ISOLATION AND CHARACTERIZATION OF PLASMIDS FROM PARENT AND VARIANT STRAINS OF *STREPTOMYCES RIBOSIDIFICUS*

Sir:

Evidence suggesting the possible involvement of plasmids in the production of antibiotics has been reported a number of times since the first report of OKANISHI *et al.* They eliminated the ability to produce kasugamycin and aureothricin by treating *Streptomyces kasugaensis* with acridine dyes or high temperature.[1] However, there are only few cases of isolation of plasmids from actinomycetes[2~7].

We report here the isolation of plasmids from *Streptomyces ribosidificus* ATCC 21294, a ribostamycin producer[8], and two of its variant strains devoid of antibiotic producibility. Comparison of the three plasmids was made by electron microscopy and DNA restriction endonuclease digestion but no differences were found. Thus the genetic expression of the plasmid in the host cell remains unknown.

Antibiotic-nonproducing variants AF-1 and AFM-1 of *S. ribosidificus* ATCC 21294 were obtained by acriflavine treatment. The 2-deoxystreptamine-negative mutant (AF-1) was described previously by KOJIMA and SATOH[9]. Frequently we were able to isolate mutant strains which are antibiotic-nonproducing, without spore formation and sensitive to self-producing antibiotic, ribostamycin. One of these strains, AFM-1 was used in the experiment. The properties of the mutant strains are shown in Tables 1 and 2.

Each of the strains was grown at 28°C for 7 days on agar slants (2% soluble starch, 0.4% yeast extract, 2% agar, pH 7.0). Mycelia on agar slants were transferred into a 500 ml Erlenmeyer flask containing 100-ml of a medium that consisted of 1% malt extract, 0.4% yeast extract and 2% glucose at pH 7.0. The flask was shaken at 28°C on a rotary shaker at 200 rpm with a 8 cm diameter orbit. The mycelium grown for 40 hours was harvested by centrifugation at 5,000×*g* for 10 minutes and washed twice with TS buffer (10 mM Tris-HCl, 0.14 M NaCl, pH 8.0). A 3 g portion of wet mycelium was resuspended in 15 ml of TES buffer (0.1 M Tris-HCl, 20 mM EDTA, 25% sucrose, pH 8.0), and 0.3 ml of a 30 mg/ml lysozyme solution was added. After incubating at 25°C for 20 minutes, the reaction mixture was immediately cooled in an ice bath. Then 18 ml of cold water and 3 ml of 1% sodium dodecylsulfate solution were added. After gentle mixing of the reaction mixture, 10 ml of 5 M sodium chloride solution were added and the mixture allowed to stand overnight at 0°C. A

Table 1. Effect of addition of 2-deoxystreptamine on ribostamycin production by mutant strains of *S. ribosidificus* ATCC 21294.

Each of the strains was grown at 28°C in production medium containing 2% soluble starch, 2.5% soybean meal, 1% wheat germ and 0.25% sodium chloride at pH 7.0. 2-Deoxystreptamine (DOS) was added at 300 μg/ml at the start. Ribostamycin production was assayed by the paper disc method using *Bacillus subtilis* as the test organism.

Strains	Addition of DOS	Ribostamycin production (μg/ml)				
		2-day	3-day	4-day	5-day	6-day
S. ribosidificus						
ATCC 21294	−	370	420	420	410	380
AF-1	−	0	0	0	0	0
AF-1	+	78	108	270	290	220
AFM-1	−	0	0	0	0	0
AFM-1	+	0	0	0	0	0

Table 2. Sensitivity to ribostamycin and kanamycin, and sporulation ability of mutant strains of *S. ribosidificus* ATCC 21294.

Sensitivity to ribostamycin (RM) and kanamycin (KM) was measured by the agar dilution method. Sporulation was determined by microscopy after growing for 10 days on agar slants containing 1% sucrose, 0.75% corn steep liquor, 0.4% K_2HPO_4, 0.2% $(NH_4)_2HPO_4$, 0.025% $MgSO_4 \cdot 7H_2O$ and 2% agar.

Strains	MIC (μg/ml)		Sporulation
	RM	KM	
S. ribosidificus			
ATCC 21294	>500	10	+
AF-1	>500	5	+
AFM-1	5	2.5	−

cleared lysate was obtained by centrifugation at 15,000 × *g* for 60 minutes. Subsequently, polyethylene glycol #1,000 was added to a final concentration of 1% and the lysate solution was kept at 0°C for 3 hours to complete the DNA precipitation. After centrifugation at 5,000 × *g* for 15 minutes, the DNA was dissolved in sarkosyl-TE buffer (0.4% sodium lauroyl sarcosinate, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). According to the method of RADOLFF *et al.*[10], an ethidium bromide-CsCl dye-buoyant density gradient centrifugation was performed to purify plasmid DNA.

As shown in Fig. 1, a plasmid DNA (lower band) which was separated from the main chromosomal DNA (upper band) was clearly observed under UV light.

The plasmid band in the tubes was collected carefully using a glass syringe. After removing ethidium bromide by *n*-butyl alcohol extraction, the plasmid DNA was dialyzed exhaustively against TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 0°C.

Electron microscopy of the plasmid from the parent strain is shown in Fig. 2. Most of the plasmid DNA molecules were revealed as covalently closed circular forms, a typical one is seen in photograph (A). Open circular forms, such as shown in the photograph (B), were rarely observed.

The contour length traced from an enlargement of the negative was 27 μm and the molecular weight based on contour length was $52 \pm 2 \times 10^6$ daltons (1.913×10^{10} daltons/cm)[12]. The contour lengths of the plasmids from strain AF-1 and AFM-1 were about the same within experimental error.

Fig. 1. Photograph of plasmid DNA of *S. ribosidificus* under UV light after separation by ethidium bromide-CsCl dye-buoyant density gradient centrifugation.

The sarkosyl-TE buffer solution of DNA (9.52 ml), obtained as described in the text, was adjusted to specific gravity 1.575 by adding 10 g solid CsCl and 1 ml of a 4.6 mg/ml ethidium bromide solution. The mixture was placed in a nitrocellulose tube and centrifugation was performed on a Beckman model L2-75B ultracentrifuge in a type 75Ti fixed angle rotor at 36,000 rpm. for 40 hours.

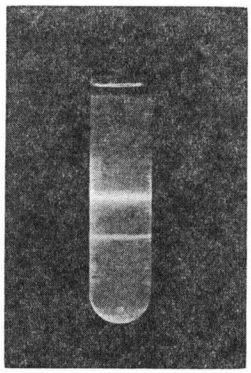


Fig. 2. Electron micrographs of plasmid DNA from *S. ribosidificus* ATCC 21294.

According to the modified KLEINSCHMIDT spreading technique[11], a cytochrome film was picked up on carbon coated 3 mm copper grids (150 mesh). The grids were rinsed in isopentane for 10 seconds, air dried, and then rotary shadowed with platinum - palladium (4 : 1) at an angle of tan. = 1/10 in a JEOL JEE4B evaporator. Electron micrographs were obtained by use of a JEOL JEM C-100 electron microscope.

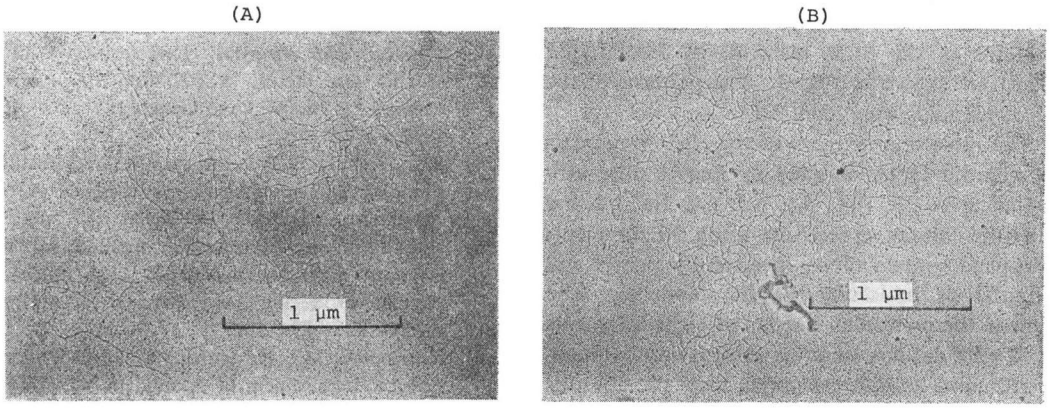


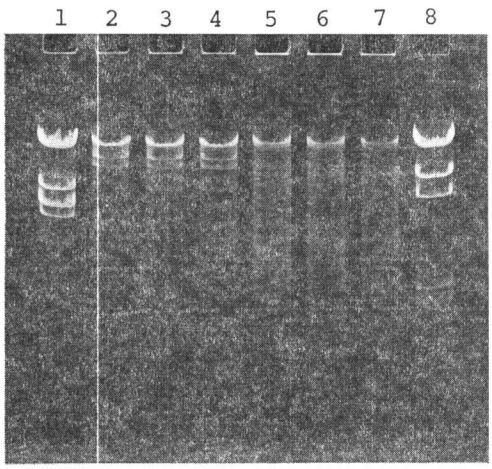
Fig. 3. The digestion patterns of the three plasmids by EcoRI and BamI.

EcoRI digested DNAs: 1, phage λcI; 2, plasmid from parent strain; 3, AF-1; 4, AFM-1. BamI digested DNAs: 5, plasmid from parent strain; 6, AF-1; 7, AFM-1. 8, HindIII digested phage λcI DNA.

Restriction endonucleases were purchased from Miles Laboratories, Ltd. and used as directed.

Electrophoresis was carried out on a vertical slab gel (11.5×13.0×0.3 cm) of 1% Agarose in E buffer (40 mM Tris-HCl, 1 mM EDTA, 5 mM CH_3-COONa, pH 8.0)[13].

After electrophoresis at a constant voltage of 50 volts at room temperature for 2.5 hours, the gel was stained for 5 minutes in E buffer containing 2 μg/ml of ethidium bromide. The bands were visualized by fluorescence under 365 nm UV light.



The plasmids from parent, strain AF-1 and AFM-1 appeared to be single components on Agarose gel electrophoresis. The plasmids were cut at eight sites by EcoRI and converted into eight fragments. Fragments hydrolyzed by BamI, HindIII, SalI and SmaI were too small to be detected on Agarose gels. The digestion patterns of the three plasmids by EcoRI and BamI are shown in Fig. 3. The patterns of the three plasmids were identical.

The molecular weights of the eight fragments of plasmid DNA digested by EcoRI were estimated to be 23, 10.0, 8.1, 5.3, 2.9, 1.5, 1.2 and 1.0×10^6 daltons respectively, using the fragments of phage lambda DNA digested by EcoRI and HindIII as molecular weight standards. The sum of the molecular weights of eight fragments is 52×10^6 daltons, and this value is approximately the same as that calculated from the contour length.

As presented above, no difference was found between three plasmids obtained from phenotypically different strains of *S. ribosidificus*. Further studies are needed to determine the relationship between the plasmid and production of the antibiotic, if any.

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