ISOLATION AND CHARACTERIZATION OF PLASMID DNAs IN ACTINOMYCETES

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Plasmid involvement in the production of various antibiotics in streptomycetes has been suggested by "curing" experiments[1~3] and, in some cases, it has been proved by genetic analysis of recombinants or by infectious transfer.[4~7] A few papers have been published on the isolation of plasmid DNA from streptomycetes, and following supercoiled DNAs (as plasmids) have been reported in antibiotic-producing streptomycetes: 3.35 μm DNA in aureothricin-producing *Streptomyces kasugaensis* M338 (At-536)[8], 18-megadalton DNA in chloramphenicol-producing *Streptomyces* sp. 3022a,[9] plasmids pSF1 and pSF2 in neomycin-producing *Streptomyces fradiae* ATCC 10745,[10] a plasmid in oxytetracycline-producing *Streptomyces rimosus*,[11] and SCP2(pSH1) in *Streptomyces coelicolor* A3(2).[12,13] We have attempted to isolate supercoiled DNAs from 32 strains of 30 actinomycete species and to determine their molecular weights by application of electron microscopy and restriction endonuclease analysis.

Organisms were inoculated into a seed medium (GGCY) consisting of 10% glucose, 0.1% glycine, 0.4% Casamino acids(Difco), 0.05% yeast extract (Difco), 0.1% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.4% trace element solution,[14] 0.2% KH_2PO_4 and 0.8% $Na_2HPO_4 \cdot 12H_2O$, and were incubated at 27°C for 3 days on a reciprocating shaker. The seed culture was inoculated into GGC medium (100 ml) which contained no yeast extract but 0.4% glycerol instead of glucose in the GGCY medium. 2′-Deoxyadenosine (0.02%) and 150 μCi of ^{3}H-thymidine were added to the medium for labeling DNA. After incubation for 24 hours at 27°C, the mycelia were harvested, suspended in 35 ml of 0.1 N NH_4OH containing 10 mM 3Na-EDTA and incubated at 37°C for 20 minutes. After centrifugation, the sedimented mycelia were suspended in 20 ml of 2×TES (TES consists of 25 mM Tris, 25 mM 2Na-EDTA and 25 mM NaCl, pH 7.4). One ml of lysozyme solution (40 mg/ml) was added, and the suspension was incubated at 37°C until the mycelia showed about 50% lysis. Three ml of 10% sodium dodecyl sulfate (SDS) was added, the suspention incubated at 37°C for 20 minutes, and centrifuged at 20,000×g for 30 minutes at 20°C to obtain a cleared lysate. To the cleared lysate, 5 M NaCl was added to a final concentration of 1.0 M. The mixture was kept in ice bath for at least 2.5 hours, and the precipitate was removed by centrifugation (1,200×g, 15 minutes, 0°C). To the supernatant fluid, RNase was added at a concentration of 50 mcg/ml and the solution incubated at 37°C for 20 minutes. After that, Pronase E solution (incubated for 1 hour at 37°C in advance) was added at 100 mcg/ml followed by incubation at 37°C for 20 minutes. Polyethylene glycol 6000 (40%) was added to a concentration of 10% and the solution kept in a refrigerator overnight to precipitate DNA. The precipitated DNA was collected by centrifugation at 1,200×g for 10 minutes, and the sedimented material was dissolved in 4.7 ml of TES buffer with careful stirring. The solution was dialyzed against TES buffer for at least 3 hours. Exactly 4.70 ml of the dialyzed solution was taken and CsCl (5.00 g) was added. Insoluble materials were removed by low speed centrifugation and 0.5 ml of ethidium bromide solution (4.8 mg/ml) was added and the solution mixed. After centrifugation at 100,000×g for 42 hours, radioactivities of gradient fractions were measured in the usual manner. The fractions containing supercoiled DNA as well as chromosomal DNA were dialyzed against DSB (10 mM Tris, 10 mM NaCl, 1 mM 2Na-EDTA, pH 7.6), and samples were analyzed by agarose gel electrophoresis to detect plasmid DNA. Supercoiled DNA in the residual sample was purified by neutral sucrose gradient centrifugation.

DNA samples for electron microscopy were prepared by the formamide technique,[15] and molecular weights of DNAs were calculated from contour lengths which were obtained by taking pBR 322 DNA as internal standard. Four restriction endonucleases (*Eco* RI, *Sal* I, *Bam* HI, and *Hind* III) were used to digest plasmid DNA, and the samples were subjected to agarose gel electrophoresis to investigate the cleavage number and

molecular weight of fragments.

Mycelia of most streptomycetes in the stationary phase of growth were lyzed easily by lysozyme, after growth in a chemically defined medium containing glycine (0.1%) and glycerol at concentrations less than 0.4%. Some strains which were only poor lyzed became sensitive to lysozyme when the mycelia were incubated in 0.1 N NH_4OH at 37°C for 20 minutes before the lysozyme treatment.

From 32 antibiotic-producing strains, supercoiled DNAs were found in 7 strains as follows: *Streptomyces ribosidificus* KCC S-0923 (ribostamycin), *Streptomyces niveus* KCC S-0599 (novobiocin), *S. kasugaensis* MB 273 (kasugamycin and aureothricin), *Streptomyces violaceus-ruber* SANK 95570 (methylenomycins), *S. fradiae* KCC S-0579 (ATCC 10745, neomycin), *Streptomyces omiyaensis* NIHJ AT-95 (chloramphenicol) and *Streptoverticillium mashuensis* KCC S-0059 (streptomycin). A satellite peak of DNA in dye-bouyant density gradient centrifugation was detected in cases of *S. ribosidificus*, *S. niveus*, *S. kasugaensis*, *S. omiyaensis* and *S. fradiae*. Typical patterns are shown in Fig. 1 A together with those of neutral sucrose gradient centrifugation (Fig. 1 C). No satellite peak was observed in *Stv. mashuensis* and *S. violaceus-ruber*, but their supercoiled DNAs were found by agarose gel electrophoresis in the fractions near fraction number 12 (Fig. 1 B). Electron microscopic photographs are shown in Fig. 2. Characteristics of each plasmid DNA are summarized in Table 1.

Two plasmids were observed in *S. fradiae* ATCC 10745 and their molecular weights were calculated to 62×10^6 and 43×10^6 daltons. These values are different from those (14.9 and 21.9 $\times 10^6$ daltons) reported by YAGISAWA *et al.*[10] *S. violaceus-ruber* examined in this experiment produced methylenomycin A as did *S. coelicolor* A3(2)[16~18]. Both may belong to the same species, since their phenotypic feature are identical. Biosynthetic steps of methylenomycin A in *S. coelicolor* A3(2) have been reported to be controlled by genes on plasmid SCP1, although this plasmid DNA has not yet been isolated.[6] In our *S. violaceus-ruber*, however, a 100×10^6 dalton plasmid (pSV1) was detected. It will be interesting to examine the relationship between pSV1 and methylenomycin A production.

Among 32 strains examined, it was found that only 7 strains had supercoiled DNA. Furthermore, supercoiled DNAs were not found in

Fig. 1. CsCl-ethidium bromide density gradient centrifugation of ^3H-labeled DNA isolated from *S. ribosidificus* KCC S-0293 (A) and *Stv. mashuensis* KCC S-0059 (B).

Neutral sucrose gradient centrifugation of ^3H-labeled DNA obtained from above density gradient centrifugation, *S. ribosidificus* (C) and *Stv. mashuensis* (D).

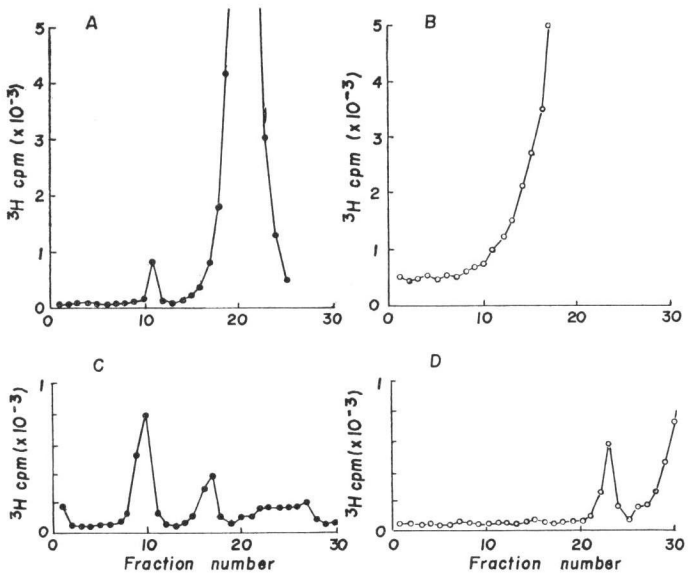

Fig. 2. Electron micrographs of plasmid DNAs.

(A) pSR1 DNA of *S. ribosidificus* KCC S-0923.
(B) pSM1 DNA of *Stv. mashuensis* KCC S-0059.
(C) pSO1 DNA of *S. omiyaensis* NIHJ AT-95.
(D) pSN1 DNA of *S. niveus* KCC S-0599.

The molecular weight of the plasmid was determined by use of the pBR322 as internal standard (2.6×10^6 daltons). Bar corresponds to 1 μm.

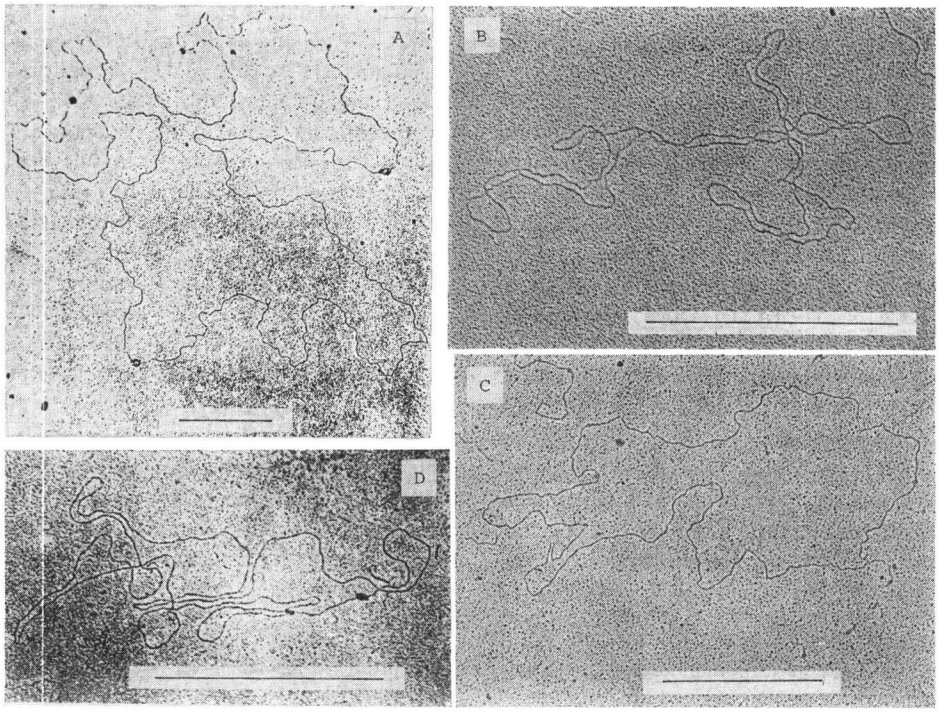

Table 1. Characterization of actiomycete plasmids.

Plasmid	Host	Numbers of plasmid fragments				Molecular weight ($\times 10^{-6}$)	
		Eco RI	*Hind* III	*Bam* HI	*Sal* I	AGE*	EM**
pSR1	*S. ribosidificus* KCC S-0923	8	4	8	+++	49	56
pSM1	*Stv. mashuensis* KCC S-0059	0	0	2	8	16	17
pSN1	*S. niveus* KCC S-0599	4	0	0	+++	20	20
pSK1	*S. kasugaensis* IMC MB273	1	0	2	1	6.7	6.7
pSO1	*S. omiyaensis* NIHJ AT-95	2	+++	2	+++	18	19
pSV1	*S. violaceus-ruber* SANK 95570***						100
Large	*S. fradiae* ATCC 10745***						62
Small	*S. fradiae* ATCC 10745***						43

* AGE: Agarose gel electrophoresis
** EM: Electron micrograph
*** These plasmid DNAs were not obtained enough amount to use for the restriction endonuclease experiments.

strains of *S. venezuelae* and *S. alboniger* with which genetical studies suggested the possible plasmid involvement in antibiotic production. The reasons why supercoiled DNA was not detected in most strains tested may be as follows: (1) Our methods may have been still unsuitable to separate plasmid DNA from cell components or insufficient to inhibit the nuclease activity of the lyzed cells; (2) During the long time of storage or maintenance, the strains used in these experiments might have lost their plasmids; (3) Some plasmids may have conformations other than supercoiled DNA.[8] For the study of these possibilities, *S. coelicolor* A3(2) may be the best material and we are attempting to detect a plasmid in this strain.

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