation procedure by Omura et al. (10).

Restriction nuclease digestion and ligation of DNA. Strain ISP5500 chromosomal DNA (20 μg) was partially cleaved with *Bcl*I to generate fragments of predominantly 3 to 10 kilobases (kb) in length and ligated with pIJ702 (3 μg) cleaved with *Bgl*II. The DNA mixture was suspended with ligation mixture (50 mM Tris-hydrochloride [pH 7.8], 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP, and 50 μg of bovine serum albumin per ml) at a DNA concentration of 200 μg/ml and ligated with T4 ligase at 4°C overnight. The ligated mixture was precipitated with 2 volumes of cold ethanol and dissolved with TE buffer (10 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA).

Protoplast transformation. The host strains were grown in 100 ml of tryptic soy broth (Difco Laboratories) supplemented with 1 g of glucose and 0.1 g of glycine. Protoplasts (4×10^9) generated by the method of Okanishi (9) were suspended in a small volume of P medium (9) and mixed with the ligated DNA in 20 μl of TE buffer. Transformation was done by the addition of 0.5 ml of 20% polyethylene glycol 1000 dissolved in T medium as described previously by Thompson et al. (17). The regeneration medium R2YEMT was an R2 agar medium supplemented with 0.5% yeast extract, 100 μg of methionine per ml, and 400 μg of tyrosine per ml. Methionine is known to have an inducible effect on tyrosinase synthesis in at least one *Streptomyces* species (6). The vector plasmid pIJ702 contained the *Streptomyces antibioticus* tyrosinase gene and the *S. azureus* thiostrepton resistance gene in a replicon based on the multicopy plasmid

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pIJ101 (E. Katz et al., personal communication; 7). Insertion of DNA into the *Bgl*II site within the tyrosinase gene of pIJ702 leads to a loss of melanin production. Clones containing recombinant plasmids were selected as thiostrepton resistant and melanin negative (Thi^r Mel$^-$) among cells containing the vector plasmid whose phenotypes were Thi^r Mel$^+$. Kanamycin-resistant clones were obtained by the following method. Regeneration plates were overlaid with 2.5 ml of soft agar supplemented with 500 μg of thiostrepton per ml. Thiostrepton-resistant and melanin-negative clones were replicated onto minimal medium agar (4) containing 50 μg of kanamycin per ml.

Sensitivity against various aminoglycoside antibiotics. Mycelia grown in tryptic soy broth were washed and suspended with 0.85% NaCl and fragmented by a cutter (Polytron Kinematica). A sample of cell suspension was plated onto minimal medium agar supplemented with various aminoglycoside antibiotics (100 μg/ml).

Detection of aminoglycoside acetyltransferase. Mycelia grown in tryptic soy broth were washed twice with TMK buffer (0.1 M Tris-hydrochloride [pH 7.8], 0.01 M $MgCl_2$, 0.06 M KCl, 0.06 M mercaptoethanol) and sonicated in an equal volume of TMK buffer. A cell extract was recovered by centrifugation at 105,000 × *g* at 4°C for 1 h. An assay mixture (1 ml) contained 0.1 M Tris-hydrochloride (pH 7.8), 10 mM magnesium acetate, 5 mM dithiothreitol, 0.5 mM acetyl coenzyme A (acetyl-CoA), 16 mM Na_2 ATP, 60 mM KCl, 0.4 mM $ZnSO_4$, 100 μg of kanamycin, and 100 μl of the cell extract. After incubation of the mixture at 37°C for 1 h, residual kanamycin was measured by the disk method with *Bacilus subtilis* ATCC 6633 as a test organism.

Preparation of S150 fraction and ribosomes. The S150 fraction and ribosomes were prepared by the method of Yamamoto et al. (18).

In vitro poly (U)-directed polyphenylalanine synthesis. Poly(U)-directed polyphenylalanine synthesis was assayed in a 200-μl reaction mixture containing 50 mM Tris-hydrochloride (pH 7.8), 60 mM NH_4Cl, 7.5 mM magnesium acetate, 1 mM ATP, 5 mM phosphoenolpyruvate (pH 7.0), 25 μM GTP, 6 μg of pyruvate kinase, 19 μg of *E. coli* phenylalanine-specific tRNA, 50 μg of poly(U), 0.4 mM spermidine, 0.5 mM phenylalanine, 3 μM L-[*U*-^{14}C]phenylalanine, S150 (150 to 300 μg of protein), and ribosomes (60 to 80 μg). The reaction mixtures were incubated at 34°C, and 50-μl samples were added to 0.8 ml of water and 150 μl of bovine serum albumin (1 mg/ml). One milliliter of 10% trichloroacetic acid was added, and the mixture was heated at 90°C for 20 min. Acid-insoluble materials were collected onto glass-fiber filters (Whatman GF/C), and radioactivity was measured with a liquid scintillation counter.

Nick translation and Southern hybridization. Nick translation of pMCP5 to obtain a ^{32}P-labeled DNA probe and subsequent hybridization to total DNA isolated from *S. lividans* 1326 and *S. kanamyceticus* ISP5500 were carried out by the method of Southern (12).

RESULTS

Isolation of a kanamycin-resistant transformant. After ligation of *S. kanamyceticus* *Bcl*I-cleaved DNA with *Bgl*II-cleaved pIJ702, about 10,000 *S. lividans* Thi^r transformants were obtained. About 2,000 of these presumptively contained recombinant plasmids, since they were Mel$^-$. One of these colonies (CMA5) was found to be kanamycin resistant by replication. CMA5, like *S. kanamyceticus* ISP5500, was resistant to at least 500 μg/ml of kanamycin, whereas *S. lividans* 1326 was inhibited by 5 μg/ml of kanamycin.

Plasmid DNA (named pMCP5) isolated from CMA5 was retransformed into strain 1326. After transformation, thiostrepton- and kanamycin-resistant (Thi^r Km^r) clones were isolated, indicating that the plasmid DNA contained the kanamycin resistance gene. This was confirmed by a curing experiment. Protoplasts were obtained from CMA5 cells and regenerated on R2YEMT agar medium. Regenerated protoplasts were replica plated onto minimal medium and medium supplemented with 50 μg of kanamycin per ml. The frequency of curing was about 90%. All of the kanamycin-sensitive clones tested (12 clones) were also thiostrepton sensitive and did not have any plasmid. On the contrary, all of the kanamycin-resistant clones (seven clones) tested still had pMCP5. Both *S. lavendulae* S985 and *S. parvulus* 2283 were transformed to kanamycin resistance by pMCP5. The resistant colonies contained plasmid DNA of the expected size.

Agarose gel electrophoresis of pIJ702 and pMCP5 cleaved with *Mlu*I and *Bcl*I is shown in Fig. 1. As pIJ702 has a single *Mlu*I cleavage site and pMCP5 was shown to have two *Mlu*I cleavage sites (Fig. 1A), a single *Mlu*I site existed in the insert. The length of the insertion fragment was about 7.5 kb. pIJ702 has four cleavage fragments with *Bcl*I, and three of these also existed in pMCP5 (Fig. 1B). The second largest *Bcl*I-cleaved fragment disappeared in pMCP5, which means that the insert was in the middle of the missing fragment. As the 1.9 to 2.0-kb band of *Bcl*I-cleaved pMCP5 was shown to be a doublet by microdensitometer tracing, pMCP5 had four additional *Bcl*I fragments instead of the missing one. Considering that junctions of the insert and the vector plasmid are not cleaved with *Bcl*I, the insert in pMCP5 contained three internal *Bcl*I sites. This might be due to partial digestion with *Bcl*I or simultaneous cloning of originally independent *Bcl*I fragments.

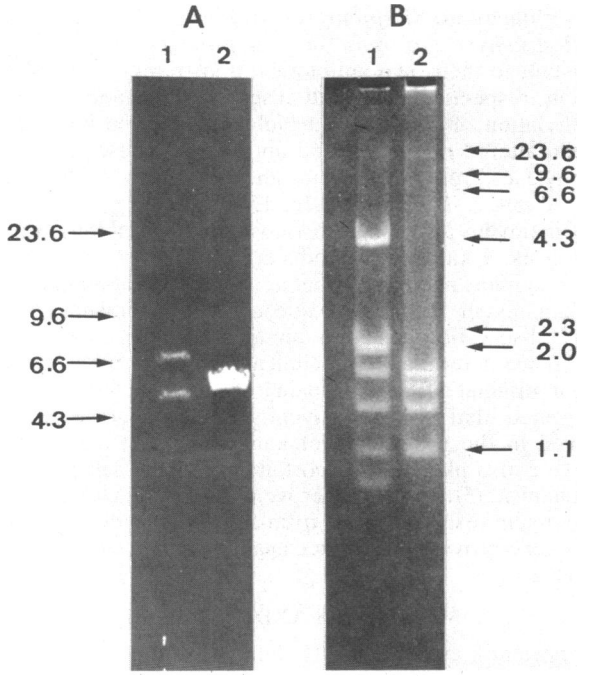

FIG. 1. Agarose gel electrophoresis of pIJ702 and pMCP5. (A) Lane 1, *Mlu*I fragments of pMCP5; lane 2, *Mlu*I fragment of pIJ702. Agarose gel concentration was 0.4%. (B) Lane 1, *Bcl*I fragments of pMCP5; lane 2, *Bcl*I fragments fo pIJ702. Agarose gel concentration was 1.5%. Bacteriophage λ DNA and plasmid PM2 digested with *Hin*dIII were used as reference markers. The numbers and arrows represent the sizes (kb pairs) and positions of marker bands.

TABLE 1. Resistance to various aminoglycoside antibiotics

Antibiotic[a]	Resistance[b]				
	ISP5500	3131	1326	CMA5	CMAC5
Kanamycin	R	S	S	R	S
Sisomicin	S	S	S	R	S
Ribostamycin	R	S	S	S	S
Tobramycin	S	S	S	R	S
Paromomycin	S	S	S	S	S
Amikacin	S	S	S	R	S
Dibekacin	R	S	S	R	S
Dihydrostreptomycin	S	S	S	S	S
Neomycin	S	S	S	S	S
Gentamicin	S	S	S	R	S

[a] Added at 100 μg/ml.
[b] R, Resistant; S, sensitive.

Resistance patterns against various aminoglycoside antibiotics. The resistance of ISP5500, 1326, and CMA5 cells against various aminoglycoside antibiotics was examined (Table 1). *S. lividans* 1326 and 3131 containing the vector plasmid pIJ702 were sensitive to aminoglycosides. The donor strain, *S. kanamyceticus* ISP5500, was highly resistant to ribostamycin and dibekacin as well as kanamycin. The resistance pattern corresponded to the substrate specificity of the aminoglycoside acetyltransferase (AAC6′) which is known to exist in ISP5500. On the contrary, CMA5 cells showed a different resistance pattern to aminoglycosides. They showed resistance to sisomicin, tobramycin, amikacin, dibekacin, and gentamicin besides kanamycin, suggesting that the resistance was not due to AAC6′. A plasmid-cured strain from CMA5 (named CMAC5) showed almost the same resistance pattern as the parental 1326 strain. These resistance patterns were confirmed by other kanamycin-resistant transformants from *S. lavendulae* S985 and *S. parvulus* 2283 (data not shown). They indicated that transformation with pMCP5 plasmid resulted in resistance to kanamycin, sisomicin, tobramycin, amikacin, dibekacin, and gentamicin. Cell extracts from strains ISP5500, 1326, and CMA5 were tested for the presence of aminoglycoside acetyltransferase. *S. kanamyceticus* ISP5500 contained the aminoglycoside acetyltransferase enzyme, but 1326 and CMA5 had no detectable activity.

In vitro poly(U)-directed polyphenylalanine synthesis. The alternative resistance mechanism of a change in the ribosomes was examined by in vitro poly(U)-directed polyphenylalanine synthesis (Fig. 2). Polyphenylalanine synthesis was markedly inhibited in the presence of kanamycin when both ribosomes and the S150 fraction were derived from

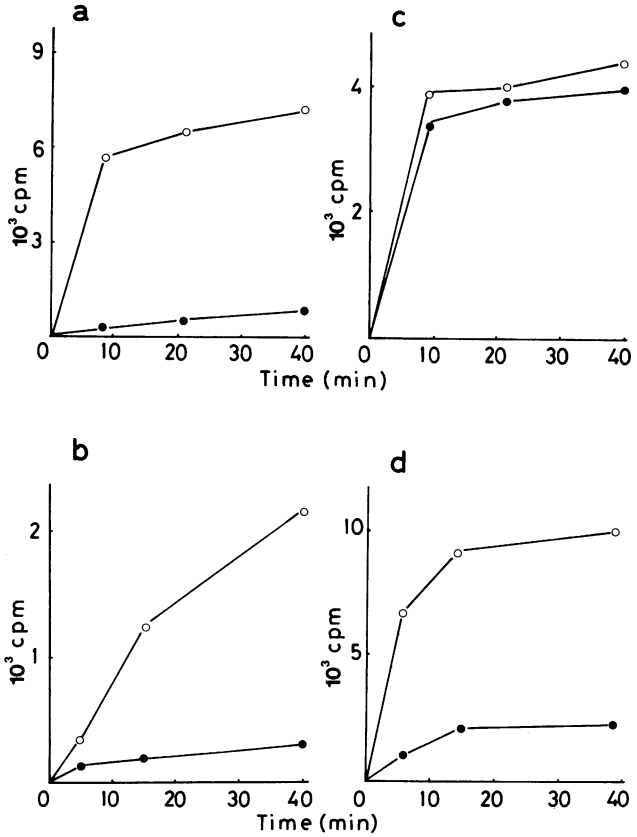

FIG. 2. Effect of kanamycin on polyphenylalanine synthesis, using ribosomes and the S150 fraction. Symbols: ○, without kanamycin; ●, with 100 μg of kanamycin per ml. Cells were grown in tryptic soy broth, and ribosomes and the S150 fraction were isolated as described in the text. (a) Both ribosomes and the S150 fraction from *S. lividans* 1326; (b) both ribosomes and the S150 fraction from *S. lividans* 3131 (1326 containing pIJ702); (c) both ribosomes and the S150 fraction from CMA5; (d) both ribosomes and the S150 fraction from *S. kanamyceticus* ISP5500.

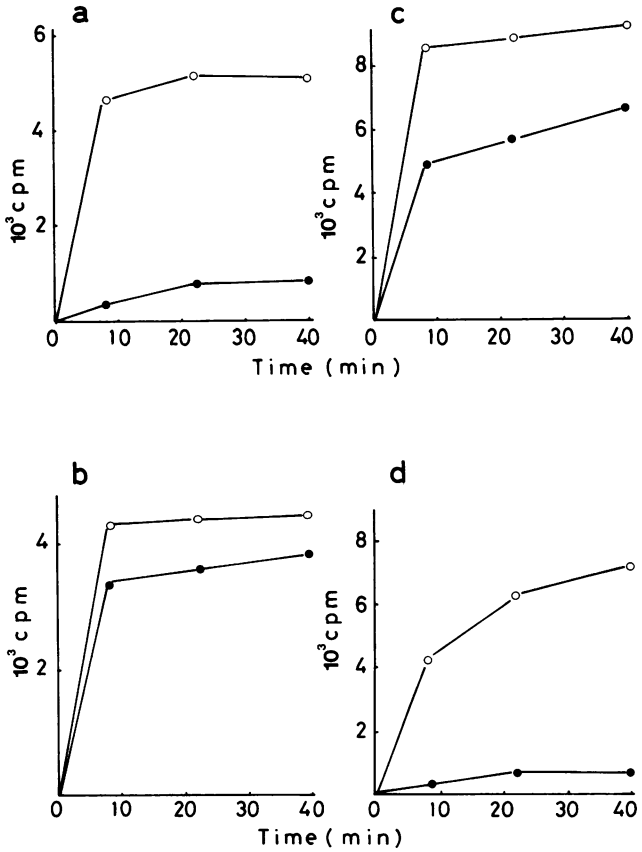

FIG. 3. Effect of kanamycin on polyphenylalanine synthesis, using ribosomes and the S150 fraction. Symbols: ○, without kanamycin; ●, with 100 μg of kanamycin per ml. (a) Ribosomes from strain 1326 and the S150 fraction from CMA5; (b) ribosomes from CMA5 and the S150 fraction from 1326; (c) ribosomes from 1326 and the S150 fraction from strain ISP5500; (d) ribosomes from ISP5500 and the S150 fraction from 1326. Assay mixtures contained acetyl-CoA (1 mM) in the case of (c) and (d).

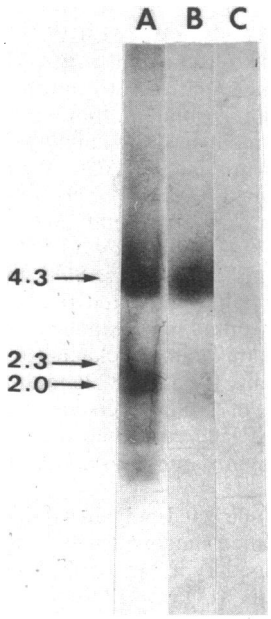

FIG. 4. Hybridization of ^{32}-labeled pMCP5 to total DNA. (A) pMCP5 digested with *Bcl*I; (B) strain ISP5500 total DNA digested with *Bcl*I; (C) strain 1326 total DNA digested with *Bcl*I. Agarose gel concentration was 0.7%. The numbers and arrows represent the sizes (kb pairs) and positions of λ DNA digested with *Hin*dIII.

strain 1326 (Fig. 2a), strain 3131 (Fig. 2b), or strain ISP5500 (Fig. 2d), whereas synthesis with S150 and ribosomes from CMA5 was scarcely inhibited (Fig. 2c). When as assay mixture contained acetyl-CoA for the expression of aminoglycoside acetylating enzyme, polyphenylalanine synthesis with S150 and ribosomes from ISP5500 was resistant to kanamycin (data not shown). These results strongly suggested that kanamycin resistance of polyphenylalanine synthesis from CMA5 was due to resistance of ribosomes. To confirm this possibility, ribosomes and the S150 fraction of ISP5500 or CMA5 were exchanged reciprocally for those of 1326, and the sensitivity of polyphenylalanine synthesis to kanamycin was examined (Fig. 3). Resistance against kanamycin was observed when ribosomes from CMA5 were combined with the S150 fraction from 1326 (Fig. 3b), whereas a combined system between ribosomes from 1326 and the S150 fraction from CMA5 was sensitive to kanamycin (Fig. 3a). On the contrary, in the case of ISP5500 and 1326, resistance against kanamycin was detected only when the S150 fraction from ISP5500 was used for polyphenylalanine synthesis in the presence of acetyl-CoA (Fig. 3c). These results showed that the resistance of CMA5 against kanamycin was due to the ribosome resistance that was gained by transformation with pMCP5.

It is curious that the kanamycin resistance mechanism manifested in cells containing pMCP5 was not detectable in ISP5500 from which DNA was derived. To pursue these observations, we carried out the following two experiments. First, we tested whether the inserted DNA originated from *S. kanamyceticus* ISP5500, using Southern hybridization. A ^{32}P-labeled probe of pMCP5 was hybridized to undigested or *Bcl*I-cleaved total DNA from *S. lividans* 1326 and *S. kanamyceticus* ISP5500 (Fig. 4). A DNA sequence homologous to pMCP5 could be detected in *Bcl*I-cleaved ISP5500 total DNA (Fig. 4, lane B), whereas no sequence homology was detected in *Bcl*I-cleaved strain 1326 total DNA (Fig. 4, lane C).

Second, we tested the kanamycin resistance of ribosomes from *S. kanamyceticus* ISP5500 grown in antibiotic-producing medium. *S. kanamyceticus* ISP5500 grown in tryptic soy broth could not produce kanamycin, whereas kanamycin was produced when cells were grown in a medium containing 2% starch, 2% maltose, 1% $NaNO_3$, 0.01% $CaCO_3$, and 3% soybean meal (pH 7.0) (11). The preceding experiments of in vitro polyphenylalanine synthesis were done with ribosomes from the three strains grown in tryptic soy broth. Then ribosomes from ISP5500 grown in the antibiotic-producing medium were tested for kanamycin resistance.

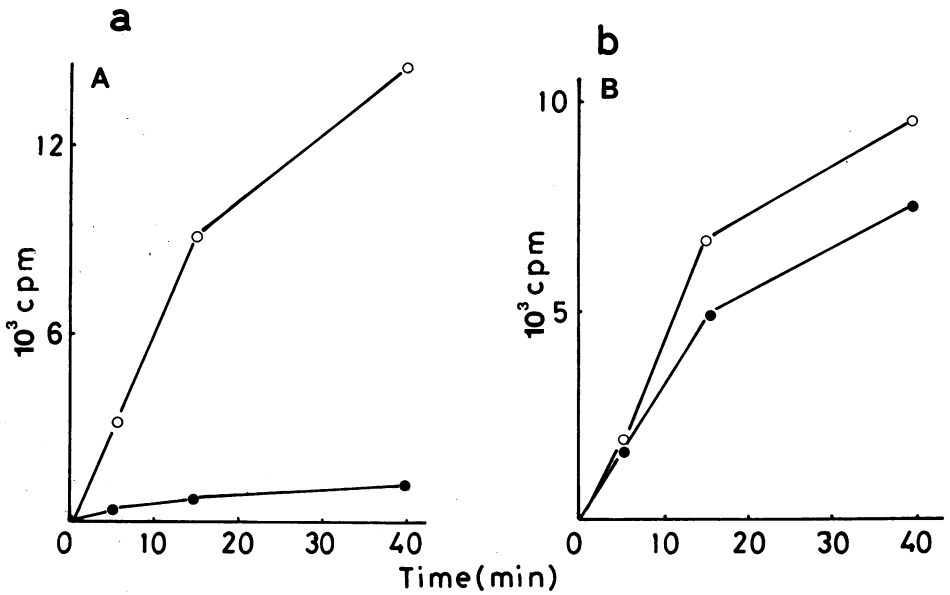

FIG. 5. Effect of kanamycin on polyphenylalanine synthesis with ribosomes and the S150 fraction. Symbols: ○, without kanamycin; ●, with 100 μg of kanamycin per ml. Strain ISP5500 cells were grown in the antibiotic-producing medium as described in the text. (A) Ribosomes from strain 1326 and the S150 fraction from ISP5500; (B) ribosomes from ISP5500 and the S150 fraction from 1326.

Polyphenylalanine synthesis was resistant to kanamycin when both ribosomes and the S150 fraction were derived from ISP5500 grown in the antibiotic-producing medium (data not shown). Furthermore, this resistance was shown to be due to ribosomes when ribosomes and the S150 fraction were reciprocally exchanged between strains 1326 and ISP5500 (Fig. 5). These results indicated that the resistance of cells containing pMCP5 against kanamycin originated from ISP5500.

DISCUSSION

Originally, we tried to clone the aminoglycoside acetyltransferase gene from *S. kanamyceticus* ISP5500 into *S. lividans* 1326. Surprisingly, the kanamycin-resistant transformant described in this paper showed a different resistance pattern to various aminoglycoside antibiotics from that of the donor ISP5500 strain. This resistance was revealed to be an acquired resistance of ribosomes. The inconsistency that the ribosomes from ISP5500 were not resistant to kanamycin was solved by using ribosomes from ISP5500 grown in antibiotic-producing medium. The cloned resistance gene was not expressed in ISP5500 grown in the kanamycin nonproducing medium, although it was expressed during growth in the producing medium. In other words, the ribosome resistance in the kanamycin-resistant transformants seems to be constitutive, whereas in the donor strain, the resistance is inducible. Hotta et al. reported that the resistance of *S. kanamyceticus* to various aminoglycosides was due to the presence of the inactivating enzyme from the results of in vitro polyphenylalanine synthesis (5). However, they used ribosomes from *S. kanamyceticus* grown in tryptic soy broth, so it is likely that they missed the resistance of ribosomes against kanamycin. Kanamycin acetyltransferase was known to develop rapidly during a lag period of the growth of *S. kanamyceticus* in the antibiotic-producing medium, but suddenly decreased at the beginning of a logarithmic-growth period, whereas kanamycin production began subsequently at the logarithmic-growth period and reached a maximum at stationary phase (11). This discrepancy of the appearance of the acetyltransferase and the kanamycin production suggested that involvement of the enzyme in the resistance to its own antibiotics is unlikely. On the contrary, the coincidence of ribosome resistance and kanamycin production reported in this paper strongly suggested the concept that alteration of ribosomes plays an important role in the self-defence of the kanamycin-producing strain against kanamycin. In addition, the cloned gene will be available to explore regulation of gene expression in streptomycetes.

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