A SURVEY OF PLASMIDS AMONG NATURAL ISOLATES OF *STREPTOMYCES*

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The possible involvement of plasmid DNA in the biosynthesis of antibiotics in Streptomycetaceae has been intensively studied, and plasmids have been isolated from many antibiotic-producing *Streptomyces*.[1~13] However no strong evidence has been found which proves that plasmids play a part in the biosynthesis of any antibiotic except for methylenomycin[14]. To study the possible role of plasmids in antibiotic production, we examined 120 newly-isolated strains of *Streptomyces* for the presence of plasmids; the antibiotic-producing abilities of each strain were determined and correlated with the presence of a plasmid. For this purpose, we have developed a method for rapid extraction of plasmids from streptomycetes cultivated on a solid medium. This method and our results concerning plasmid occurrence and antibiotic biosynthesis are reported in this note.

The strains were isolated from 25 soil samples collected in various areas in the Southwest of France. From each soil sample, we retained only morphologically different *Streptomyces* isolates.

Each strain was grown at 27°C for 5 days on 25 ml of GAPY medium consisting of glucose 10 g, soluble starch 20 g, yeast extract 5 g, soya peptone 5 g, $CaCO_3$ 1 g, agar 15 g, and made up to 1 liter of distilled water. Mycelia and spores were suspended in 5 ml of TES buffer (15% sucrose, 50 mM tris-HCl and 20 mM EDTA; pH 8) by scraping the surface of the agar with a pipette. The cellular suspension was centrifuged at 12,000 $\times g$ for 10 minutes and the pellet was suspended in 3 ml of TES buffer containing 10 mg/ml of lysozyme, and incubated at 30°C for 15 minutes. Diethylpyrocarbonate (50 μl) was added after lysozyme treatment to inhibit possible DNase activity. The protoplasts were burst by the addition of 2 ml of 10% (w/v) sodium dodecylsulfate (SDS) solution at 90°C. Lysis, as evidenced by increased viscosity, was completed by raising the temperature to 60°C. The chromosomal DNA of the lysates was denatured by adding 500 to 700 μl of a 1 N NaOH solution. The final pH of 12.6 was controlled with a sensitive pH indicator paper. The solution was mixed gently and kept at room temperature for up to 15 minutes. The pH was lowered to 8.5 by addition of 2 ml of 2 M tris-HCl (pH 7) and the tube was gently inverted. After 15 minutes 2 ml of 5 M NaCl were added and the mixture was allowed to stand for 2 hours at 0°C. The precipitate was removed by centrifugation at 25,000 $\times g$ for 30 minutes and the supernatant was treated with pancreatic RNase at 50 μg/ml for 30 minutes at 37°C. DNA was precipitated with cold ethanol and redissolved in 100 μl of TEN buffer (50 mM tris-HCl, 5 mM EDTA and 50 mM NaCl, pH 8). The final plasmid DNA extract (40 μl) was mixed with 8 μl of a staining solution (10% Ficoll and 0.1% bromophenol blue) and analyzed by agarose gel electrophoresis.

We have applied this method to isolate the plasmids from strains of known *Streptomyces* species such as *S. lividans* and *S. rimosus* in which the engineered plasmids pIJ41, pIJ350 and pIJ364 (kindly provided by D. HOPWOOD)[15] has been introduced by protoplast transformation. In each case a plasmid band was detected by gel electrophoresis. Among the 120 wild-type *Streptomyces* strains analyzed, plasmid DNA bands have been reproducibly detected in 21 strains (17.5%) and occasionally in others. These findings are similar to those described by other authors.[5,6,10,12] Fig. 1 shows the electrophoresis of DNA extracted from the plasmid-positive strains. For some strains, multiple bands are detected on the agarose gel electrophoresis of DNA extracts. Experiments are in progress to determine whether these bands are different forms of plasmid DNA or different plasmids. The bands indicating approximate 20×10^6 dalton (Slots 4, 5, 7, 8, 9, 10 and 22) might be possible to be the fragments of chromosomal DNA.

The molecular weights of these plasmids were estimated from their migration on gels relative to standard plasmid DNA (Table 1).

Strains bearing large plasmids are the most

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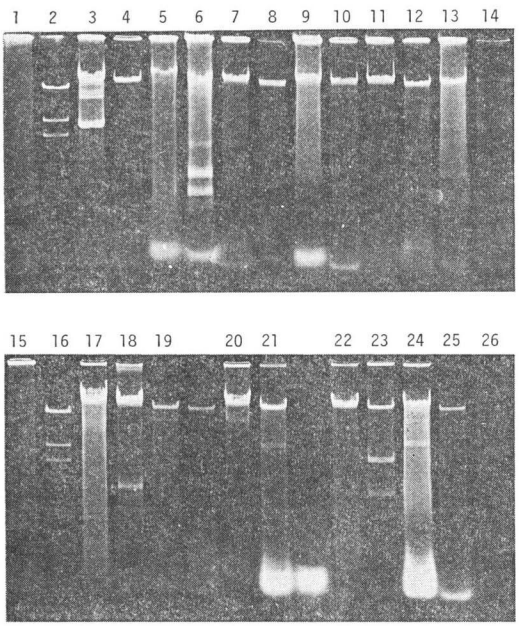
Fig. 1. Agarose gel electrophoresis (0.6%) of DNA extracts from the plasmid-containing strains.

DNA species were separated by electrophoresis in 0.6% agarose (Sigma) using tris-acetate buffer (40 mM tris-base, 20 mM sodium acetate and 2 mM EDTA; pH 8) for 3 hours at 80 volts constant voltage.

Slot 1: pC194 (*Bacillus subtilis* plasmid), 2: phage lambda DNA digested with *Hin*d III, 3: strain 14 G_1, 4: 14 H_1, 5: 14 CMC_1, 6: 14 B_1, 7: 7 A_6, 8: 5 F_5, 9: 4 E_{10}, 10: 2 D_7, 11: 2 A_1, 12: P III, 13 Nat, 14: Rub, 15: pC 194, 16: phage lambda DNA digested with *Hin*d III, 17: 12 D_9, 18: 22 D_2, 19: 20 E_8, 20: 20 E_9, 21: 18 D_{50}, 22: 14 E_5, 23: 14 D_3, 24: 14 D_{50}, 25: 15 G_3, 26: Rub.



frequently detected. Based on repeated observations of the intensity of the bands on the gel, the number of plasmid copies appears to be higher for the small plasmids than for the large ones.

The method of plasmid extraction described here allows rapid and efficient screening of a number of strains cultivated on a solid medium. The advantage of this method is that growth in glycine-containing liquid culture as commonly used is not necessary; good lysis is obtained for all strains. Pigment is not completely eliminated in this method of plasmid isolation, but it does not interfere with plasmid detection.

The antibiotic activities of the 120 strains have been determined by the agar piece method[16] using sensitive indicator microorganisms including *Escherichia coli*, *Bacillus subtilis*, *Streptococcus pneumoniae* and *Saccharomyces cerevisiae*. On the basis of inhibition zones on any of the indicator strains, the streptomycetes have been considered to be producers or non-producers. From Table 2 it can be deduced that the production of antibiotics is no higher in the population of plasmid-bearing streptomycetes than in the strains where no plasmid was detected.

A common observation in screening programs for the selection of new microorganisms is that morphologically different streptomycetes isolated from a given soil sample may produce identical antibiotic activities. This observation could best be explained by the fact that these distinct strep-

Table 1. Molecular weights of DNA plasmids isolated from wild-type *Streptomyces* strains.

Strains	Molecular weights* ($\times 10^6$)	Strains	Molecular weights* ($\times 10^6$)
14 B_1	2.5	2 A_1	18.0
Nat	4.1	14 E_5	≥20.0
14 G_1	4.6	20 E_9	≥20.0
14 D_3	4.6	2 D_7	≥20.0
22 D_2	4.8	4 E_{10}	≥20.0
P III	7.0	5 F_5	≥20.0
7 A_6	9.6	14 CMC_1	≥20.0
Rub	9.6	14 H_1	≥20.0
18 D_{50}	13.0	15 G_3	≥20.0
20 E_8	13.0	14 D_{50}	≥20.0
12 D_9	16.0		

* Molecular weights were obtained by agarose gel electrophoresis analysis, and correspond to the plasmid band migrating the farthest on each gel (presumed to be the covalently closed circular form). Molecular weights have been estimated with reference plasmids having known size (pIJ41, pIJ350 and pIJ364).

Table 2. Plasmid and antibiotic activity in 120 wild-type *Streptomyces* strains.

	Antibiotic activity		
	+	−	Total
Plasmid containing strains	13	8	21
Plasmid-less strains	79	20	99
Total	92	28	120

+: Antibiotic activity detected against one or several indicative strains used.
−: No antibiotic activity detected.

tomycetes from the same geographical origin harbor the same plasmid which carries essential genes for the production of an antibiotic. During this study we found a case in which six different streptomycetes isolates from a single sample were identical on the basis of antibiotic activities determined using a dozen microbial tester species. Repeated attempts to find a plasmid in these strains failed in all but one case. However, the possibility that a common plasmid is integrated into the chromosomal DNA of various hosts cannot be ruled out.

Conversely, we found seven plasmid-containing strains among fourteen streptomycetes which were selected from another soil sample. Using a variety of indicator strains including various representative Gram-negative and Gram-positive bacteria and several different yeasts and fungi, no evidence for a common molecule with an antibiotic activity could be found in the five producers among the seven plasmid-containing strains.

Thus, our results do not favor the hypothesis of the general involvement of plasmid-born genes in the biosynthesis of antibiotics in streptomycetaceae.

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